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Short communication

Development of an HPLC method for determination of metabolic compounds in myocardial tissue

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Abstract

The determination of adenine nucleotides and creatine compounds has great importance in the characterization of ischemic myocardial injury and post-ischemic recovery. It was developed by an HPLC method for the quantification of creatine (Cr), creatine phosphate (CrP), hypoxanthine (HX), AMP, adenosine (Ad), ADP and ATP in isolated perfused rat hearts. The chromatographic conditions were: RP 18 column; mobile phase composed by KH_2PO_4 (215 mM), tetrabutylammonium hydrogen sulfate (2.3 mM), acetonitrile (4%) and KOH (1 M 0.4%); flow rate 1 ml min^{-1}; temperature 25 °C; injection volume 20 μl; detection at 220 nm and height peak (HP) as the integration parameter. The method was validated by means of linearity and sensitivity evaluations, using calibration curves done with five concentration levels of each compound. The limits of quantification (LOQ) were also determined. The system precision was calculated as the coefficient of variation for five injections for each compound tested. The purity of the peaks was established using enzymatic peak shift analysis with hexokinase and creatine kinase and also comparing HP at various wavelengths. Frozen hearts were homogenized with a mechanical homogenizer for 3 min at 0 °C added with 5 ml of 0.4N $HCLO_4$. After precipitation with 0.8 ml of 2 M KOH the extract was shaked for 2 min and later centrifuged at 0 °C for 10 min. The supernatant was kept on ice, filtrated and injected into the HPLC system. The results show that the method for the determination of Cr, CrP, HX, AMP, Ad, ADP and ATP by HPLC here described has good linearity, LOQ, precision, specificity and is simple and rapid to perform.
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Keywords: Adenine nucleotides; Creatine compounds; High performance liquid chromatography; Validation; Myocardial injury

1. Introduction

Quantification of creatine compounds, hypoxanthine and adenine nucleotides in myocardial tissue has great importance for determining changes in its energetic state. For example, it is useful for characterizing ischemic and anoxic contractile injury of myocardium and its metabolic recovery induced by reperfusion or reoxygenation, respectively [1]. Reperfusion of an ischemic heart showed partial or complete recovery of myocardial high-energy phosphate contents, depending upon the period during which the heart had

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been exposed to ischemia [2]. Different methods were used to measure metabolic compounds: enzymatic [3,4], high-performance liquid chromatography with ultraviolet light detection (HPLC-UV) [5–13], gradient HPLC [14–16] or ion-exchange methods [17,18] have been performed with this purpose.

In the present work, an isocratic HPLC method with ion–pair system and replacing of ion exchange of previous ones, was optimized and validated for the simultaneous determination of these compounds in extracts from frozen clamped myocardial tissue. This method was developed for studying the recovery from ischemia-reperfusion dysfunction following up the contractile behavior and high-energy phosphate levels.

2. Experimental

2.1. Equipment

The chromatographic system was composed of a Konik KNK 500G chromatographer, with a double piston serial pump, a programmer for the microprocessor KNK 029-375 (Konik, Barcelona, Spain), a Rheodyne 7125 sample injector with a fixed loop of a 20 μl capability (Rheodyne, Cotati, CA, USA) and a helium bubbling degasificator KNK 029-254. A Lichrocart RP 18 reverse phase column of 250 mm × 4 mm i.d. and a guard column Lichrospher 100 RP 18, both with a particle size of 5 μm (Merck, Darmstadt, Germany) were used. A variable wavelength UV-Vis detector model 204 (Linear NV, USA), was used and the integrator employed was a Datajet model SP 4600 (Spectra Physics, San Jose, CA, USA).

For the preparation of samples, Eppendorf research micropipettes of 5000 μl, 20–200 μl, 100–1000 μl were used. The myocardial tissue was frozen by means of liquid nitrogen and conserved in a Freezer Revco (Revco Scientific, Asheville, NC 28804-9777, USA) until used. It was homogenized by a mechanical homogenizer (Virtis 23, Research, Gardiner, NY 12525). The resultant extracts were centrifuged with a refrigerated centrifuge (Hermle Z 323 K, Hermle Labortecknik, Germany).

