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Note

Improved method for high energy nucleotide analysis of canine cardiac muscle using reversed-phase high-performance liquid chromatography

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High-energy nucleotide analysis is gaining increased use in laboratories investigating the biochemical manifestations of cardiac ischemia. Many investigators have utilized the depletion of high-energy nucleotides and subsequent accumulation of degradation products associated with ischemia to study changes in cardiac cell metabolism, structure and function [1, 2]. High-performance liquid chromatography (HPLC) enables a sensitive separation of these compounds based on their stoichiometric differences. Unfortunately, the heterogeneity of high-energy nucleotides and their metabolites ordinarily makes their separation in a single isocratic assay difficult and time consuming. The methodology described in the present report provides excellent resolution of high-energy nucleotides and their major degradative products from totally ischemic canine cardiac muscle with minimal assay time using isocratic elution with a very economical buffer system.

MATERIALS AND METHODS

Chromatographic equipment

Sample application and solvent delivery were accomplished by a variable-volume injection system (Model U6K, Waters Assoc., Milford, MA, U.S.A.) in conjunction with a reciprocating pump (Model 6000A, Waters Assoc.). Each sample was manually injected via a 25-μl Hamilton syringe (Hamilton, Reno, NV, U.S.A.). The separation system consisted of an untreated Radial Pak-A reversed-phase column (C_{18}, 10 μm, 8 mm I.D., Waters Assoc.) and a radial compression module (Model RCM 100, Waters Assoc.) operated at 175 bar. A double-beam UV detector (Model 441, Waters Assoc.) monitored the absorbance of the eluents at 254 nm. A data module (Model 730, Waters

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Assoc.) integrated the detected response, providing peak areas and retention times for each sample.

Buffer preparation

An amount of 11.5 g of HPLC grade ammonium dihydrogen phosphate (Fisher Scientific, Fair Lawn, NJ, U.S.A.) weighed on a Cahn TA-450 Balance (Cahn Instruments, Cerritos, CA, U.S.A.) was brought up to 1 l using ultrapure, reagent-grade water (Milli-Q, Millipore, Bedford, MA, U.S.A.). The pH was adjusted to 5.5 (Accument Model 750 selective ion analyzer, Fisher Scientific) with 3 *N* ammonium hydroxide (A.C.S. grade, Mallinckrodt, Paris, KY, U.S.A.) diluted with ultrapure water. The buffer was filtered and degassed using a Millipore Solvent Clarification Kit with a 0.45-μm aqueous filter (Type HA, Millipore). Fresh buffer solution was prepared prior to each group of sample runs.

Sample extraction and preparation

Canine cardiac tissue was divided into subendocardial, midmyocardial and subepicardial slices weighing between 75 and 150 mg. Reliable results, however, have been obtained with samples as small as 25 mg in subsequent studies. Each tissue slice was weighed quickly on a Cahn Model DTL microbalance and placed in 1.5 ml of 3.6% perchloric acid (70%, ACS, Fisher Scientific) at 0.5°C and then immediately homogenized using a Tri-R stirrer. Weighing and transfer to the perchloric acid required 10—15 sec. Following homogenization, tissue was allowed to extract for 30 min at 0.5°C, followed by centrifugation at 850 *g* for 20 min at 0.5°C. The supernatant was neutralized with potassium carbonate—potassium hydroxide to a pH of 5.0—6.0 and frozen until analysis.

Standard preparation

Standards for ATP, ADP, AMP, inosine, hypoxanthine and xanthine were prepared by dissolving high-quality pure standards (Sigma, St. Louis, MO, U.S.A.) in ultrapure water to approximate concentrations. The exact concentration was then determined using a double beam spectrophotometer (Model 250, Gilford Instruments, Oberlin, NJ, U.S.A.).

Column equilibration and rejuvenation

Before assays were performed, the column was flushed at least ten times with 100% methanol, followed by a thorough flushing with 2% methanol in ultrapure water. The column was allowed to equilibrate with ammonium phosphate buffer until response factors, as determined by the Data Module 730, were repeatable.

After sample runs were terminated, the column was flushed with 2% methanol in ultrapure water to prevent build-up of ammonium salts and then flushed with 100% methanol to wash out any remaining organic compounds and to inhibit bacterial growth.

