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Application and validation of an ion-exchange high-performance liquid chromatographic method for measuring adenine nucleotides, creatine and creatine phosphate in mouse brain

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SUMMARY

We have modified and applied an ion-exchange high-performance liquid chromatographic method for measuring adenine nucleotides (adenosine monophosphate, adenosine diphosphate and adenosine triphosphate) as well as creatine and creatine phosphate in brain tissue. There was a linear relationship between the area of each peak and the amount of standard injected onto the column in the concentration range 0.5–25 nmol per 50 μl. The concentrations of creatine phosphate and creatine were not stable in a standard mixture for 20 h at 4°C unless the pH of the standard mixture was adjusted to neutral. We therefore strongly recommend the neutralization of all standard mixtures and samples before storage. The measurements of adenine nucleotides, creatine and creatine phosphate in control mouse brain determined by this method agreed well with an enzymic method of nucleotide measurement. Furthermore, both methods detected similar decreases in the concentrations of adenosine triphosphate and creatine phosphate, together with concomitant increases in the concentrations of adenosine diphosphate, adenosine monophosphate and creatine when mice were placed under anoxic conditions (either 30 s or 2 min); these changes were greater after 2 min of anoxia than after 30 s of anoxia.

INTRODUCTION

Adenosine triphosphate (ATP) is the main source of chemical energy in living matter and plays a fundamental role in mammalian brain function [1]. Constant levels of ATP are maintained by the creatine phosphokinase [2] and adenylate kinase [3] reactions; creatine phosphokinase catalyses a reversible

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transfer of phosphate between creatine phosphate (PCr) and adenosine diphosphate (ADP) to form ATP and creatine (Cr); adenylate kinase catalyses the formation of ADP from ATP and adenosine monophosphate (AMP). Accurate methods for the determination of the concentrations of these compounds in brain tissue would help to elucidate their role in brain function.

High-performance liquid chromatography (HPLC) is a powerful technique for the separation and quantitation of nucleotides, nucleosides and bases [4]. Nucleotides, such as AMP, ADP and ATP, can be separated on a reversed-phase column with a UV detector set to 254 nm [5]. However, PCr cannot be detected in this system. Harmsen et al. [6] modified a previously described method [7] to separate PCr and nucleotides on an ion-exchange column with detection at 210 nm. They applied the method to the measurement of concentrations of nucleotides in myocardial tissue [6,8]. The method has not, to our knowledge, been used to quantitate these metabolites in brain tissue.

Simpson and Brown [4] recommend in their review that any HPLC assay should be tailored to meet the requirements for the specific analytes in a particular sample. We have therefore modified the method of Harmsen et al. [6] for the measurement of adenine nucleotides, Cr and PCr in brain tissue and have validated it by comparison with enzymic measurements.

EXPERIMENTAL

Apparatus

The HPLC system (Merck-Hitachi) consisted of an L-6200 intelligent pump (low-pressure gradient system), an L-5000 LC controller and an L-4200 UV–VIS variable-wavelength detector. This was used in association with a Gilson Model 231-401 autosampler with a Rheodyne valve (7010). All data were collected and stored with a Nelson Analytical Series 900 interface, and peak integration was carried out with Nelson 2600 chromatography software and a Hewlett-Packard Vectra PC.

The ion-exchange column used in these experiments was a Whatman (Clifton, NJ, U.S.A.) Partisil 10 SAX (250 mm×4.6 mm I.D.). This was used in association with a small guard column (40 mm×4.5 mm I.D.) filled with pellicular anion exchanger.

Reagents

Potassium dihydrogen phosphate (KH_2PO_4 G.R.), phosphoric acid (H_3PO_4 G.R.) and potassium hydroxide (KOH) were purchased from Merck (Darmstadt, F.R.G.). Standard adenine nucleotides, Cr and PCr were purchased from Serva Biochemicals (Heidelberg, F.R.G.). The standards were dissolved in purified water, and the pH was adjusted to neutral. Pure water was prepared using a Millipore UF system. All other reagents were of reagent grade and purchased from reputable sources.

HPLC procedure

Buffer was made freshly each day, filtered through a 0.45-μm Millipore filter and degassed. Buffer A consisted of 0.01 *M* H_3PO_4 adjusted to pH 2.85 with 1 *M* KOH; buffer B was 0.75 *M* KH_2PO_4 adjusted to pH 4.40 with 1 *M* KOH.