Drugs and reagents were weighed on a Mettler Toledo AG 204 balance (Mettler, Greinfensee, Switzerland).

2.2. Materials

Standard drugs creatine (Cr), creatine phosphate (CrP), hypoxanthine (HX), AMP, adenosine (Ad), ADP, ATP, hexokinase (EC 2.7.1.1), creatine kinase (EC 2.7.3.2) and tetrabutylammonium hydrogen sulfate (TBAHS) were obtained from ICN Biomedicals Inc., OH, USA. acetonitrile, HPLC grade (Fischer), sterile water for injection (Roux Ocefa, Bs. As. Argentina), potassium dihydrogen phosphate (J.T. Baker, Mexico), perchloric acid (Merck, Darmstadt, Germany), potassium hydroxide (J.T. Baker, Mexico). Millipore membranes type HV, 50 mm (Millipore, Bedford, MA, USA) and 13 mm (MSI, Westborough, MA, USA) diameters and a pore size of 0.45 μm were used to filter the mobile phase and the samples and standard, respectively.

2.3. Chromatographic conditions

The chromatographic separation of Cr, CrP, HX, AMP, Ad, ADP and ATP was performed using a C18 reversed-phase column (250 mm × 4 mm i.d., particle size 5 μm). The mobile phase was composed of 215 mM potassium dihydrogen phosphate, 2.3 mM TBAHS, 4% acetonitrile, 0.4% potassium hydroxide (1 M) and the flow rate was 1 ml min^{-1}. The sample injection volume was 20 μl and during isocratic acquisition the components were monitored at 220 nm. All instruments and the columns were operated in laboratory at room temperature (23–25 °C).

2.4. Standard solutions

Standard stock solutions were Cr (3 mg ml^{-1}), HX (0.4 mg ml^{-1}), AMP (3 mg ml^{-1}), Ad (1 mg ml^{-1}) and ADP (1 mg ml^{-1}) prepared in 0.4 M perchloric acid. These solutions were stored at −20 °C until 1 month, and used as references for peaks quantification. Fresh dilution was made before each assay, adding mobile phase in order to obtain 90 μg ml^{-1} (Cr), 9 μg ml^{-1} (HX), 60 μg ml^{-1} (AMP), 4 μg ml^{-1} (Ad) and 5 μg ml^{-1} (ADP) concentrations. Standard solutions of ATP (0.4 mg ml^{-1}) and CrP (1 mg ml^{-1}) and their respective dilutions, 20 μg ml^{-1} and 30 μg ml^{-1}, were prepared daily in the same way as described for the sample preparation.

2.5. Sample preparation

Hearts were excised from Wistar rats weighing 200–250 g, anesthetized with pentobarbital overdose. They were rapidly perfused by employing the Langendorf technique through aorta and coronary arteries. Spontaneous contractions were avoided by surgery. A latex balloon was placed into the left ventricle and connected to a Statham Pb 23 Db pressure transducer associated to a Beckman polygraph R511A. The muscles were electrically stimulated with pulses of 5 V to 5 ms at 1 Hz in the right ventricle and apex by a 611 Stimulator Phipps & Bird Inc. All the muscles were perfused at a constant rate (6 ml min^{-1} g^{-1} at 30 °C) with a Krebs-C solution containing 1 mM $MgCl_2$, 125 mM NaCl, 0.5 mM NaH_2PO_4, 7 mM KCl, 2 mM $CaCl_2$, 25 mM $NaHCO_3$ and 6 mM dextrose, bubbled with 95% O_2–5% CO_2 to a pH of 7.3–7.4.

