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An HPLC method for determination of inosine and hypoxanthine in human plasma from healthy volunteers and patients presenting with potential acute cardiac ischemia

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Abstract

A simple and sensitive high-performance liquid chromatography (HPLC) method utilizing ultraviolet (UV) detection was developed for the determination of inosine and hypoxanthine in human plasma. For component separation, a monolithic C_{18} column at a flow rate of 1.0 mL/min with an aqueous mobile phase of trifluoroacetic acid (0.1% TFA in deionized water pH 2.2, v/v) and methanol gradient was used. The method employed a one-step sample preparation utilizing centrifugal filtration with high component recoveries (∼98%) from plasma, which eliminated the need of an internal standard. The method demonstrated excellent linearity (0.25–5 μg/mL, $R > 0.9990$) for both inosine and hypoxanthine with detection limits of 100 ng/mL. This simple and cost effective method was utilized to evaluate potential endogenous plasma biomarker(s), which may aid hospital emergency personnel in the early detection of acute cardiac ischemia in patients presenting with non-traumatic chest pain.

Keywords: Inosine; Hypoxanthine; Biomarker; HPLC; Acute cardiac ischemia; Monolithic column

1. Introduction

According to a recent report by the World Health Organization (WHO, 2002 data), approximately 32 million myocardial infarctions (MI) occurred worldwide resulting in more than 12 million deaths [1]. Cardiovascular disease is the leading cause of mortality in the world and includes MI, which can be presaged by acute cardiac ischemia [2–5]. In a patient suspected of having an MI or on-going acute cardiac ischemia, standard diagnostic procedures include patient history and physical exam, an electrocardiogram (ECG) and sequential assessment of biomarkers of myocardial damage [6–8].

Current test methods for endogenous cardiac biomarkers (e.g. cardiac troponin I, creatine kinase-MB and myoglobin) include LC–MS analysis [9,10] and fluorescence immunoassay [11–14]; however elevation of these protein biomarkers reflect some level of myocardial necrosis, and are typically elevated in a diagnostic range several hours after acute myocardial infarction. Inosine (9-β-D-ribofuranosylhypoxanthine, MW 268 Da nucleoside) and hypoxanthine (1,7-dihydro-6H-purin-6-one, MW 136 Da purine) are endogenous non-protein plasma constituents normally found at low concentrations (e.g. ∼200–400 ng/mL) in human plasma resulting from dietary and endogenous purine metabolism [15].

Using the mouse model, recent research performed by our group demonstrated that inosine levels increased from cardiac tissue subjected to constant conditions of oxidative stress (e.g. acute cardiac ischemia or myocardial infarction) [16]. The purpose for development of this test method was to facilitate

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evaluation of a hypothesis that humans undergoing acute cardiac ischemia will have elevated blood levels of ATP catabolic products (e.g. inosine and hypoxanthine) in the blood, until medical treatment can succeed in restoring adequate blood flow to the oxygen deprived myocardium.

Current methods for plasma level measurement of selected ATP catabolic by-products such as inosine, hypoxanthine, xanthine and uric acid, in plasma utilize HPLC-UV with sample preparation steps including solid phase extraction [15], protein precipitations (e.g. ethanol or TCA) as well as some methods requiring use of an internal standard [17,18]. High-performance liquid chromatography (HPLC) with ion pairing reagents [19–21] or protein precipitation and enzyme catalyzed luminescence detection [22] have also been used. One HPLC method utilized centrifugal filtration for sample preparation; however their method did not completely resolve hypoxanthine and xanthine components at concentration levels five times lower than our patient hypoxanthine concentration levels, and with reported column degradation after 3 months of use [23]. None of these techniques, however, offers as simple a determination for inosine and hypoxanthine (can also evaluate uric acid, adenosine and xanthine) in human plasma as the method detailed in the present communication. The method utilizes centrifugal membrane filter technology and does not require the use of an internal standard. In addition, this method employs a recently introduced HPLC column technology (Onyx™ monolithic column, Phenomenex® Inc. 2005 market introduction) [24], which provided sufficient component resolution and sensitivity for measurement of inosine and hypoxanthine in human plasma samples, from healthy volunteers and emergency room patients presenting with chest pain with and without acute cardiac ischemia.

