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SIMPLE STEP GRADIENT ELUTION OF THE MAJOR HIGH-ENERGY COMPOUNDS AND THEIR CATABOLITES IN CARDIAC MUSCLE USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The majority of high-energy nucleotides and their catabolites are separated in a single 13-min run using reversed-phase high-performance liquid chromatography. Economical step gradient elution of these compounds with a Nova Pak-A C_{18}, 5-μm, 10 cm × 8 mm column accomplishes this separation with recoveries of 94—100% and sensitivities of 1–5 pmol. Furthermore, the total adenine nucleotide pool can now be quantitated precisely in a simple and easily automated procedure.

INTRODUCTION

Adenine nucleotides play a critical role in the regulation and integration of cellular metabolism. Adenosine 5′-triphosphate (ATP) is the major carrier of chemical energy in all living species. During muscle contraction, this chemical energy is converted to mechanical energy through a series of reactions during which myosin-bound ATP is hydrolyzed, resulting in an altered orientation of actin and myosin and the formation of cross-bridges. The ubiquitous involvement of adenine nucleotides in the metabolism, active transport and mechanical work of myocardial cells makes their accurate measurement essential for investigating the biochemical, structural and functional manifestations of cardiac ischemia [1].

High-performance liquid chromatography (HPLC) provides a sensitive and efficient means for quantitating nucleotides. However, the heterogeneity of high-energy nucleotides and their degradation products ordinarily makes their

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simultaneous assays difficult and time-consuming. Previously, our laboratory developed an inexpensive isocratic elution system to rapidly and accurately quantitate adenine nucleotides and some of their catabolites [2]. The present paper describes a new methodology which has markedly increased our ability to identify and quantitate precisely the majority of high-energy nucleotides and their breakdown products present in cardiac tissue using a very economical step gradient buffer system. Accurate determinations of ATP, adenosine 5′-diphosphate (ADP), adenosine 5′-monophosphate (AMP), adenosine 3′,5′-cyclic monophosphate (cAMP) adenosine, guanosine 5′-triphosphate (GTP), inosine 5′-monophosphate (IMP), inosine, hypoxanthine, xanthine, urate, nicotinamide-adenine dinucleotide (NAD) and nicotinamide-adenine dinucleotide phosphate (NADP) can now be accomplished within a 13-min run using the technique described. Furthermore, the ability of the Nova Pak-A reversed-phase column to resolve IMP from ATP and ADP makes this methodology useful for the study of ischemic metabolism in a variety of tissues.

EXPERIMENTAL

Chromatographic equipment

Sample injection was accomplished by a Waters Intelligent sample processor (WISP, Model 710B, refrigerated, Waters Assoc., Milford, MA, U.S.A.). A reciprocating pump (Model 6000A, Waters Assoc.) in conjunction with a solvent selector (Model 101, Alltech Assoc., Deerfield, IL, U.S.A.) performed the step gradient solvent delivery. The chromatographic column was an untreated Nova Pak-A (C_{18}, 5 μm particle size, 10 cm × 8 mm, Waters Assoc.) operated in a radial compression module (Model RCM 100, Waters Assoc.) at 175 bar. A UV detector (Model 441, Waters Assoc.) monitored the absorbance of the eluents at 254 nm. A data module (Model 730, Waters Assoc.) integrated the detected response, providing peak areas and retention times for each sample. The solenoid switch of the solvent selector was controlled by the timed event functions of the data module.

Buffer preparation

A 100 m*M* solution 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 prepared using 1 l of ultrapure, reagent-grade water (Milli-Q, Millipore, Bedford, MA, U.S.A.). The pH was adjusted to 5.5 (Accumet selective ion analyzer, Model 750, Fisher Scientific) with 3 *M* ammonium hydroxide (ACS grade, Mallinckrodt, Paris, KY, U.S.A.) diluted with ultrapure water. This buffer formed the first "step" (1) of the solvent system.

Solvent 2, 6% (v/v) methanol in water, was made by mixing HPLC-grade methanol (Fisher Scientific) with ultrapure water. The third step (3) contained 40% (v/v) HPLC-grade methanol in ultrapure water. All solvents were carefully filtered and degassed using a Millipore solvent clarification kit with 0.45-μm disposable filters (Millipore). Fresh solutions were prepared prior to each group of sample analysis.