Muscles were perfused for about 30 min (control condition). After the control perfusion, other muscles were submitted to a period of 45 min ischemia (by stopping the flow and keeping the muscle submerged in Krebs solution). At the end of each experiment, rat hearts (control or ischemic) were quickly frozen with liquid nitrogen cooled clamps, then fragmented and frozen at −80 °C. Ventricle samples were homogenized with 5 ml 0.4 M perchloric acid by mechanic homogenization at high speed during 10 min. The extracts were precipitated with 0.8 ml 0.2 M KOH by shaking during 2 min. All steps were carried out on ice. After centrifugation (3000 × *g* at 0 °C for 10 min), the supernatant was filtered and an aliquot of 20 μl was injected into the HPLC system.

2.6. Assay validation

The analytical method was validated by evaluating linearity through the calibration curve obtained with five concentration levels of standard for each analyzed compound.

By analyzing the linear regression of the peak-height versus concentration, the determination coefficient, the intercept and the slope were calculated. To confirm the adjustment to linear pattern, a residual analysis and the Student's test were carried out.

The limits of quantification (LOQ) for each analyzed compound was defined as the lowest concentration of compound in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions.

The system precision for each analyzed compound was determined as the coefficient of variation (CV) for five injections of the same standard solution.

The purity of the various different peaks was established by using two methods: the peak-height ratios of standard solutions and extracts at different wavelengths, and the enzymatic peak shift analysis for ATP and ADP [19]. Known enzymatic reactions, in which an enzyme added to the extract specifically converts one nucleotide into another, were used. The latter method showed the simultaneous disappearance of one substrate and appearance of the corresponding product. This technique uses the specificity of enzymatic reactions on a particular nucleotide, such as the hexokinase (HK) in an excess of glucose, to identify the ATP peaks, as follows:

(a) *The addition of 3 IU* HK in the presence of glucose abolished the ATP peak and increased the ADP one from extracts and standards in buffer solution at pH 7.6 when they were incubated during 30 min at 30 °C:

$$\text{ATP} + \text{glucose} \xrightarrow{\text{HK}} \text{ADP} + \text{glucose-6-phosphate}$$

(b) *The addition of 0.8 IU* Creatine kinase (CrK) in the presence of CrP and Mg^{+2} abolished the ADP peak and increased the ATP one from extracts and standards in buffer solution at pH 7.0 when they were incubated for 20 min at 28 °C:

$$\text{ADP} + \text{CrP} \xrightarrow[\text{Mg}^{+2}]{\text{CrK}} \text{ATP} + \text{Cr}$$

3. Results and discussion

Fig. 1a shows a typical chromatogram with the separation of a mixed standard solution that contains the seven substances assayed (with their respective retention times, R_t) and Fig. 1b other obtained by extracting frozen perfused ventricles (control condition). The total running time for the assay was completed within 18 min, as it can be seen from the R_t. The separation was clear and let an easy identification of the peaks.

A linear response was observed for each compound at the concentration range analyzed as it is shown

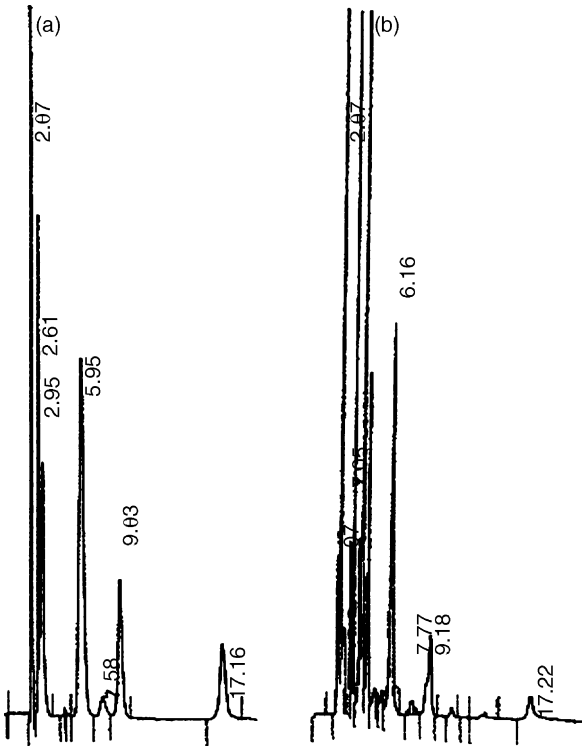

Fig. 1. (a) Typical chromatogram of the separation of a standard solution containing Cr (R_t 2.07), CrP (R_t 2.61), HX (R_t 2.95), AMP (R_t 5.95), Ad (R_t 7.58), ADP (R_t 9.03) and ATP (R_t 17.16) (R_t: retention time in minutes); (b) chromatogram obtained by extracting frozen perfused ventricles (control condition).