2. Experimental

2.1. Chemicals and blank plasma

Hypoxanthine and xanthine were purchased from Acros Organics (Fair Lawn, NJ, USA) and adenosine, inosine and uric acid were purchased from Sigma–Aldrich (St. Louis, MO, USA) with all chemicals being ACS reagent grade or higher purity. For mobile phase preparation, trifluoroacetic acid (TFA) was reagent grade, methanol was Optima HPLC grade and both were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Ultrapure distilled and deionized water (18 MΩ cm) used for all HPLC work was prepared in-house using PureLab® Ultra water purification system (US Filter, Lowell, MA, USA) and 0.2 μm filtered prior to use. Blood bank human blank plasma used for preparation of controls was provided by VCU Medical Center, Richmond, VA, USA.

2.2. HPLC equipment and mobile phase

The HPLC–DAD (diode array detector) equipment consisted of a Hewlett-Packard (HP) Model 1090 HPLC system (Agilent Technologies, Palo Alto, CA, USA). The analytical column used was a Phenomenex® Onyx™ monolithic C_{18}, 20 cm × 4.6 mm I.D., 130 Å column coupled to an Onyx™ C_{18} guard column, 5 cm × 4.6 mm I.D. (Torrance CA, USA). The guard column was replaced after each analytical run of approximately 50 samples. The mobile phase consisted of aqueous trifluoroacetic acid (0.1% TFA in deionized water, pH 2.2, v/v) and methanol gradient. The mobile phase gradient was programmed with time course as follows (99:1 0.1% TFA in deionized water:methanol (v/v) at 0 min and held for 3 min; 70:20 0.1% TFA in deionized water:methanol (v/v) at 10 min; 5:90 0.1% TFA in deionized water:methanol (v/v) at 11 min and held 2 min, and 99:1 0.1% TFA in deionized water:methanol (v/v) at 14 min).

The mobile phase was continuously degassed using helium sparging and used at a flow rate of 1.0 mL/min. Typical HPLC operating pressure at gradient time 0 min conditions was approximately 84 bar with ambient column temperature. An injection volume of 15 μL of the prepared plasma sample was accomplished using the HP Model 1090 autosampler. Component detection was achieved using the HP Model 1090 DAD detector with data collection at the optimal UV wavelength absorption of 250 nm for both inosine and hypoxanthine. The detector was operated at high sensitivity set point with a 1 s response time. A 345 kPa backpressure regulator (SSI, State College, PA, USA) was coupled to the detector outlet to prevent mobile phase outgassing. Data acquisition and component computations were performed using TotalChrom™ Workstation software (Perkin Elmer™, Norwalk, CT, USA).

2.3. Standard and control preparation

Stock standards of adenosine, inosine, hypoxanthine, xanthine and uric acid (100 μg/mL) were prepared in deionized water and stored at 4 °C. Working standards to establish HPLC retention times of adenosine, xanthine and uric acid components were prepared at 2.5 μg/mL concentrations in deionized water. Working standards of inosine and hypoxanthine (250, 500, 1000, 3000 and 5000 ng/mL) were prepared in deionized water. All working standards were stored at −70 °C and stable for at least 6 months. Working controls of inosine and hypoxanthine (250, 2000 and 4000 ng/mL) were prepared using pooled hospital blood plasma ($n = 3$ donated lots) which were evaluated individually and confirmed to lack detectable levels of inosine and hypoxanthine components.

It is possible the levels of inosine and hypoxanthine in blood bank plasma were not detectable due to the time (>10 days) the plasma was stored refrigerated (4 °C) prior to expiration and availability for laboratory experimental use. Without freezing the plasma or utilizing plasma enzyme inhibitors, xanthine oxidase and purine nucleoside phosphorylase found in plasma may metabolize the normally low levels of inosine and hypoxanthine to their end product uric acid. Following preparation of control samples, they were immediately frozen at −70 °C, to prevent endogenous plasma purine nucleoside phosphorylase from converting inosine to hypoxanthine prior to formal sample analysis.