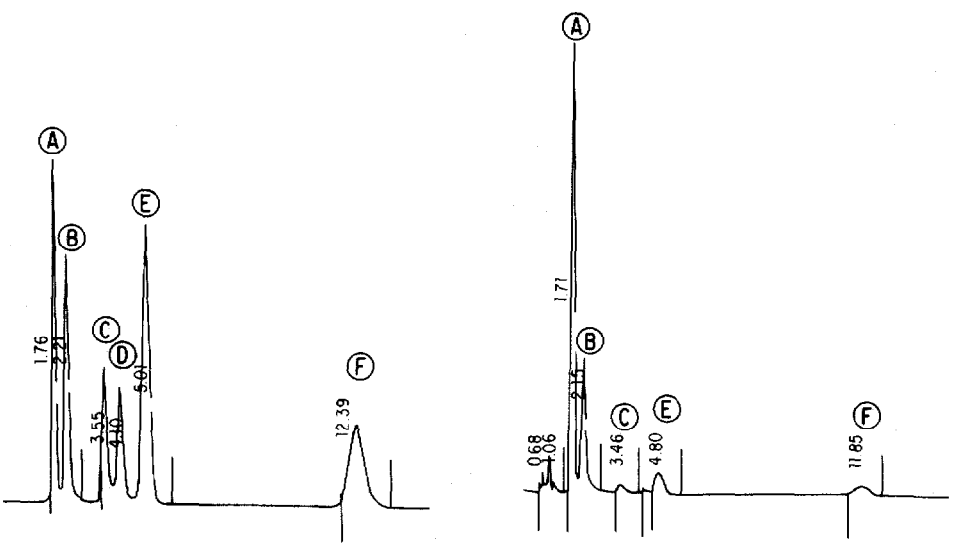

Fig. 1. Chromatogram showing the resolution of standards: A, adenosine 5′-triphosphate (ATP); B, adenosine 5′-diphosphate (ADP); C, hypoxanthine (HYP); D, xanthine (XAN); E, adenosine 5′-monophosphate (AMP); F, inosine (INO). Radial Pak-A column (C_{18}, 10 μm, 8 mm I.D.), ammonium dihydrogen phosphate buffer (0.1 *M*, pH 5.5, ambient temperature), flow-rate 4 ml/min.

Fig. 2. Control canine cardiac tissue. Peaks and chromatographic conditions as given in Fig. 1.

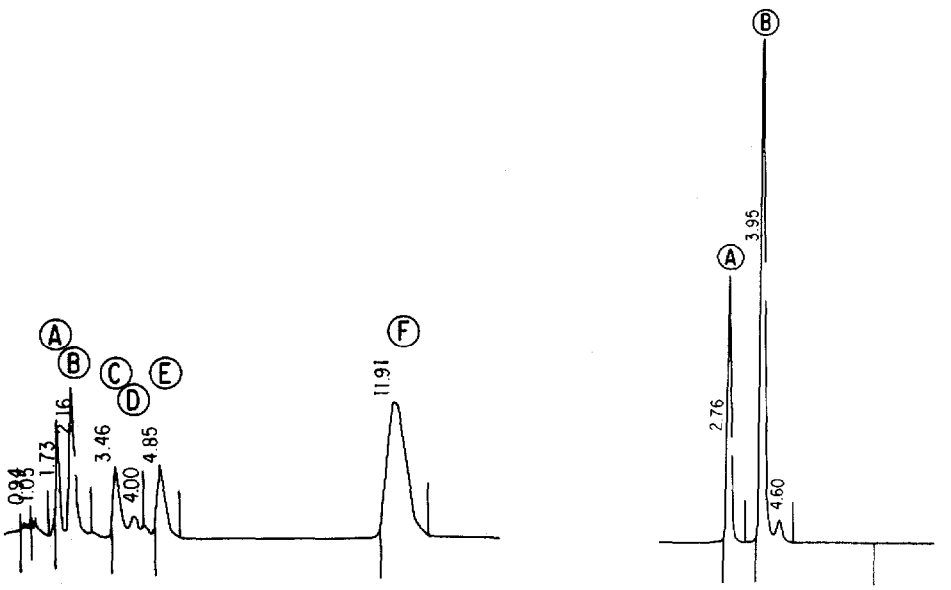

Fig. 3. Canine cardiac tissue after 60 min of total ischemia. Peaks and chromatographic conditions as given in Fig. 1.

Fig. 4. Chromatogram for standards A, phosphocreatine (CP); and B, creatine (C). Flow-rate, 1 ml/min; otherwise chromatographic conditions as given in Fig. 1.