in Table 1. The coefficient of determinations, the intercepts and slopes with their respective 95% confidence intervals, the sum of residuals and the results of Student's test are consistent, confirming that the model used for this analytical method was linear.

Table 2
Adenine nucleotides and creatine compounds determination by an HLPC method

Compounds	LOQ ($\mu g\,ml^{-1}$)	LOQ ($\mu mol\,g^{-1}$)[a]	SP (CV, %; $n = 5$)
Cr	0.75	0.27	0.78
CrP	0.5	0.11	1.00
HX	0.2	0.07	0.87
AMP	0.4	0.05	0.44
Ad	0.4	0.07	0.29
ADP	0.39	0.04	1.39
ATP	0.49	0.04	1.52

Limits of quantification (LOQ) expressed as $\mu g\,ml^{-1}$ and $\mu mol\,g^{-1}$ (dry weight), and system precision (SP) expressed as CV (%).

[a] Calculated with mean values of muscle dried weight and with a mean ratio of wet weight (Ww)/dry weight (Dw).

Table 3
Adenine nucleotides and creatine compounds determination by an HLPC method

Compounds	λ_1/λ_2	Standard[a]	Extract[a]
Cr	220/210	0.21	0.21
CrP	220/230	0.57	0.63
HX	275/220	0.69	0.67
AMP	220/254	0.54	0.55
Ad	220/254	0.49	0.49
ADP	220/254	0.53	0.54
ATP	220/254	0.52	0.55

Purity of the various different peaks, established by the peak-height ratio method of standard solutions and extracts at different wavelengths (λ).

[a] Mean from three determinations with CV <2%.

Table 1
Parameters of linearity obtained for the adenine nucleotides and creatine compounds determination by an HLPC method

Compounds	Concentration range ($\mu g\,ml^{-1}$)	a[a]	b[b]	r^2[c]	ΣRI[d]	t_r[e]
Cr	46.5–230	0.607 ± 3.16	0.791 ± 0.02	0.9982	1.84×10^{-13}	40.78
CrP	5–45	17.96 ± 9.45	6.84 ± 0.41	0.9928	7.10×10^{-14}	20.33
HX	3–15	−19.88 ± 13.88	31.11 ± 1.39	0.9949	3.41×10^{-13}	24.18
AMP	15–77	0.57 ± 2.95	1.05 ± 0.06	0.9909	9.59×10^{-14}	18.06
Ad	1.2–11	2.31 ± 1.47	10.48 ± 0.24	0.9991	8.7×10^{-14}	54.71
ADP	2–5	0.11 ± 6.67	9.61 ± 2.82	0.9927	2.13×10^{-14}	20.19
ATP	6–52	8.57 ± 6.04	4.15 ± 0.21	0.9961	1.24×10^{-13}	27.68

[a] Intercept ± 95% confidence intervals.
[b] Slope ± 95% confidence intervals.
[c] Determination coefficient.
[d] Sum of residuals.
[e] $t_{0.05(3)} = 3.1$.