2.4. Sample conditions

Following hospital approval, blood was obtained from hospital emergency room patients ($n = 20$), in vacutainer™ tubes containing heparin as per hospital emergency room protocols for patients presenting with chest pain and potential MI or acute myocardial ischemia. Sample tubes were centrifuged at ~1000 × *g* for 10 min with plasma drawn off and split into tubes for hospital clinical testing and one tube immediately frozen at −20 °C (transferred to −70 °C for storage) for inosine and hypoxanthine analysis. Plasma samples from healthy blood donors (male and female, both genders > 18 years of age) were purchased from ProMedDx (Norton, MA, USA) which used an IRB approved specimen collection protocol and stored frozen at −70 °C. Prior to HPLC analysis, plasma samples were thawed to ambient temperature, mixed thoroughly by inversion and centrifuged at 1000 × *g* for 10 min to eliminate fibrinous material.

2.5. Sample preparation

Samples were prepared for HPLC analysis by pipetting 250 μL of plasma into a polypropylene Microcon® YM-10 (10,000 molecular weight cutoff, MWCO) centrifugal filter tube (Millipore, Bedford MA, USA). The sample tubes were capped and centrifuged at 14,000 × *g* for 15 min at ambient lab temperature. The clear filtrates were transferred to deactivated glass HPLC autosampler vials (Waters®, Milford MA, USA) with 15 μL injected into the HPLC system for analysis.

3. Results and discussion

3.1. HPLC conditions optimization

Several types of C_{18} columns were evaluated for resolving adenosine, inosine, hypoxanthine, xanthine and uric acid from other plasma components. Due to minimal sample preparation using the centrifugal membrane filter, the ideal HPLC column should have high efficiency for resolving inosine and hypoxanthine components from components in the plasma matrix. Conventional HPLC columns such as Synergi Polar-RP C_{18} (15 cm × 3.0 mm I.D. × 4 μm packing) and Hypersil ODS C_{18} (15 cm × 3.2 mm I.D. × 3 μm packing) were evaluated versus the recently marketed HPLC column technology, the Onyx monolithic C_{18} column (10 cm × 4.6 mm I.D.). The monolithic column provided superior chromatographic resolution of components as described later in Section 3.4 with a low system backpressure of approximately 84 bar (gradient time zero conditions and flow rate of 1 mL/min). It should be emphasized that both conventional HPLC columns were evaluated at operating flow rates of ~0.6 mL/min and with system pressures that were approximately twice as high as when using the monolithic column. The supplier of the monolithic column cited advantages of high component efficiencies (resolution) and low system backpressure with use of the new monolithic column technology. We observed that both of these stated advantages over the two conventional mid-bore diameter HPLC columns evaluated were clearly demonstrated.

The mobile phase aqueous component, 0.1% TFA in deionized water, provided a pH of 2.2 which also provided good peak shape (e.g. uric acid component, p*K*a ~5.8) from components of interest from the endogenous plasma components (MW < 10,000 Da) obtained from the YM-10 sample preparation. Optimization and adjustment of the acid strength improved the separation between hypoxanthine (RT 5.2 min) and uric acid (RT 5.7 min). Initial use of aqueous 0.05% TFA did not provide component baseline resolution while aqueous 0.1% TFA offered complete component baseline resolution at the expense of increased column retention times. The mobile phase organic modifiers (e.g. acetonitrile versus methanol) were evaluated to determine which organic solvent would provide the best chromatographic separation from endogenous plasma components and at the same time being most cost effective. Methanol was chosen as the organic modifier as it provided symmetrical component peak shapes and good selectivity from other endogenous plasma components; however the HPLC system backpressure was somewhat higher when using methanol with the methanol gradient increasing from 1 to 90%. Methanol is also more cost effective for routine HPLC analysis because of its lower procurement cost.