Standard preparation

Reference solutions of ATP, ADP, AMP, adenosine, inosine, hypoxanthine, xanthine and NAD were prepared by dissolving high-quality pure standards (Sigma, St. Louis, MO, U.S.A.) in ultrapure water to approximate concentrations. The exact concentration for each reference solution was then determined using a UV—visible double-beam spectrophotometer (Lambda 5, Perkin-Elmer, Norwalk, CT, U.S.A.) and peak identities were confirmed by absorbance ratioing.

Sample extraction and preparation

Canine cardiac tissue was divided into subendocardial and subepicardial slices weighing approx. 50 mg. Each tissue slice was quickly weighed on a Cahn Model DLT microbalance and placed in 1.0 ml of 3.6% perchloric acid (70%, ACS grade, Fisher Scientific) at 0.5°C and then immediately homogenized using a Tri-R stirrer with a precooled pestle (Model S63C, Tri-R Instruments, Rockville Centre, NY, U.S.A.). Samples weighing between 5 and 25 mg, such as from thin-needle biopsies, were assayed in the same manner with similar results. Weighing and transfer to the perchloric acid required 10–15 s. Following homogenation, tissue was allowed to extract for 30 min at 0.5°C, centrifuged at 850 *g* for 15 min at 0.5°C (Centra-7R, International Equipment Company, Needham Heights, MA, U.S.A.). The supernatant was neutralized with a potassium carbonate—potassium hydroxide solution to a pH of 6.0–6.5 and frozen at —70°C (So-Low Environmental Equipment, Cincinnati, OH, U.S.A.) until analysis.

Solute stability and extraction efficiency

Sample solutes stored in 1.5-ml, sealed, conical tubes (Sarstedt, Princeton, NJ, U.S.A.) at —70°C were reassayed one year after initial quantitation with no appreciable changes in the adenine nucleotide pool content. Extraction efficiencies, as determined by subjecting known amounts of reference solutions to the entire sample extraction and quantitation procedure, were as follows: ATP, 95%; ADP, 98%; AMP, inosine, hypoxanthine, 97%; adenosine, 100%; xanthine, 94%; and NAD, 96%.

The detection limits, defined as having a signal-to-noise ratio of 5, were 5 pmol for GTP, urate, IMP, inosine, AMP and adenosine and 1 pmol for ATP, ADP, AMP, hypoxanthine, xanthine, NADH and NAD.

Column equilibration and regeneration

Before assays were performed, the column was flushed with ten times the column volume of 100% methanol followed by thorough flushing with 2% methanol in ultrapure water. Afterwards, the column was allowed to equilibrate with ammonium phosphate buffer until response factors for successive analyses of the reference solution as determined by the data module Model 730, differed by less than 1%. This level of precision was generally obtained with the third reference analysis.

After a series of sample analyses were completed, the column was flushed with 2% methanol in water to prevent build-up of buffer salts, and then flushed and stored in a 50:50 mixture of methanol and acetonitrile (HPLC grade,

Fisher Scientific) to expel any organic compounds that might be retained on the column and inhibit bacterial growth.

Step gradient formation

Timing for the solvent switching device was determined by the following scheme. The separation was allowed to proceed in an isocratic fashion with 100 m*M* ammonium phosphate (Solvent 1) until ATP, ADP, hypoxanthine, xanthine and AMP were separated. At this point, Solvent 2 was introduced to the column, eluting NAD. Solvent 3 was introduced after the NAD to elute inosine and adenosine. Shortly after the appearance of adenosine, the column was re-equilibrated with Solvent 1 to achieve maximum resolution of ATP and ADP in the following analysis.

RESULTS

Quantitation

Calibration chromatograms for the standards ATP, ADP, AMP, adenosine, inosine, hypoxanthine, xanthine and NAD were generated by injecting 20 μl of a reference mixture of known concentration. Fig. 1 is a representative calibration chromatogram showing excellent resolution of the standards when collectively assayed. The appearance of each solvent front is indicated on the baseline below. Although not quantitated in the present application, cAMP, GTP, IMP, urate and NADP are often of interest and are added to the standard mixture in Fig. 2 to demonstrate their separation. Reduced nicotinamide-adenine dinucleotide (NADH) which has been subjected to the acid extraction

