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Simultaneous detection of nucleotides, nucleosides and oxidative metabolites in myocardial biopsies

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Abstract

A new simple, simultaneous matrix HPLC methodology was developed to facilitate better peak separability and resolution for the determination of levels of myocardial tissue nucleotides, nucleosides and oxidative metabolites. The components of interests were ATP, AMP, ADP, IMP, hypoxanthine, xanthine, adenosine, inosine, NAD, and NADH, which are used to establish myocardial cellular energy status and effectiveness of cardioprotection. Their detection was achieved using a 4-μm spherical bead, 300 × 3.9 mm I.D. Nova-Pak C_{18} column in a 12% methanol mobile phase solvent selection, ion-pairing reagents 1.47 m*M* TBAP (tetrabutylammonium phosphate) and 73.5 m*M* KH_2PO_4, at a pH of 4.0. The extraction method was modified for rapid determination to ensure diminished acid labile NADH effects. Comparisons of peak retention (k), resolution (R_s) of solvents of varying concentrations and pH adjustment facilitated this method. This isocratic single run determination allows for simple, simultaneous rapid quantification and identification of alterations in high-energy phosphates, nucleoside degradation products and NAD/NADH levels associated with myocardial ischemia, with excellent reliability.

Keywords: Nucleotides; Nucleosides

1. Introduction

Myocardial ischemia, either induced at the time of cardiac surgery or from coronary occlusion, results in myocytes becoming adenosine triphosphate (ATP) depleted. Intracellular ATP is essential for myocardial contraction and relaxation and ATP depletion is an important factor contributing to the incomplete recovery of ventricular function following ischemia and reperfusion. However, absolute ATP content does not predict or correlate with myocardial recovery, because the decrease in ATP during ischemia is not accompanied by a rise in adenosine-diphosphate (ADP), as ADP is quickly transformed to adenosine-monophosphate (AMP) and inosine-monophosphate (IMP). The dephosphorylation of AMP and IMP by 5′-nucleotidase results in the formation of adenosine which is deaminated to inosine by adenosine deaminase. Since these resultant nucleosides, adenosine and inosine and their degradative components, hypoxanthine and xanthine, pass through the myocyte membrane, they enter the interstitial space and are washed out during reflow and rendered unavailable for the nucleotide salvage pathway.

Previous studies have demonstrated that total

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nucleotide precursor availability in the peri-myocyte cellular milieu, during and immediately after ischemia is an important determinant and predictor of post-ischemic myocardial recovery. Additionally, the status of the oxidative metabolites, nicotinic adenine dinucleotide (β-NAD) and its reduced form NADH, as an electron-accepting system are also known to reflect myocardial energy potential. These compounds are therefore important indicators of myocardial ischemic tolerance. Their measurements in ischemic myocardial tissue are essential research and clinical tools to evaluate the biochemical mechanism of ischemic myocyte cellular injury and degradation of myocardial function. Attempts have been made to simply and easily measure and quantify these metabolites. However, these previous methods have been cumbersome, requiring multiple runs, or unreliable with coelution problems as single runs. This report describes a method of quantification utilizing a higher resolution column, the Nova-Pak C_{18}, for single run isocratic peak determinations by methanol mobile phase separation of nucleotides, nucleosides and oxidative metabolites [1–7], to easily capture information about myocardial metabolic processes of salvage and synthesis of high energy compounds and overall energy status during cardiac ischemia.

2. Experimental

2.1. Reagents and standards

Trichloroacetic acid (TCA), pairing ion-reagent tetrabutylammonium phosphate (TBAP), tri-*n*-octylamine and standards were obtained from Sigma (St. Louis, MO, USA). Potassium phosphate monobasic HPLC grade was purchased from Fisher Scientific (Pittsburgh, PA, USA). Freon was purchased from Matheson Richards (Jolient, IL, USA).

2.2. Column description

A Nova-Pak C_{18} 4-μm spherical radii bead, dimension 300 × 3.9 mm I.D. (part No. 11695) was used. To eliminate particulate contamination a RCSS Guard-PAK™ precolumn was placed before each column.

2.3. Instrumentation

A Waters 484 tunable absorbance detector was set to 254 nm, the standard proximity wavelength of maximum absorbance for the nucleotides and nucleosides of interest. Sample automation was programmed using a Waters 712 WISP injector. A Waters 510 two pump system allowed flexibility for acetonitrile–methanol buffer concentration adjustments for best peak separation. Pump control, chromatogram data acquisition and conversion of analog to digital signal were accomplished by connecting a watchdog board from the PUMP/WISP to NEC PowerMate 1 Personal Computer. Peak external standard area under the curve comparisons were performed by MAXIMA 820 software package.