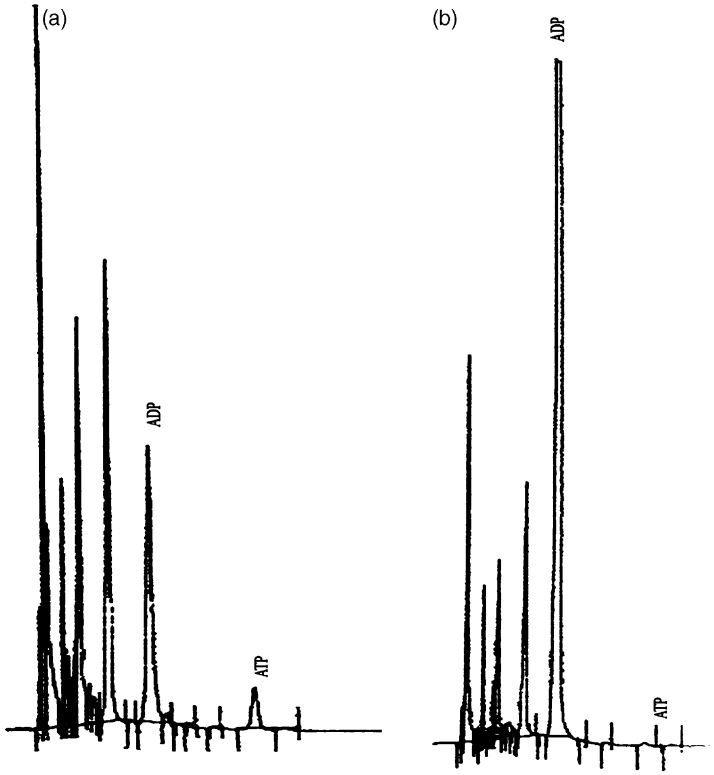

Fig. 2. Chromatograms of extracts from myocardial tissue before (a) and after (b) the reaction with HK. The ATP peak disappeared and the ADP one increased proportionally in size.

The LOQ expressed (as $\mu g\,ml^{-1}$) and $\mu mol\,g^{-1}$ (dry weight) and the system precision for each analyzed compounds expressed as CV (%) are shown in Table 2.

The purity of the peaks, established by the peak-height ratio method for standard solutions and extracts at different wavelengths, is demonstrated by the results shown in Table 3, which yielded consistent purity of all peaks.

On the other hand, the extract chromatograms from myocardial tissue before and after the HK reaction, see Fig. 2a and b, showed the purity of the ATP peak,

Table 4
Adenine nucleotides and creatine compounds determination by an HPLC method

DAM	Cr	CrP	HX	AMP	Ad	ADP	ATP
Control perfusion ($n = 5$)	46.2 ± 5.0	25.4 ± 3.5	3.0 ± 0.4	5.4 ± 0.8	1.5 ± 0.3	6.6 ± 1.1	11.8 ± 2.0
Ischemia 45 min ($n = 6$)	33.4 ± 2.8*	6.2 ± 2.2*	9.8 ± 1.8*	7.2 ± 0.5	1.1 ± 0.1	2.1 ± 0.3*	1.2 ± 0.4*

Results obtained with the developed analytical method (DAM). Mean values ± S.E.M. (*$P < 0.05$ vs. control perfusion) of metabolic compounds in myocardial tissue under control perfusion and ischemia conditions:

$$\text{dry weight}\,(\mu mol\,g^{-1}) = \frac{\text{HP Ext. Conc. St. Vf. RWw/Dw}}{\text{HP St.WeightMusc. WM}}.$$

HP Ext.: extract height peak; HP St: standard height peak; Conc. St.: concentration standard; Vf.: last volume extract; RWw/Dw: relation wet weight (Hw)/dry weight (Dw); WeightMusc.: weight of the muscle; WM: compound weight molecular.