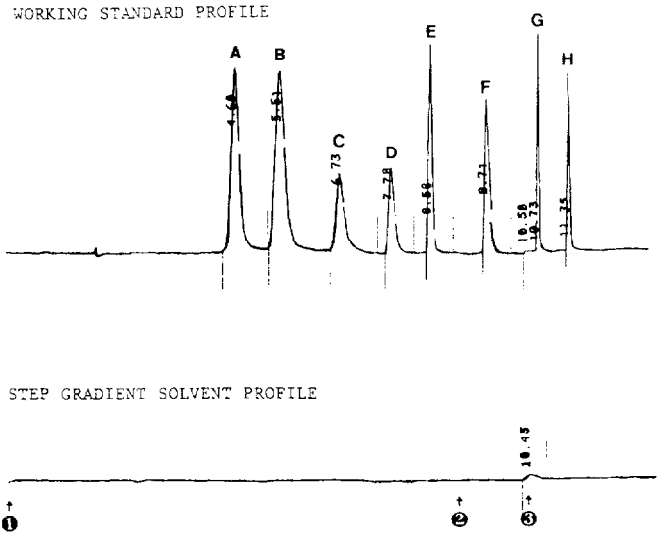

Fig. 1. Step gradient elution of the working standard mixture. Baseline: (1) 100 m*M* ammonium dihydrogen phosphate buffer, pH 5.5; (2) 6% methanol in water; (3) 40% methanol in water. Nova Pak-A column (C_{18}, 5 μm particle size, 10 cm × 8 mm), ambient temperature, flow-rate 1.5 ml/min; detector sensitivity 0.05 a.u.f.s. at 254 nm. Peaks: A = ATP; B = ADP; C = hypoxanthine; D = xanthine; E = AMP; F = NAD; G = inosine; H = adenosine.

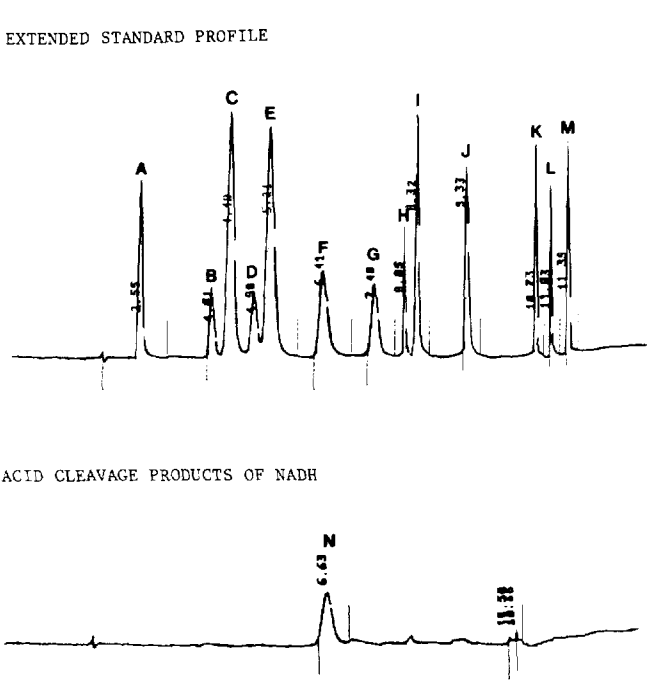

Fig. 2. Resolution of an extended standard mixture. NADH which has been subjected to the tissue extraction procedure is pictured below. Chromatographic conditions as given in Fig. 1. Peaks: A = GTP; B = urate; C = ATP; D = IMP; E = ADP; F = hypoxanthine; G = xanthine; H = NADP; I = AMP; I = NAD: K = inosine; L = cAMP; M = adenosine; N = APR.

procedure is shown below the expanded standard mixture in Fig. 2 to demonstrate the origin of adenosine 5′-diphosphoribose (APR), which often coelutes with hypoxanthine in acid extractions of tissue.

For tissue extract chromatograms, frozen samples were thawed, centrifuged at 13 000 *g* for 2 min (Model 235B, Fisher Scientific), and then 50 μl of the supernatant loaded into limited-volume inserts for automatic injection by the WISP. Injection volume was routinely 20 μl, and all runs were performed in duplicate at 1.5 ml/min. Figs. 3 and 4 are chromatograms of canine cardiac tissue obtained at the control period and after 60 min of ischemia following cardioplegic arrest, illustrating the ischemic degradation of adenine nucleotides during cardiopulmonary bypass.