The method of Harmsen et al. [6] was modified in order to achieve a more satisfactory separation. The column was allowed to equilibrate with buffer A at a flow-rate of 2.0 ml/min. The UV detector was set to a wavelength of 210 nm. The column was eluted with buffer A for 5 min after the injection of sample (50 μl), and then the gradient was started with 2.5% buffer B per min until 100% was reached (45 min). However, because it was possible to separate all peaks on the chromatogram with 40 min, the pump program was interrupted after 40 min and the column was washed with buffer A for 5 min to prepare it for the next injection. Peak identification was performed using external standards, and concentrations were calculated on the basis of peak areas.

Nucleotide determination in mouse brain

White male mice (NMRI) weighing ca. 25 g were used for the experiment. The mice, some of which had been subjected to either 30 s or 2 min of anoxia (95% nitrogen–5% carbon dioxide environment), were killed by a blow to the nape of the neck and the heads were removed and frozen quickly in liquid nitrogen. The brain was removed in pieces with a dentist's drill under liquid nitrogen, weighed and placed in a freeze dryer for 40 h. The tissue was then processed for nucleotide extraction as previously described [9]: the tissue was homogenized with 0.33 *M* perchloric acid (10 ml/g wet weight) and centrifuged at 10 000 *g* for 10 min at 4 °C to remove the precipitated protein; the supernatant was neutralized with 2 *M* KOH before storage at −80 °C and measurement. The concentrations of ATP, ADP, AMP, Cr and PCr were determined either by HPLC or, with the exception of Cr, by enzymic conversion into the oxidized form of nicotinamide-adenine dinucleotide (NAD) followed by spectrophotometric measurement [9].

RESULTS AND DISCUSSION

The gradient of 4% buffer B per min originally applied by Harmsen et al. [6] for the measurement of myocardial tissue extracts did not allow adequate separation of all of the nucleotides in brain tissue extracts. In contrast, the modified gradient chromatography system of 2.5% buffer B described in this paper resulted in an adequate separation of the adenine nucleotides in a standard mixture (Fig. 1) and in an extract of mouse brain (Fig. 2). There was a small but consistent rise in the baseline of the chromatogram during elution; this was probably due to unknown UV-absorbing impurities in commercial phosphates; these impurities are first retained on the column and then eluted later in the gradient [10]. Furthermore, commercial refilling of the ion-ex-

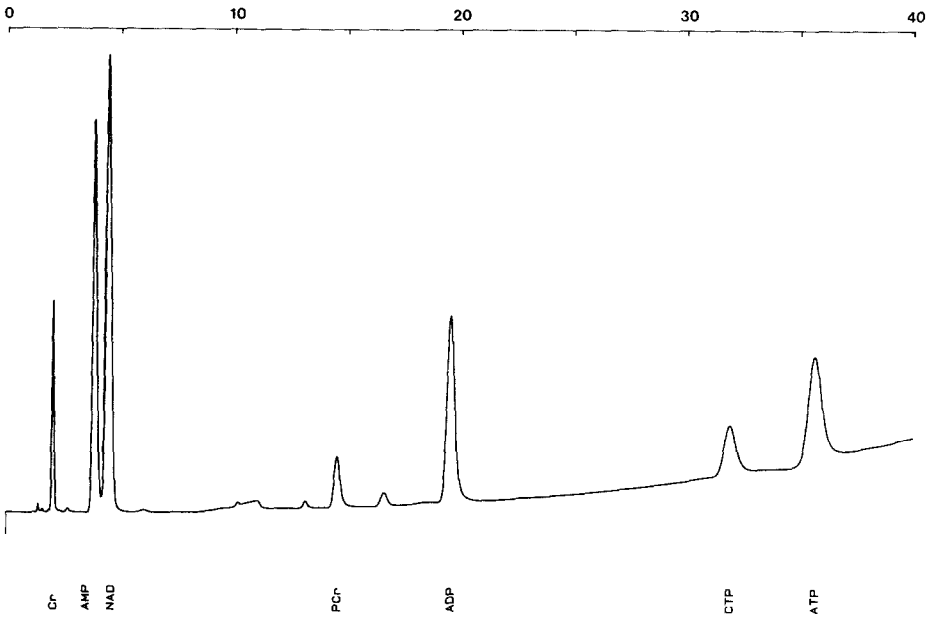

Fig. 1. Ion-exchange HPLC separation of a standard mixture of nucleotides, Cr and PCr (10 nmol per 50 μl). The chromatogram is plotted on a vertical scale of 0–110 mV and a horizontal scale given in min.

change column resulted in a poorer separation of AMP and NAD. We therefore always used new columns.