2.4. Chromatographic conditions

A mobile phase consisting of 12% methanol, ion-pairing reagent 1.47 m*M* TBAP(tetrabutylammonium phosphate), 73.5 m*M* KH_2PO_4, adjusted to pH 4.0 by titration with phosphoric acid, was prepared using deionized organic adsorption ultrapure water. Column pressures were a constant 24.82 MPa. Temperatures were held constant in overnight runs at 23.5–24.0°C. Pump flow-rates were 1.0 ml/min during equilibration and during automated isocratic acquisition.

2.5. Sample separation

Heart specimens were rapidly frozen in liquid N_2, lyophilized for 24–48 h (VirTis Freezemobile 6, Virtis Company, Gardiner, NY, USA), 10–20 mg duplicate left ventricle sections of dry tissue were weighed into a glass tissue grinder and 800 μl of 0.73 mol/l TCA was added. The solution was then homogenized for 15 s; vortex-mixed briefly and poured into 1.5-ml Eppendorf tubes, and immediately placed in a minature centrifuge (Tomy Seiko, Tokyo, Japan). Samples were centrifuged for 10 s. Supernatant (400 μl) was removed and added to an Eppendorf tube containing equal volumes of tri-*n*-octylamine and Freon (50:50, v/v) then vortex-mixed for 15 s and centrifuged for 10 s. Limited volume inserts were frozen at −70°C for later batch

injection into the HPLC system via the programable WISP.

3. Results and discussion

The purpose of this method was two-fold: (1) to establish a separation of major components of high-energy myocardial metabolism, that is, nucleotides (ATP, AMP, ADP, IMP) and degradative components, nucleosides, under isocratic conditions, and (2) to simultaneously identify and quantify NAD and NADH at the same corresponding wavelength by similar extraction preparation methods.

When examining the effects of myocardial substrate utilization, a direct measurement of nucleotides and nucleosides can indicate whether optimal myocyte preservation has been accomplished. For example, during induced cold ischemia for cardiac surgery, increasing glucose concentration is not felt to enhance myocardial protection and may detrimentally affect recovery. However, using warm arrest, increased glucose availability as substrate could enhance post-ischemic metabolic and functional recovery; however, profound hyperglycemia may increase patient susceptibility to neurologic injury. Therefore, to study an optimal dose response effect of glucose upon functional recovery following warm arrest, 59 isolated retrograde perfused rabbit hearts received multidose cardioplegia containing increasing glucose, and underwent 120 min of warm 34°C global ischemia. Experimental functional results are shown in Table 1. Additionally, to further understand the metabolic mechanism of this pattern of functional recovery, 20 hearts receiving 0 or 22 m*M* glucose (best recovery) had ATP, total nucleotides (ATP, ADP, AMP, IMP) and nucleosides (adenosine, inosine, hypoxanthine, xanthine) measured by this new method of HPLC. Pre-ischemic values were equal, but 15 min after reflow, ATP was 8 ± 1 μmol/g vs. 15 ± 2 μmol/g, total nucleotides were 23 ± 3 μmol/g vs. 50 ± 4 μmol/gm and total nucleosides were 8 ± 2 μmol/g vs. 15 ± 2 μmol/g in 0 vs. 22 m*M* glucose groups, respectively, all significantly enhanced. We concluded that moderate glucose doses resulted in the best functional and metabolic recovery. Next, we compared hearts preserved at lower temperatures with and without glucose. These low temperature experiments indicated no difference in myocardial energy status; adenylate energy charge or ATP levels for hearts subjected to a cold (4°C) 2-h global ischemic period with or without the presence of glucose. The complete HPLC quantification of these identifiable constituents in similar isolated perfused rabbit hearts is shown in Table 2. These biochemical results, as shown by this new HPLC method, correlated well with the equivalent functional recovery noted, as demonstrated by left ventricular developed pressure. From this information, decisions can be made about the effectiveness of glucose substrate utilization at different cardiac storage temperatures.