When determinations of phosphocreatine (CP) are desired, a simultaneous recording on a second channel can be made by connecting an extended wavelength module (EWM, Waters Assoc.) to the Model 441 detector with one channel operating at 254 nm and the other at 229 nm. Although the λ_{max} for CP is 214 nm, its absorbance at 229 nm is sufficient for accurate quantitations. Fig. 3 shows the dual-channel recording for a single sample analysis.

Application

Presently, with the exception of very short operations, high-energy phosphate levels decline with even the best forms of cardioplegic protection. Breakdown products of the adenine nucleotides (adenosine, inosine and hypoxanthine) easily cross the cell membrane and are washed away upon reper-

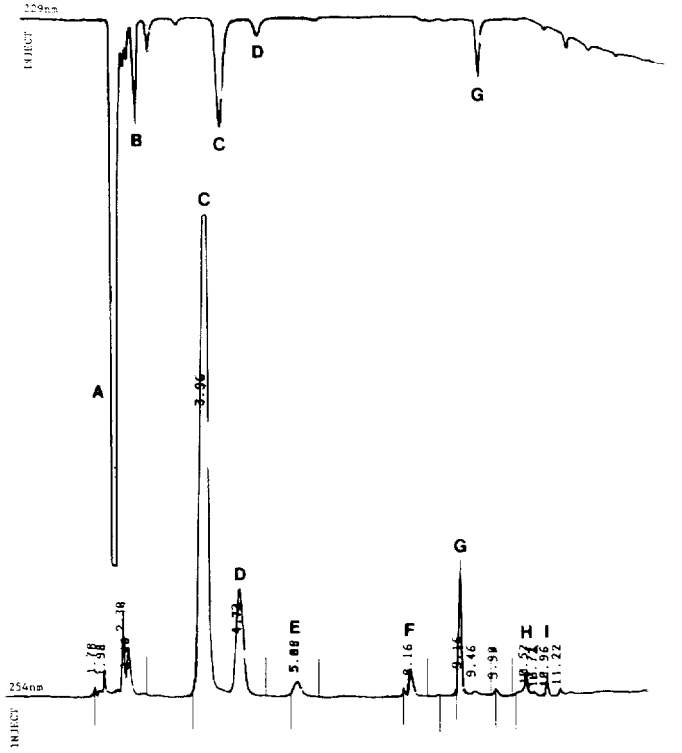

Fig. 3. Dual-channel recording of control canine cardiac tissue (approx. 1 min of ischemia). Channel 1 recorded at 254 nm and channel 2 at 229 nm (extended-wavelength module detector sensitivity 0.1 a.u.f.s.). Otherwise chromatographic conditions as given in Fig. 1. Peaks: A = phosphocreatine; B = creatine; C = ATP; D = ADP; E = APR; F = AMP, G = NAD; H = inosine; I = adenosine.

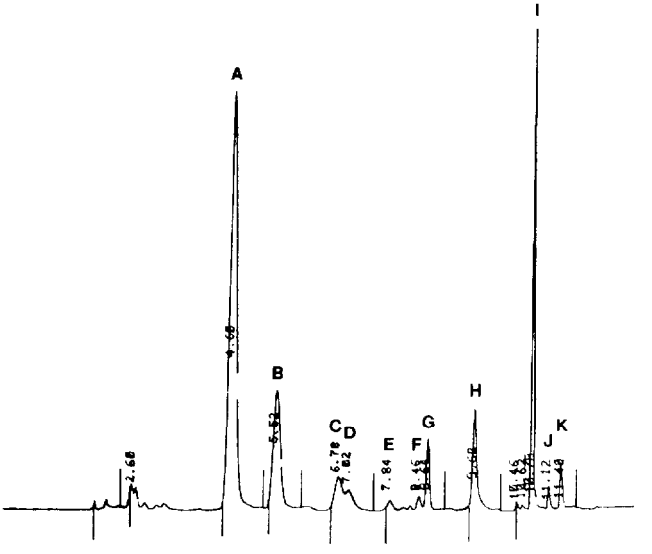

Fig. 4. Canine cardiac tissue after 60 min of ischemia following cardioplegic arrest. Chromatographic conditions as given in Fig. 1. Peaks: A = ATP; B = ADP; C = hypoxanthine; D = APR; E = xanthine; F = NADP; G = AMP; H = NAD; I = inosine; J = cAMP; K = adenosine.

fusion. Since these precursors are no longer available for salvage pathways, even moderate losses of ATP may take several days to replenish [3]. However, if adenosine deamination and transport out of the cell are prevented, this nucleoside could theoretically be rapidly rephosphorylated to ATP at the time of reperfusion. The described methodology was developed to assess the effect of preserving intracellular adenosine levels on ATP rephosphorylation.