The peak-area reproducibility for the different nucleotides in a standard mixture injected hourly onto the same column over a period of 20 h was between 2.3 and 3.6% (Table I). The retention time reproducibility of the same samples is shown in Table II. Although the retention time reproducibility over 20 h was good, other experiments showed that small day-to-day fluctuations in the system, or differences in batches of columns, resulted in slight changes in the retention times; please note for example that the retention time of creatine phosphate in the standard mixture in Fig. 1 is slightly different from the retention time of creatine phosphate in the brain tissue extract in Fig. 2. Therefore, because the peak identification and integration depended on external standards, a standard mixture was injected daily and the retention times adjusted accordingly.

There was a linear relationship between the area of the peak and the amount of nucleotide injected onto the column. The relationship covered the concentration range 0.5–25 nmol per 50 μl for ATP, ADP, Cr and PCr and 0.25–25 nmol per 50 μl for AMP. The compounds of interest could be separated into two groups on the basis of the slopes of the area–concentration curves; ATP, ADP and AMP were in one group and Cr and PCr formed a second group. We

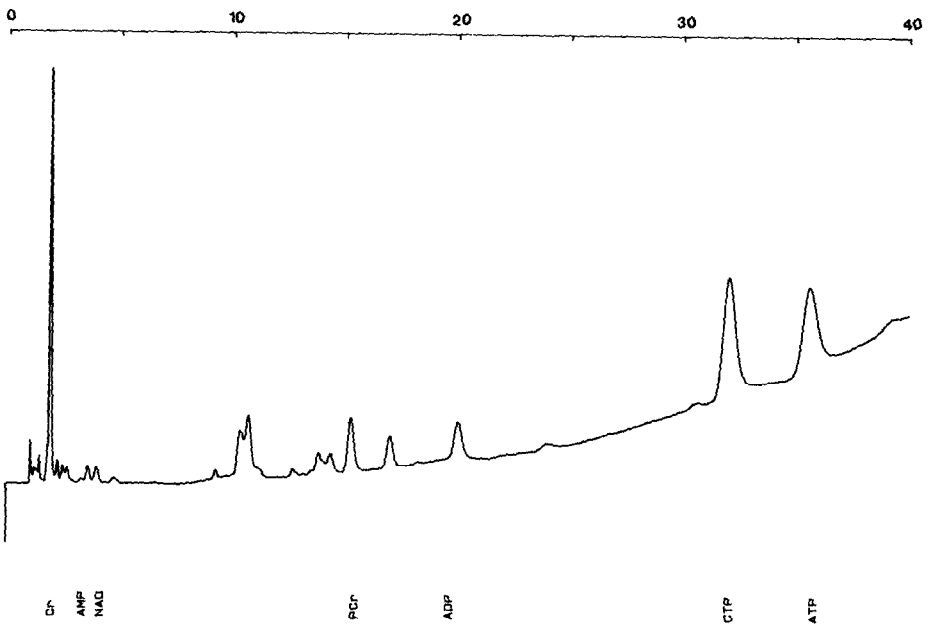

Fig. 2. Ion-exchange HPLC separation of an extract of mouse brain. The chromatogram is plotted on a vertical scale of 0–45 mV and a horizontal scale given in min.

TABLE I

REPRODUCIBILITY OF PEAK AREAS FOR INJECTIONS OF A STANDARD MIXTURE (10 nmol/50 μl)

Nucleotide	Mean peak area (integration units)	Coefficient of variation (%)
ATP	923 943	2.3
PCr	318 297	3.6
ADP	980 142	2.2
AMP	1043 397	2.7
Creatine	235 627	2.5

had originally attempted to use CTP as an internal standard. However, it was not possible to use CTP to calculate the concentration of Cr and PCr because CTP has a slope similar to ATP, ADP and AMP only.

In some experiments the pH of the standard mixture was not adjusted to a neutral pH before storage. Although the concentration of ATP (as well as ADP and AMP) remained stable over a 24-h period at 4 °C under these conditions, the concentration of PCr decreased and the concentration of Cr increased (Fig. 3). This suggests that PCr is hydrolysed to Cr in an acidic standard mixture.