The retention times (min) and coefficient of variation (C.V.) (%) observed for each compound are hypoxanthine (3.12 ± 0.01, 0.45), xanthine (3.31 ± 0.01, 0.32, adenosine (3.90 ± 0.01, 0.16), NAD (4.14 ± 0.01, 0.18), IMP (4.60 ± 0.03, 0.57), AMP (5.13 ± 0.01, 0.18), inosine (5.77 ± 0.01, 0.20),

Table 1
Glucose functional results at 34°C

Glucose (m*M*)	*n*	*DP* (%)	−d*P*/d*t* (%)	CF (%)	MVO2 (%)
0	10	45 ± 3	45 ± 3	67 ± 4	66 ± 4
1.4	8	51 ± 8	54 ± 8	71 ± 5	55 ± 9
5.5	8	67 ± 2[a]	74 ± 2[a]	68 ± 3	68 ± 4
11	7	68 ± 3[a]	77 ± 4[a]	65 ± 2	71 ± 3
22	10	74 ± 3[a]	86 ± 5[a]	69 ± 5	73 ± 5
44	8	71 ± 4[a]	79 ± 5[a]	67 ± 5	68 ± 4
88	8	66 ± 2	75 ± 3[a]	61 ± 4	65 ± 3

Developed pressure (*DP*), d*P*/d*t*, coronary flow (CF) and myocardial oxygen consumption (MVO2) were compared as a percentage of pre-ischemia vs. 45 min after reflow (mean ± S.E.M.).
[a] = $p<0.05$ vs. 0 m*M* glucose.

Table 2
Glucose metabolic and functional results at 4°C

Constituent	Concentration (μmol/g dry tissue)	
	0 m*M* (64 ± 22% *DP*)	22 m*M* glucose (61 ± 6% *DP*)
ATP	7.43 ± 2.73	8.30 ± 0.68
AMP	0.45 ± 0.15	0.49 ± 0.13
ADP	3.54 ± 0.82	3.69 ± 0.56
IMP	0.03 ± 0.02	0.03 ± 0.02
TNN[a]	11.44 ± 3.54	12.49 ± 0.75
Hypoxanthine	0.09 ± 0.04	0.07 ± 0.01
Xanthine	0.65 ± 0.31	0.58 ± 0.13
Adenosine	0.15 ± 0.03	0.17 ± 0.05
Inosine	0.19 ± 0.16	0.21 ± 0.14
TDN[b]	1.11 ± 0.43	1.03 ± 0.13
NAD	2.69 ± 0.22	2.66 ± 0.37
NADH	0.38 ± 0.19	0.56 ± 0.10
NAD/NADH ratio	7.58 ± 2.61	5.04 ± 1.24
AEC[c]	0.799 ± 0.031	0.814 ± 0.023

Values are mean ± S.D. for sixteen rabbit hearts at 15 min reperfusion following 120 min of cold cardioplegic ischemia with and without 22 m*M* glucose supplementation. % left ventricular developed pressure = 1% recovery of left ventricular function as compared to prior to ischemia. No statistical differences exist by ANOVA Scheffe-*F* test in nucleotide, nucleoside, NAD or NADH content.

[a] TNN = total non-diffusible nucleotides (ATP + AMP + ADP + IMP).

[b] TDN = total diffusible nucleotides (hypoxanthine+xanthine+adenosine+inosine).

[c] AEC = adenylate energy charge = (ATP + 0.5·ADP)/(ATP + ADP + AMP).

ADP (7.67 ± 0.03, 0.44), ATP (16.48 ± 0.06, 0.34) and NADH (18.86 ± 0.24, 1.30), respectively. Results are mean ± S.D. for eight hearts. Stability of sample extracts was less than 0.05% C.V. for à 24-h room temperature storage (including one freeze-thaw cycle) carriage sample run, except for xanthine at 0.09%. Linearity of standards, r^2, was as follows; hypoxanthine (0.962), xanthine (0.965), adenosine (0.960), NAD (0.991), IMP (0.977), AMP (0.966), inosine (0.977), ADP (0.966) ATP (0.988) and NADH (0.951). An important distinct advantage in the separation of components by isocratic methods is the absence of baseline drift during the chromatographic run. Although internal standard ratio assessments are the best evaluation of peak purity, a serial dilution of known external standard peak integration is then compared to unknowns for quantification of products. Additionally, although it is thought that rapid NADH conversion to NAD occurs ex vivo, the effective changes can be minimized by rapid neutralization and immediate injection upon thawing. Furthermore, increases in tissue concentration may be utilized in order to detect NADH levels below 1 μmol/g dry tissue. Finally, in low concentrations NADH, stability increases as pH increases. Stability and non-decay of NADH at 23°C has been measured at 2 h with a pH of 4–5 [8], therefore we utilized a final pH of 5.4 for the injection neutralized solution. Consequently, an excellent maximum coefficient of variation of only 1.3% was observed for NADH retention times and NAD levels were quite comparable to other reported standard values [9]. The findings in this series of experiments, using this new and simple technique, are of clinical relevance in terms of investigating the best temperatures for cardiac storage and optimal cardiac preservation solutions. Rapid and simple detection of high energy phosphates, their degradation products and oxidative metabolites provide a simple investigative means by which biochemical mechanisms can be elucidated.