RESULTS

Quantitation

Calibration chromatograms for the standards ATP, ADP, AMP, inosine (INO), hypoxanthine (HYP), and xanthine (XAN) were generated by injecting 20 μl of a standard mixture of known concentration determined as previously described. Fig. 1 is a representative chromatogram showing good resolution of the standards when collectively assayed.

Likewise, frozen tissue samples were thawed, centrifuged at 4000 *g* for 30 min and then 20 μl of the supernatant were injected. Figs. 2 and 3 are chromatograms of canine cardiac tissue obtained at the control period and after 60 min of total in vitro ischemia, respectively, illustrating the metabolic degradation of high-energy phosphates and the subsequent accumulation of metabolites.

TABLE I

SUBENDOCARDIAL, MIDMYOCARDIAL AND SUBEPICARDIAL HIGH-ENERGY NUCLEOTIDE DEPLETION AND DEGRADATIVE METABOLITE ACCUMULATION OVER TIME DURING TOTAL ISCHEMIA IN VITRO, 37°C (n = 10)

Values are given in μmol/g wet weight.

Compound		Control (3—5 min)			15 min			30 min	
		Sub-endo	Mid	Sub-epi	Sub-endo	Mid	Sub-epi	Sub-endo	Mid
ATP	Mean	5.517	5.438	5.458	3.927	4.140	4.271	2.764	3.134
	S.D.	1.018	1.029	0.976	0.570	0.698	0.819	0.447	0.636
	S.E.	0.339	0.343	0.325	0.180	0.221	0.259	0.141	0.201
ADP	Mean	1.282	1.432	1.424	1.136	1.116	1.273	0.986	1.038
	S.D.	0.204	0.232	0.234	0.193	0.174	0.188	0.153	0.161
	S.E.	0.068	0.077	0.078	0.061	0.055	0.059	0.048	0.051
AMP	Mean	0.195	0.239	0.214	0.160	0.144	0.180	0.183	0.192
	S.D.	0.092	0.105	0.121	0.053	0.049	0.069	0.075	0.090
	S.E.	0.029	0.033	0.038	0.017	0.016	0.022	0.024	0.028
HYP	Mean	0.145	0.150	0.124	0.298	0.321	0.279	0.571	0.507
	S.D.	0.025	0.035	0.037	0.096	0.090	0.073	0.160	0.124
	S.E.	0.014	0.020	0.021	0.055	0.052	0.042	0.092	0.071
XAN	Mean	—	—	—	0.115	0.097	0.088	0.123	0.118
	S.D.	—	—	—	0.021	0.030	0.047	0.065	0.049
	S.E.	—	—	—	0.012	0.017	0.027	0.037	0.029
INO	Mean	0.248	0.353	0.270	1.572	1.437	1.125	2.566	2.384
	S.D.	0.127	0.113	0.165	0.646	0.535	0.566	0.725	0.608
	S.E.	0.048	0.043	0.062	0.244	0.202	0.214	0.274	0.230
ΣAd		6.994	7.109	7.096	5.223	5.400	5.724	3.933	4.364
Σ {NS+B}—{ADO}		0.393	0.503	0.394	1.985	1.855	1.492	3.260	3.009
Total		7.387	7.612	7.490	7.208	7.255	7.216	7.193	7.373

Phosphocreatine (CP) and creatine (C), additional indicators of cardiac muscle energy stores, can also be quantitated using this buffer system and a wavelength of 214 nm. Fig. 4 is a calibration chromatogram for the standards CP and C.

Applications

The described method was used in a study to determine whether a transmural progression of metabolic changes occurs during in vitro total ischemia. Following rapid excision of a canine heart, subendo-, midmyo-, and subepicardial samples were obtained at control (3—5 min following excision) and 15-min intervals for determination of high-energy nucleotides and their major degradative metabolites. Data obtained from ten dogs are summarized in Table I and show a progressive depletion of nucleotides with a consequent