A mobile phase gradient was used for reproducible separations of the structurally similar purines (hypoxanthine, uric acid) and nucleosides (inosine, adenosine). Since the mobile phase organic constituent is critical to controlling component elution times (initial 1% methanol composition at gradient time zero), the use of protein precipitation technique using solvents such as acetonitrile or methanol (typically 1:1 or 2:1, organic:plasma ratio) was eliminated from consideration. The structurally similar components injected using organic solvent precipitation were not chromatographically resolved due to band broadening effects from the added organic modifier.

Different column oven temperatures (e.g. ambient lab of 20, 30 and 40 °C) were evaluated without significant chromatographic improvement (component resolution, peak shape), thus ambient temperature was utilized for the analysis. At higher column temperatures (e.g. 40 °C), component co-elution for both early (hypoxanthine, uric acid) and late components (inosine, adenosine) was observed.

3.2. Linearity, limits of quantitation and detection, computations

The plasma method was linear throughout the concentration range of 0.25–5 μg/mL for inosine (mean correlation coefficient of 0.9991, $n = 10$) and hypoxanthine (mean correlation coefficient of 0.9998, $n = 10$) with all standard back-calculated values within 5% of their nominal amount. The limit of detection (LOD) for each component of the method was ~100 ng/mL. The LOD was determined using a fortified amount of each component in pooled blood blank plasma at 100 ng/mL ($n = 3$) and calculation from each component's standard curve (component peak heights had greater than three times s/n than blank plasma background). For plasma component calculations and

Table 1
Combined intra- and inter-day accuracy and precision for inosine and hypoxanthine in plasma controls

Component	Fortified concentration (ng/mL) ($n = 15$)	Calculated mean concentration (ng/mL) ($n = 15$)	Error (%)	R.S.D. (%)
Inosine	250	243	−2.8	8.1
Inosine	2000	1966	−1.7	4.9
Inosine	4000	3914	−2.2	3.6
Hypoxanthine	250	265	6.0	7.5
Hypoxanthine	2000	2044	2.2	5.5
Hypoxanthine	4000	3931	−1.7	2.2

Controls demonstrated excellent accuracy ± 6% and precision ± 8.1% throughout the plasma concentration range.

reporting results, normal linear regression utilizing external standardization and peak height was used with the lowest standard calibrator (0.25 μg/mL) used as the limit of quantitation (defined as combined accuracy and precision within 20% of the nominal amount).

3.3. Accuracy, precision and recovery

The accuracy and precision for the method was determined by evaluation of replicate prepared plasma control samples at 250, 2000 and 4000 ng/mL (Table 1). The combined intra-day (within day) and inter-day (between day) accuracy of the method was reported as the percent error of nominal fortified amounts versus measured component concentrations. The combined intra-day and inter-day precision of the method was reported as percent relative standard deviation (% R.S.D.). The method demonstrated excellent accuracy (±6%) and precision (±8.1) for both components in plasma ($n = 15$ at each component concentration level).

Absolute recovery for the plasma method was evaluated by comparing extracted fortified controls prepared in pooled blood blank plasma versus unextracted standards prepared in deionized water ($n = 3$ at 250, 2000 and 4000 ng/mL). The absolute recovery for the plasma method was determined to be >98% for both inosine and hypoxanthine. In addition, the standards and controls used for all HPLC analysis were prepared and handled identical to patient and volunteer subject samples, thus controlling for potential errors in sample handling, micropipetting and YM-10 component extraction recovery.

3.4. Chromatography

Fig. 1 illustrates chromatograms of 2000 ng/mL hypoxanthine (RT ∼5.3 min), uric acid (RT ∼5.8 min), xanthine (RT ∼7.2 min), adenosine (RT ∼10.7 min) and inosine (RT ∼10.9 min) in deionized water for marking component retention times; limit of quantitation and lowest plasma standard of 250 ng/mL hypoxanthine and inosine; pooled blank plasma from the VCU Health Systems Hospital blood bank; prepared plasma from a healthy female subject; and prepared plasma from a hospital emergency room female patient exhibiting symptoms of chest pain and acute myocardial ischemia (Figures A, B, C, D and E, respectively). The method demonstrated excellent chromatographic selectivity with no endogenous plasma interferences at the retention times of hypoxanthine and inosine with sufficient sensitivity for both components of interest using conventional UV detection and an analytical run time of ∼21 min (allows mobile phase gradient equilibration). To extend column lifetime, the analytical column was flushed after each analytical run (∼50 injections) for 1 h at 1.0 mL/min with acetonitrile: deionized water (90:10, v/v) to eliminate potential retained nonpolar substances from the column.