A further example of the usefulness of this new HPLC method for evaluating cardioprotection is in the area of preconditioning. Preconditioning is thought to be a series of complex adaptive changes induced in myocardium by brief episodes of reversible ischemia followed by reperfusion that augments later ischemic tolerance. There is evidence that preconditioning is induced by multiple signaling pathways, including adenosine a1 receptors and adrenergic receptors, which have a final common pathway G-protein modulation of protein kinase C induction and translocation. Recently, the pharmacologic induction of preconditioning, via protein kinase C (PKC) translocation, has been achieved with direct PKC agonists of the diacylglycerol family. However, whether functional improvement or nucleotide status following myocardial stunning is altered by preconditioning with PKC agonists remains controversial. This present study undertook to test if pretreatment with the specific PKC agonist 1,2-*sn*-dioctanoylglycerol could improve myocardial function following global ischemia. To study this hypothesis, isolated rabbit hearts, maintained at 34°C, received

Table 3
Preconditioning functional results

	n	DP (%)	+dP/dt (%)	−dP/dt (%)	CF (%)	MVO2 (%)
CTL	10	38 ± 3	31 ± 5	39 ± 3	67 ± 4	66 ± 4
DMSO	5	36 ± 9	35 ± 7	46 ± 8	85 ± 7	86 ± 7
DOG	5	58 ± 2[a]	55 ± 5[a]	67 ± 5[a]	73 ± 6	79 ± 9

Isolated rabbit hearts, maintained at 34°C, received either standard cardioplegia (CTL) or had 1,2-*sn*-dioctanoylglycerol (DOG) or its vehicle DMSO, administered continuously for 15 min at 100 mmol, 25 min prior to ischemia. Hearts were then subjected to 120 min of global ischemia. Functional recovery was compared as in Table 1.
[a] $p<0.05$ vs. CTL.

either standard cardioplegia alone or had 1,2-*sn*-dioctanoylglycerol or its vehicle dimethyl sulphoxide (DMSO), administered continuously for 15 min at 100 mmol, 25 min prior to ischemia. Hearts were then subjected to 120 min of global ischemia. Functional recovery was compared as above and is shown in Table 3. Shown in Table 4 are the HPLC results of isolated rabbit hearts subjected to this preconditioning drug treatment. One can see the direct changes in high energy phosphates available and adenylate energy charge when hearts were subjected to ischemia. However, it can be noted that preconditioning treatment with 1,2-dioctanoyl-*sn*-glycerol did not alter or degrade metabolites and one can assume cellular energetics were not enhanced on a nucleotide basis. In a chromatogram from this series of experiments, shown in Fig. 1, following ischemia and reperfusion, all compounds of interest were identified, except for IMP (level below detectable range <0.01 μmol/g dry tissue). This new method allowed crowding to be fully distinguished when observed in detail. Alternatively, an end of ischemia biopsy of myocardial tissue, prior to reperfusion, shown in Fig. 2, depicts the condition of degradative compound accumulation. The rise in adenosine and the fall in high energy content is typical of this period of myocardial ischemia. Upon reperfusion the adenosine is washed out of the cells or taken up in the purine salvage pathway.

The use of HPLC for the determination of nucleotides, nucleosides, and oxidative metabolites in bio-

Table 4
Preconditioning biochemical results

Constituent	Concentration (μmol/g dry tissue)		
	Baseline	Post DOG	Reflow
ATP	9.64 ± 0.61	18.60 ± 1.31	4.40 ± 2.61
AMP	0.27 ± 0.11	0.66 ± 0.13	0.36 ± 0.15
ADP	3.75 ± 0.41	4.15 ± 0.45	2.19 ± 0.40
TNN	23.67 ± 0.96	23.66 ± 0.86	6.95 ± 2.40
TDN	0.53 ± 0.14	0.56 ± 0.12	0.59 ± 0.35
AEC	0.907 ± 0.012	0.887 ± 0.015	0.770 ± 0.096

Biopsy results in twelve hearts subjected to a 10-min infusion of "preconditioning" analogue, 1,2-dioctanoyl-*sn*-glycerol, as pretreatment before ischemia and reperfusion. Results are in mean ± S.D. TNN = total non-diffusible nucleotides (ATP + AMP + ADP + IMP), TDN = total diffusible nucleosides (hypoxanthine+xanthine+adenosine+inosine). AEC=adenylate energy charge = (ATP + 0.5·ADP)/(ATP + ADP + AMP).