Following median sternotomy, healthy mongrel dogs were placed on cardiopulmonary bypass and received either control (Normosol R + 15 mequiv./l potassium) or experimental cardioplegia (treated group) administered at 25°C just as the heart was rendered totally ischemic for 60 min. The experimental solution was identical to the control solution except it contained erythro-g-(2-hydroxy-3-nouye)adenine (EHNA, Burroughs-Wellcome, NC, U.S.A.), a potent adenosine deaminase blocker, plus dipyridamole (Persantine, Boehringer Ingelheim, Ridgefield, CT, U.S.A.), an adenosine transport blocker. Control biopsies were obtained after the initiation of cardiopulmonary bypass, but prior to the administration of cardioplegia. Additional samples were obtained after 60 min of ischemia (C60) and at 15 min of reperfusion (W15). Table I is a summary of the data showing inosine accumulation ($P < 0.005$) during 60 min of cardioplegic arrest and its subsequent loss during reperfusion in the control group. Table II shows adenosine accumulation ($P < 0.005$) in the treated group which is not washed out upon restoration of flow. These results

TABLE I

DEPLETION OF ATP DURING CARDIOPULMONARY BYPASS: CONTROL CARDIOPLEGIA AT 25° C

Energy compound		Level (μmol/g wet weight)		
		Control	After 60 min ischemia	After 15 min reperfusion
ATP	Mean	4.89	3.52	4.00
	S.D.	0.59	0.80	1.17
	S.E.	0.24	0.28	0.41
ADP	Mean	1.49	1.10	1.05
	S.D.	0.26	0.23	0.23
	S.E.	0.09	0.08	0.08
AMP	Mean	0.18	0.17	0.13
	S.D.	0.04	0.07	0.03
	S.E.	0.02	0.03	0.01
Adenosine	Mean	0.01	0.01	0.00
	S.D.	0.01	0.01	0.00
	S.E.	0.01	0.01	0.00
Inosine	Mean	0.22	0.89*	0.19
	S.D.	0.05	0.48	0.11
	S.E.	0.03	0.28	0.06

*$P < 0.005$ (non-paired t-test, treated cardioplegia versus untreated).

TABLE II

ADENOSINE PRESERVATION DURING CARDIOPULMONARY BYPASS: EXPERIMENTAL CARDIOPLEGIA (EHNA, PERSANTINE) AT 25° C

Energy compound		Level (μmol/g wet weight)		
		Control	After 60 min ischemia	After 15 min reperfusion
ATP	Mean	5.13	3.12	4.95*
	S.D.	0.71	0.96	1.22
	S.E.	0.27	0.36	0.46
ADP	Mean	1.12	1.21	0.84
	S.D.	0.23	0.23	0.19
	S.E.	0.09	0.09	0.07
AMP	Mean	0.15	0.28	0.13
	S.D.	0.07	0.25	0.06
	S.E.	0.03	0.09	0.02
Adenosine	Mean	0.02	0.83**	0.57
	S.D.	0.01	0.38	0.25
	S.E.	0.01	0.14	0.10
Inosine	Mean	0.02	0.04	0.06
	S.D.	0.01	0.01	0.03
	S.E.	0.01	0.01	0.01

*$P < 0.025$ (non-paired t-test, treated cardioplegia versus untreated).
**$P < 0.005$ (non-paired t-test, treated cardioplegia versus untreated).

show a statistically significant difference in ATP levels with the treated group showing consistently higher levels at 15 min of reperfusion ($P < 0.025$) [4]. This striking regeneration of ATP following cardiopulmonary bypass is believed to be secondary to adenosine rephosphorylation in the treated group.