3.5. Sample preparation, optimization and filtrate stability

Sample preparation evaluations using protein precipitation and centrifugal membrane filters were conducted. As previously described in Section 3.1, organic solvent precipitation was not useful due to resulting poor chromatographic resolution of structurally similar components. TCA was not evaluated due to the hazards of using the strong acid and the resulting sample dilution effect potentially affecting overall method sensitivity. The centrifugal membrane filter is commonly used to concentrate peptides, proteins and nucleic acids for proteomic and genomic determinations [25]. Since the molecular weights of our components are all less than 300 Da, our approach to using this technique was to inject the filtrate which would contain the low molecular weight components that transfers across theYM-3 or YM-10 cellulose membrane cutoff filters. This essentially removes most peptides and all proteins from the sample to be injected as they are retained by the cellulose membrane cutoff filter, thus improving method selectivity. Method sensitivity is also improved because there is no sample dilution effect since no solvent is added.

Evaluations to optimize sample preparation conditions using the YM-10 (10,000 Da MWCO) and YM-3 (3000 Da MWCO) centrifugal filter were conducted. With the centrifugal force set at 14,000 × g (recommended by YM-10 supplier) and using 250 uL of prepared plasma control samples, the centrifuge spin time was varied from 5, 15, 30 and 60 min. The 5 min spin time did not provide enough time to adequately separate plasma proteins from the aqueous matrix (salts, small peptides and substances less than 10,000 Da) with an insufficient amount of sample filtrate recovered. The 15, 30 and 60 min centrifugal spin times resulted in maximum recovery of sample filtrate. However, the 60 min spin filtrate samples were significantly warmer than ambient lab temperature most likely due to warming effects of the sample tubes friction with air from the centrifugal spin. Thus to eliminate potential component degradation due to heat from spinning 60 min and to shorten sample preparation time, a spin time of 15 min was used for all analyses as described in Section 3.3.