TABLE II

REPRODUCIBILITY OF RETENTION TIMES FOR INJECTIONS OF A STANDARD MIXTURE (10 nmol/50 μl)

Nucleotide	Mean retention time (min)	Coefficient of variation (%)
ATP	36.6	0.1
PCr	16.1	0.1
ADP	21.3	0.1
AMP	4.2	1.1
Creatine	2.0	0.4

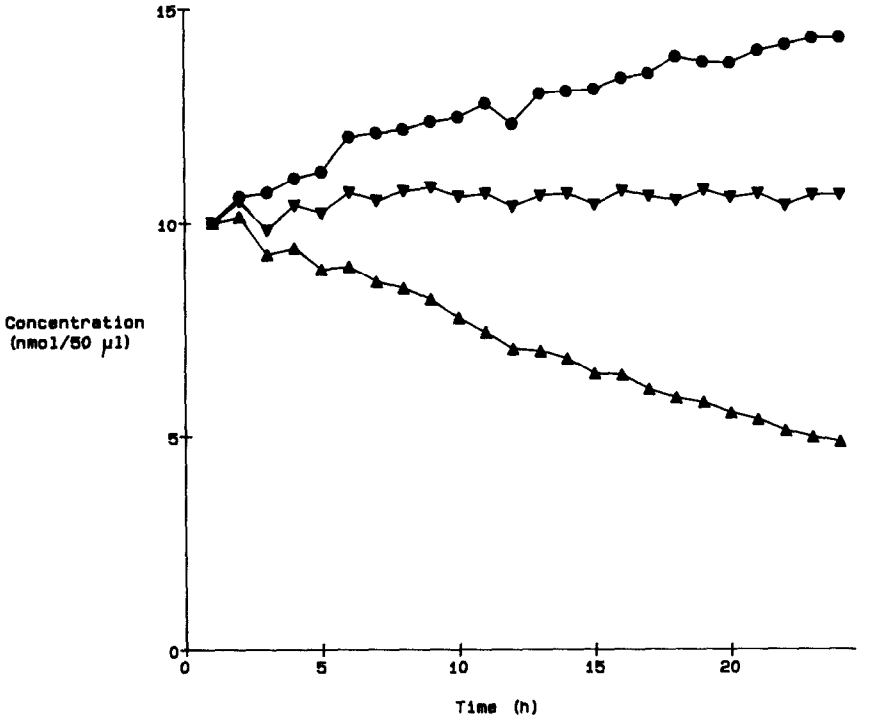

Fig. 3. Effect of acidic pH and time on the stability of standard Cr (●), PCr (▲) and ATP (▼) when stored at 4 °C. Each point represents one injection.

Fig. 4 shows the results of a similar experiment performed with a neutralized standard mixture; the concentrations of ATP, Cr and PCr remained stable over the entire 20-h period. This observation has important implications for storage of standards that contain PCr. Almost all publications dealing with HPLC

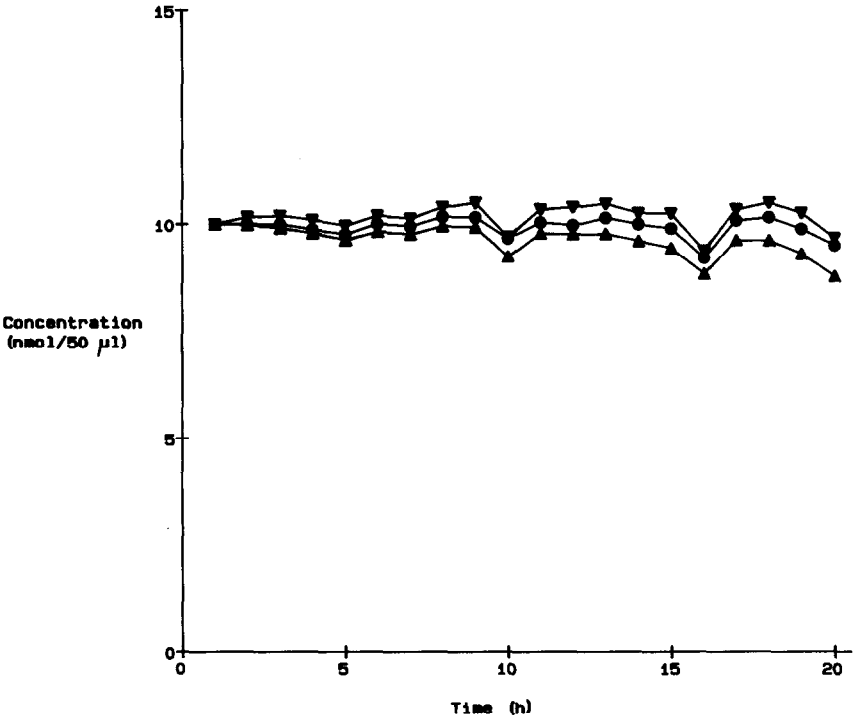

Fig. 4. Effect of neutral pH and time on the stability of standard Cr (●), PCr (▲) and ATP (▼) when stored at 4°C. Each point represents one injection.