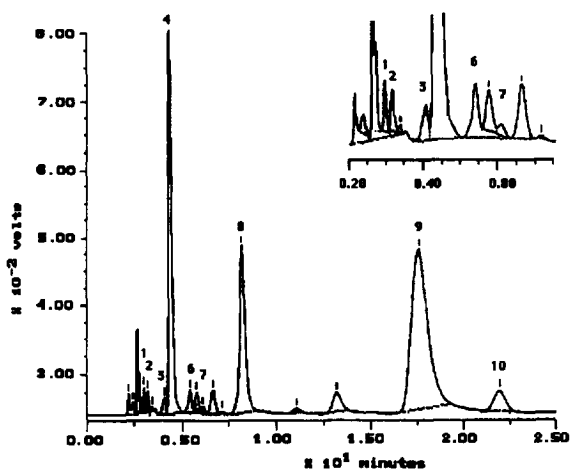

Fig. 1. Langendorff preparation of rabbit left ventricle was biopsied at 15 min reperfusion following 2 h of global ischemia (34°C, St. Thomas cardioplegic arrest). Prior to ischemia, heart was given a 10-min infusion of DAG analogue, 1,2-dioctanoyl-*sn*-glycerol (DOG), as pretreatment against ischemic damage. Reduction of high energy phosphates from baseline were measurable as noted in Table 2. Flow-rate was 1.0 ml/min using 4-μm Nova-Pak C_{18}. Precision of peak detection of early eluters is depicted in inlay. Peaks: 1 = hypoxanthine; 2 = xanthine; 3 = adenosine; 4 = β-NAD; 6 = AMP; 7 = inosine; 8 = ADP; 9 = ATP; 10 = β-NADH.

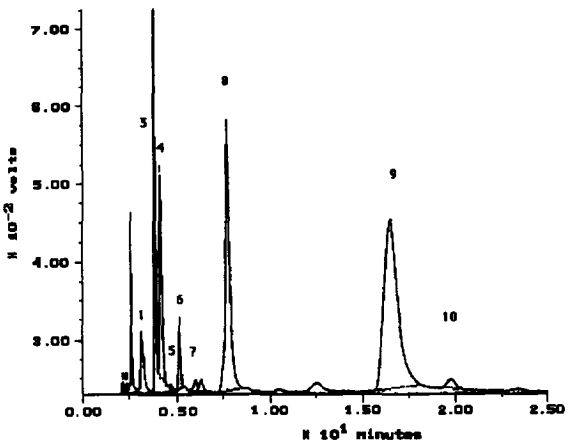

Fig. 2. Separation by ion-pair reversed-phase HPLC of in vivo canine left ventricular biopsy at the end of 110 min of ischemia during myocardial preservation with perfluorocarbon. Biopsy was immediately taken prior to aortic cross release and indicates accumulation of adenosine (peak 3). Detection was on a Nova-Pak C_{18} column at 254 nm, absorbance units full scale = 0.005. Peaks: 1 = hypoxanthine; 3 = adenosine; 4 = β-NAD; 5 = inosine mono-phosphate; 6 = AMP; 7 = inosine; 8 = ADP; 9 = ATP; 10 = β-NADH.

logical samples has been performed for more than 20 years. Methods have included reversed-phase mode, ion-exchange mode and ion-pair reversed-phase techniques for their determination. Each of these methods has advantages and limitations for myocardial sampling [10–13]. Other techniques such as high-performance capillary electrophoresis [14] and liquid chromatography–mass spectrometry [15] are now also being applied to biologic systems. However, we have described a new, simple ion-pair isocratic HPLC method for rapid determination of nucleotide and nucleosides, simultaneously with oxidative metabolites (NAD and NADH). The distinct advantages of this method are (1) the ability to perform all separations with one mobile phase, (2) the lack of baseline drift as observed in gradient elutions, (3) the absence of the prolonged equilibration time that is necessary in gradient elutions, and (4) adjustments in wavelength of detection are not requisite for component detection and quantifications. Analysis of experimental tissue from hearts demonstrates usefulness of this single-channel, one wavelength, isocratic method. This technique can further simplify and automate the detection of the metabolites involved in myocardial ischemia, for elucidation of mechanisms in cardiopreservation.

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