	45 min			60 min			Rigor initiation		
Sub-epi	Sub-endo	Mid	Sub-epi	Sub-endo	Mid	Sub-epi	Sub-endo	Mid	Sub-epi
3.218	1.923	2.400	2.620	1.492	1.673	1.862	0.950	1.234	1.487
0.364	0.472	0.361	0.452	0.386	0.629	0.529	0.424	0.542	0.657
0.115	0.149	0.114	0.143	0.173	0.281	0.236	0.134	0.171	0.208
1.056	0.937	0.960	1.081	0.810	0.862	1.000	0.793	0.798	0.947
0.138	0.144	0.161	0.244	0.170	0.197	0.219	0.211	0.188	0.190
0.043	0.046	0.051	0.077	0.076	0.088	0.098	0.067	0.059	0.060
0.216	0.217	0.157	0.217	0.224	0.333	0.356	0.383	0.252	0.288
0.157	0.104	0.054	0.102	0.073	0.158	0.165	0.218	0.105	0.148
0.050	0.033	0.017	0.032	0.033	0.071	0.074	0.069	0.033	0.047
0.449	0.777	0.670	0.538	0.820	1.021	0.929	1.194	1.028	0.790
0.088	0.181	0.194	0.178	0.359	0.470	0.465	0.280	0.325	0.147
0.051	0.105	0.112	0.103	0.254	0.332	0.329	0.162	0.188	0.085
0.143	0.136	0.099	0.111	0.297	0.238	0.122	0.243	0.161	0.176
0.050	0.057	0.040	0.031	0.246	0.048	0.069	0.048	0.052	0.042
0.029	0.033	0.023	0.018	0.174	0.034	0.049	0.028	0.030	0.024
1.903	3.281	3.032	2.857	3.283	3.362	2.990	4.095	3.806	3.401
0.520	0.419	0.701	0.396	1.333	0.752	0.567	0.329	0.355	0.645
0.197	0.158	0.265	0.150	0.770	0.434	0.328	0.116	0.125	0.228
4.490	3.077	3.517	3.918	2.526	2.868	3.218	2.126	2.284	2.722
2.495	4.194	3.801	3.506	4.400	4.621	4.041	5.532	4.995	4.367
6.985	7.271	7.318	7.424	6.926	7.489	7.259	7.658	7.279	7.089

accumulation of metabolites during the course of in vitro total ischemia. The results show a statistically significant gradient of ATP depletion, with the subendocardium consistently showing accelerated energy utilization compared to the subepicardium ($P < 0.05$). Ultrastructural evidence of irreversible injury first appeared in the subendocardium at the onset of ischemic contracture and occurred when ATP levels declined to less than 1 μmol/g wet weight. In summary, these data show that during total ischemia in vitro, cell death begins in the subendocardium at the onset of ischemic contracture and progresses towards the subepicardium over time. These changes occurred independent of variations in collateral flow or wall tension [3].

DISCUSSION

Analysis of high-energy nucleotides and their major metabolites is essential for assessing the ability of chemical and mechanical interventions to delay or reverse myocardial ischemic cell injury and for delineating the pathogenesis of ischemic cell death. Precise collective analyses of these compounds can be accomplished using reversed-phase HPLC [4—6].

The myocardial adenine nucleotide content (ΣAd), the sum of ATP, ADP, and AMP, and the nucleoside and base content (ΣNS + B), the sum of adenosine (ADO), INO, HYP, and XAN, represent the major nucleotides and their catabolic byproducts in myocardial ischemia. During ischemia, the fall in ΣAd is accounted for primarily by the marked accumulation of ΣNS + B while the total adenine pool remains essentially unchanged (Table I). The apparent recovery of more than 100% of the initial pool may be partially attributed to the catabolism of guanidine, uridine and cytidine, and late in the ischemic time course, to NAD [7]. However, in totally ischemic cardiac tissue, the contribution of ADO to the adenine pool is very slight. ADO, which is produced by the dephosphorylation of AMP by 5′-nucleotidase in the sarcolemma, can easily diffuse across the cellular membrane and is rapidly deaminated to INO by the large quantities of adenosine deaminase present in the myocardium [8]. We elected not to quantify ADO chromatographically, eliminating the need for gradient elutions and thereby reducing the equilibration delay between sample runs.

The compounds CP and C are of interest occasionally, and as noted, can readily be separated and quantified using a wavelength of 214 nm, although 80% of CP is normally lost in less than 1 min of severe ischemia [7].

These results show that ammonium dihydrogen phosphate buffer in conjunction with a radial compression system performs excellent separation of adenine high-energy nucleotides and their major metabolites. Ammonium phosphate has the advantages of being inexpensive and easy to prepare, with low viscosity, high surface tension, a low UV cutoff point and a large buffering capacity. Given the volume of work inherent in metabolic studies, this method offers efficient, precise and economical determinations.

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