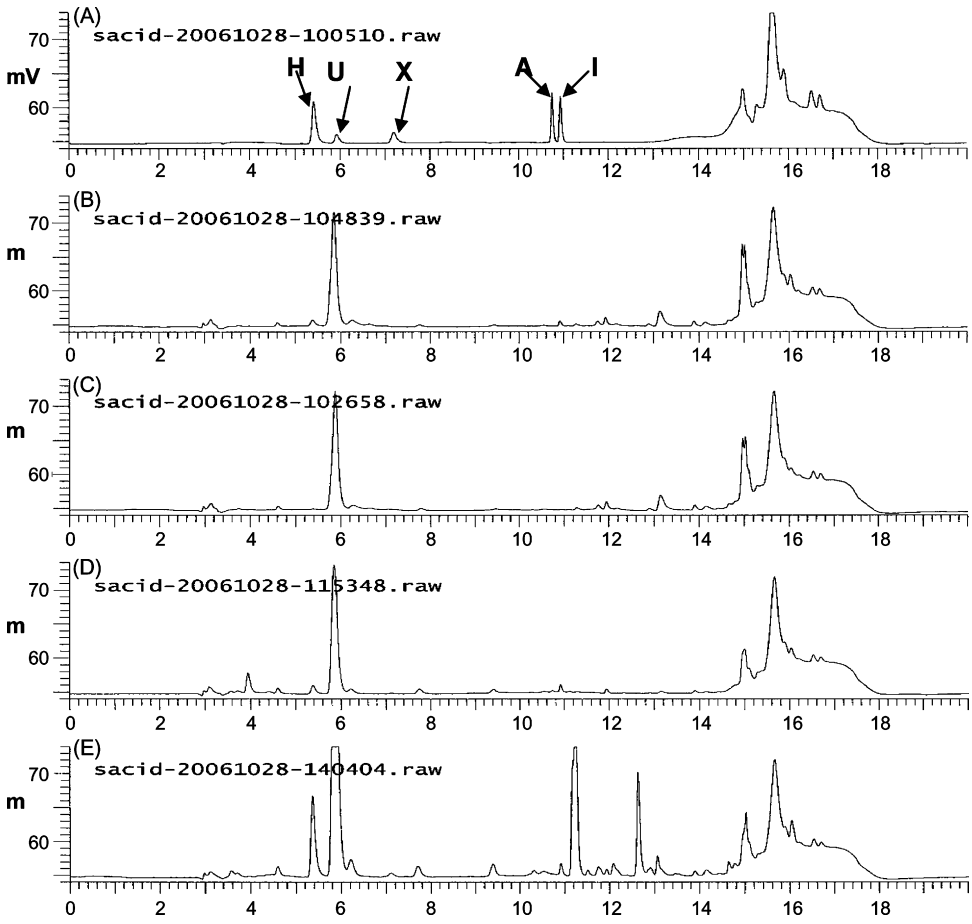

Fig. 1. Chromatograms illustrating (A) 2000 ng/mL hypoxanthine (RT ∼5.3 min), uric acid (RT ∼5.8 min), xanthine (RT ∼7.2 min), adenosine (RT ∼10.7 min) and inosine (RT ∼10.9 min) in deionized water, (B) low standard of 250 ng/mL hypoxanthine and inosine in blank plasma, (C) blank plasma, (D) plasma sample from healthy female subject and (E) plasma sample from hospital emergency room female patient.

Results for the YM-3 filter evaluation demonstrated longer spin times were required (∼45–60 min) at 14,000 × g versus the 15 min spin using the YM-10 filter. The YM-3 filtrate did not offer better filtration of smaller plasma peptides (<10K Da), as observed on chromatograms, than was already achieved using the YM-10 filter. However, using either YM-3 or YM-10 filter effectively removed the purine nucleoside phosphorylase enzyme (nominal weight ∼90–94 kDa protein, [26,27]) thus eliminating the potential for inosine to hypoxanthine metabolism in the sample filtrate. The filtrates were stored frozen (−70 °C) after HPLC analysis with both inosine and hypoxanthine components demonstrating stability for greater than 3 months.

3.6. Plasma purine nucleoside phosphorylase activity

Purine nucleoside phosphorylase (PNP, EC 2.4.2.1) is an enzyme that rapidly metabolizes inosine to hypoxanthine in blood ($t_{1/2}$ < 5 min due to red blood cells). This enzyme has low activity in plasma and is normally found in human cardiac muscle, GI tract, spleen, brain and red blood cells [28,29]. Therefore, to better estimate an ischemic heart's effluxed inosine during periods of acute cardiac oxidative stress, venous blood samples should be kept cold (ice) and prepared immediately. Either the blood sample should be immediately inhibited (e.g. peldesine, competitive inhibitor [28]) or the metabolite hypoxanthine should be simultaneously determined with inosine to better estimate the level of acute cardiac ischemia. In whole blood or plasma samples, hypoxanthine will not be further metabolized to xanthine as the human enzyme xanthine oxidase (XO), which is require for hypoxanthine to xanthine conversion, has low activity in plasma [30] and being typically found in human tissue (liver, small intestine) and other bodily fluids (milk, colostrum). A plasma (heparinized) sample is recommended for inosine and hypoxanthine determination in that the approximate 30 min clot time required for a serum sample would allow significant conversion of inosine to hypoxanthine in the collection tube, which would contain PNP from the red blood cell and plasma matrix.