DISCUSSION

Nucleotide separation has traditionally been achieved by anion-exchange chromatography [5–7]. Strong anion exchangers provide excellent resolution of the nucleosides and nucleotides but require long run times and are unable to resolve the nucleobases in a simultaneous run [8–10]. The speed and reproducibility of reversed-phase packings has resulted in much work being done to perfect sensitive resolution of ATP and ADP along with their degradative products [2, 11–13]. Paired-ion chromatography (PIC), particularly the PIC reagent tetrabutyl ammonium phosphate, is increasingly used to achieve better resolution of the nucleotides while retaining the advantages of reversed-phase packing materials [14–16]. Others have used preliminary purification runs to improve reversed-phase nucleotide separations [17]. Step gradient elution with the 3–5 μm octadecylsilane columns now available makes possible both rapid and sensitive separations of the total adenine nucleotide pool plus several other compounds of interest in ischemic metabolism. In addition, the

expense and contaminating peaks from impurities associated with paired-ion chromatography are eliminated along with the need for preliminary purification runs.

By using step gradient elution, the widely differing separation requirements of these compounds can be incorporated in a single reversed-phase analysis. The nucleotides require isocratic elution with high ionic strength to maximize their resolution. Any presence of an organic modifier in the mobile phase will compromise the resolution of both ATP from ADP and hypoxanthine from xanthine. Ordinarily, IMP cannot be separated from ATP and ADP under reversed-phase, isocratic conditions [12]. Since IMP accumulation during ischemia is limited in cardiac tissue, the error it introduces is relatively small. However, in skeletal muscle and other tissues with higher AMP deaminase activities, IMP is a prominent ischemic intermediate [17]. The Nova Pak-A cartridge provided excellent separation of IMP from ATP and ADP with this buffer system (see Fig. 2).

A pH of 5.5 further ensures total separation of hypoxanthine from xanthine. Unfortunately, the hypoxanthine peak in most tissue sample extracts also contains APR which frequently goes unnoticed. APR is the major product formed by acid cleavage of NADH during the tissue extraction and, consequently, is present in significant concentrations. Although APR cannot be completely resolved from hypoxanthine at ambient temperature, hypoxanthine concentrations can be estimated with this method (Fig. 4). However, precise quantitations of hypoxanthine and, thus, summations of the total adenine nucleotide pool, can be made by incubating the column system at 35°C. The increased temperature allows complete resolutions of APR from hypoxanthine without compromising the resolution of the other components.

Unlike Brown et al. [18] we did not detect formation of ADP from NADH cleavage as evidenced by Fig. 2. However, an acid cleavage product was found which eluted with 40% methanol. This substance is probably nicotinamide mononucleotide (NMN), but did not appear in significant quantities in our tissue samples and, thus, did not interfere with the other nucleosides and bases quantitated in this application.

Elution of the nucleosides and bases is dependent upon the amount of organic modifier present and can be easily manipulated with a methanol gradient. Frequently overlooked, however, is the fact that the retention of NAD is greatly affected by the presence of an organic solvent, and a linear organic gradient often results in the coelution of NAD and inosine. Since NAD is a major constituent of most acid extract tissue preparations, coelution of the two peaks causes a substantial error in inosine values. A solution containing only 6% methanol is sufficient to rapidly elute the NAD. Maintaining this concentration until the separation of NAD is complete ensures that the two peaks will be resolved. Large amounts of organic modifier can then be added to elute adenosine and other highly retentive compounds rapidly.

Fine tuning this sequence can easily be accomplished with an inexpensive step gradient system, eliminating the need for a gradient controller and a second solvent pump. Furthermore, the baseline problems often encountered with step gradient elution do not occur with this method because of the relatively low amount of organic solvent used for the majority of the analysis.

The use of a dual-channel recorder not only allows quantitation of phosphocreatine and creatine in a simultaneous run, but also provides a second determination of the nucleotides which can be used for ratioing purposes. Although others have developed methods for simultaneous determination of phosphocreatine and adenine nucleotides [8] this dual-wavelength recording offers the advantage of quantitating most of the high-energy phosphates and their catabolites present in myocardial tissue. The ability to quantitate the total adenine nucelotide pool in this application allowed assessment of the degree of adenosine transport and deamination blockade, along with the rephosphorylation potential of myocardial adenosine during reperfusion.

In view of the volume of work inherent in metabolic studies, this step gradient solvent system used in conjunction with the Nova Pak-A column provides an excellent alternative to existing methodologies for efficient, precise and economical determinations of the major high-energy compounds and their degradation products.

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