assay of nucleotides recommend adjusting perchloric acid tissue extracts to a neutral pH but do not mention similar treatment of standard mixtures.

Table III shows a comparison of the HPLC and spectrophotometric determinations; there was good agreement between the two methods for ATP. However, the concentrations of PCr and AMP determined spectrophotometrically were lower than those determined by HPLC. Furthermore, the ADP values determined spectrophotometrically were higher than those determined by HPLC. Nevertheless, the concentrations of ATP, ADP and Cr that we obtained by HPLC were within the ranges previously reported: 2.3–3.1 μmol/g wet weight for ATP; 0.21–0.56 μmol/g wet weight for ADP; and 4.8–7.63 μmol/g wet weight for Cr [11]. The concentration of AMP is higher than literature values, 0.01–0.05 μmol/g wet weight, and the concentration of PCr was much lower than literature values, 4.0–6.5 μmol/g wet weight [11]. This may be because in our experiments the mouse brain was immersed in liquid nitrogen. The freezing time is much longer with this technique than during direct, in situ application of liquid nitrogen [13,14], thereby resulting in some hydrolysis of PCr to Cr. The overall energy change, (ATP+0.5 ADP)/

TABLE III

EFFECT OF IN VIVO ANOXIA ON THE CONCENTRATIONS OF NULEOTIDES, Cr AND PCr, TOGETHER WITH ENERGY CHANGE (RATIO), IN MOUSE BRAIN: A COMPARISON OF ENZYMIC AND HPLC MEASUREMENTS

Each result is the mean of three different experiments with duplicate determinations (the percentage coefficient of variation is shown in parenthesis).

Nucleotide	Concentration (μmol/g wet weight)					
	Control		30 s anoxia		2 min anoxia	
	Enzymic	HPLC	Enzymic	HPLC	Enzymic	HPLC
ATP	2.40	2.41	0.75	0.71	0.11	0.15
	(3.75)	(3.73)	(2.82)	(8.45)	(9.09)	(6.67)
PCr	1.93	2.76	0.27	0.25	0.08	0.18
	(1.04)	(4.71)	(7.41)	(4.00)	(12.5)	(22.2)
ADP	0.94	0.59	1.28	0.87	0.64	0.35
	(5.32)	(3.39)	(2.34)	(6.90)	(7.81)	(17.1)
AMP	0.10	0.17	1.26	1.62	2.12	2.62
	(40.0)	(17.6)	(4.76)	(4.32)	(1.42)	(0.38)
Creatine	–	5.96	–	7.78	–	7.44
		(0.67)		(0.51)		(2.28)
Energy change	0.83	0.85	0.42	0.36	0.15	0.10

(ATP+ADP+AMP), calculated according to Atkinson [12], was slightly lower (Table III) than published data using in situ application of liquid nitrogen, 0.92–0.95 [14,15]. Nevertheless, we believe that this does not detract from the main aim of the present work, namely the application and validation of an HPLC method for a simple quantitative estimate of the concentrations of adenine nucleotides, Cr and PCr in brain tissue.

Anoxia (either 30 s or 2 min), as expected, caused a decrease in the concentrations of ATP and PCr together with a concomitant rise in the concentrations of ADP, AMP, Cr. These changes were greater after 2 min of anoxia than after 30 s of anoxia and were also evident when the results were expressed as energy change.

In conclusion, we have described in this brief communication the application of a reproducible and sensitive HPLC separation of adenine nucleotides, Cr and PCr for brain tissue extracts using an ion-exchange column and gradient elution. The results of this method agree well with those obtained from much more laborious and slower spectrophotometric methods. The method permits the accurate, simultaneous determination of the concentrations of compounds that are very important in brain energy metabolism. In addition, as a technical note, we strongly recommend that all standard mixtures and samples are neu-

tralized before storage or measurement, because PCr is very susceptible to hydrolysis, even in enzyme-free extracts.

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