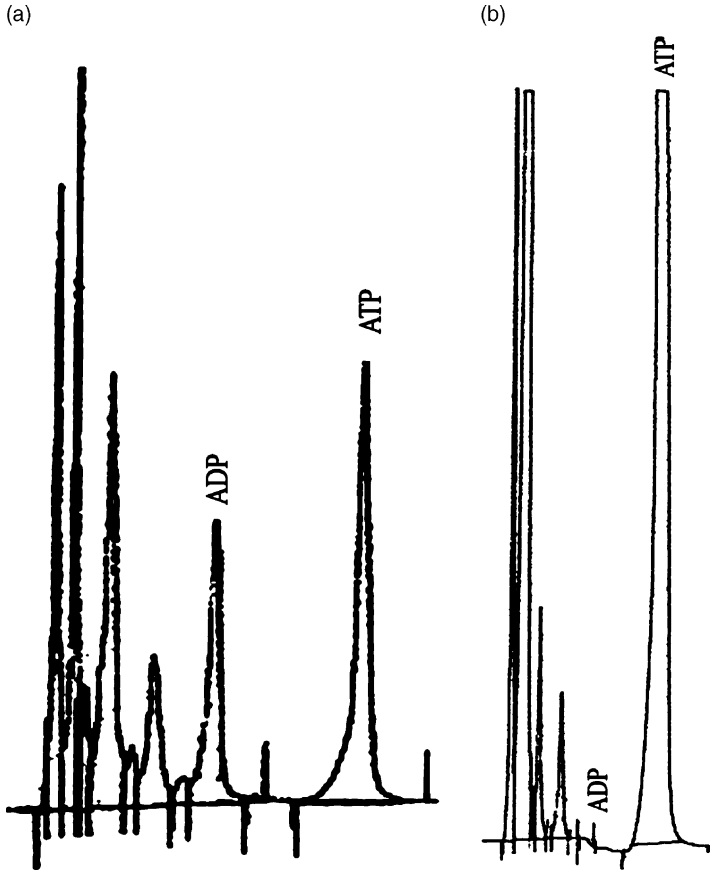

Fig. 3. Chromatograms of extracts before (a) and after (b) the reaction with CrK which abolished the ADP peak and increased the ATP one.

which disappeared while the ADP one increased proportionally in size.

Moreover, Fig. 3a and b shows the extract chromatograms of before and after the reaction with CrK, which abolished the ADP peak and increased the ATP one, demonstrating the purity of the ADP peak.

Table 4 shows the mean contents of metabolic compounds in myocardial tissue from hearts exposed to either, control perfusion and 45 min ischemia, expressed as μmol g^{-1} (dry weight), which were obtained with the developed analytical method. Our values are in general comparable to those presented by Sellevold et

Table 5
Results reported in bibliography, expressed as mean μmol g^{-1} (dry weight) $\pm$ S.E.M., with other HPLC methods in perfused and ischemic hearts (see references).

Reference	Cr	CrP	HX	AMP	Ad	ADP	ATP
Sellevold et al. [1] control perfusion	46.2 ± 1.1	35.5 ± 1.0	[a]	1.4 ± 0.1	[a]	4.6 ± 0.1	26.6 ± 0.4
Sellevold et al. [1] ischemia (20 min)	65.8 ± 2.4	1.9 ± 0.2	[a]	8.7 ± 0.7	[a]	7.9 ± 0.6	4.7 ± 0.3
Harmsen et al. [20] control perfusion	[a]	27.5 ± 2.8	[a]	0.5 ± 0.2	[a]	4.8 ± 0.3	22.9 ± 1.2
Harmsen et al. [20] ischemia (20 min)	[a]	13.4 ± 1.9	[a]	1.4 ± 0.6	[a]	5.8 ± 1.1	15.8 ± 2.8
Humphrey et al. [21] control perfusion	38.2 ± 1.5	23.6 ± 0.9	0.03 ± 0.01	1.1 ± 0.1	0.05 ± 0.01	5.7 ± 0.2	22.5 ± 0.6
Humphrey et al. [21] (ischemia, 25 min)	70.1 ± 2.1	2.5 ± 0.3	1.4 ± 0.1	12.3 ± 0.5	4.3 ± 0.2	4.6 ± 0.2	3.1 ± 0.4

[a] Not supplied.

al. [1], Harmsen et al. [20] and Humphrey et al. [21] (Table 5).

Although the ATP content was slightly low in control condition it was comparable to the ones in ischemic condition mentioned in the literature.

4. Conclusion

The developed method to determine Cr, CrP, HX, AMP, Ad, ADP and ATP by HPLC described here has good linearity, LOQ, precision and specificity and is simple and rapid to perform. The importance of the procedure for extraction of metabolic compounds from frozen cardiac tissue is highlighted.

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