Several evaluations (n = 3 samples at each condition) were performed to evaluate inosine metabolism by PNP activity in plasma stored at 4 °C (refrigerator), −20 and −70 °C. Results of the 4 °C evaluation can be seen in Fig. 2; plasma fortified with inosine only at 2000 ng/mL and without PNP enzyme inhibitor is metabolized rapidly to hypoxanthine (∼70% in 24 h); plasma fortified with 250 ng/mL of inosine and hypoxanthine and without PNP enzyme inhibitor is also metabolized rapidly to hypoxanthine (∼70% in 24 h); however the plasma fortified with 2000 ng/mL of inosine and hypoxanthine and without a PNP enzyme inhibitor, is metabolized less rapidly to hypox-

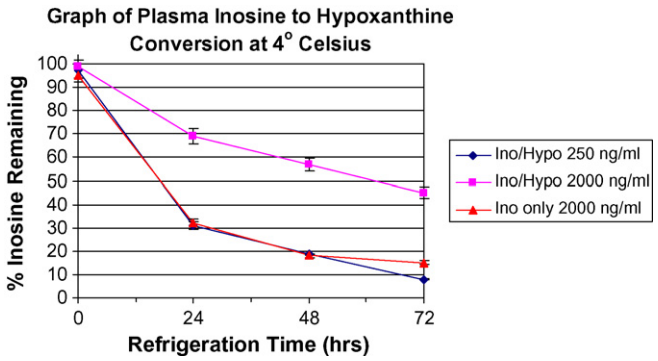

Fig. 2. Graph of mean percent inosine remaining after plasma PNP metabolism when stored at 4 °C. Square symbols represent fortified amounts of 2000 ng/mL of inosine and hypoxanthine in blank plasma ($n = 3$), diamond symbols represent fortified amounts of 250 ng/mL of inosine and hypoxanthine in blank plasma ($n = 3$) and triangle symbols represent fortified amount of 2000 ng/mL inosine only in blank plasma ($n = 3$).

anthine (~30% in 24 h) and slightly less than 50% after 72 h. Results of storing fortified plasma samples at −20 °C immediately after preparation indicated a reduced rate of inosine to hypoxanthine conversion (~30% after 8 months) with storage at −70 °C almost completely deactivating the PNP enzyme (<5% inosine conversion after 3 months).

A possible explanation for the plasma hypoxanthine concentration dependence for the conversion rate of inosine to hypoxanthine would be product inhibition (PNP $K_{eq} \approx 0.04$ mM) [31]. This low K_{eq} indicates that thermodynamically, inosine synthesis is favored over product conversion to hypoxanthine. When the venous sample plasma concentration of hypoxanthine is present at higher levels (e.g. 2000 ng/mL), the conversion of inosine to hypoxanthine by plasma PNP decreases in the absence of significant XO enzyme activity, which converts hypoxanthine to xanthine and uric acid for biological elimination (therefore XO activity ultimately increases PNP activity as it reduces hypoxanthine product inhibition of PNP). It was also determined that the total amount of inosine and hypoxanthine fortified into the pooled plasma was recovered, thus verifying the lack of significant XO activity in human plasma and supports our recommendation of simultaneous determination of both inosine and hypoxanthine components. A preliminary investigation to show the utility of the method is shown in Fig. 1D (healthy control with 350 ng/mL inosine and 373 ng/mL hypoxanthine) and 1E (potential acute cardiac ischemia patient with 641 ng/mL inosine and 3987 ng/mL hypoxanthine). These figures demonstrate an increase in both inosine and hypoxanthine concentrations in one patient having presented with chest pain and undergoing evaluation for acute cardiac ischemia.

4. Conclusions

A sensitive and selective method has been developed for evaluation of inosine and hypoxanthine in human plasma. The method employed a one-step sample preparation for plasma (no organic solvents or solid phase extraction cartridges required) with high analyte recoveries, which eliminated the need for an internal standard. In addition, this method utilized recently introduced HPLC monolithic column technology, which provided sufficient selectivity and sensitivity for measurement of these components. The method was employed without significant methodological problems in the evaluation of plasma samples obtained from healthy volunteers and hospital emergency room patients presenting with chest pain and potential acute myocardial ischemia.

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