Journal of Chromatography, 496 (1989) 441–449
Biomedical Applications
Elsevier Science Publishers B V , Amsterdam — Printed in The Netherlands

CHROMBIO 4970

Note

Influence of perchloric acid on ion-pair high-performance liquid chromatography of nucleotides

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(First received March 15th, 1989, revised manuscript received July 21st, 1989)

High-performance liquid chromatography (HPLC) is a powerful technique for the separation and quantification of nucleotides and other high-energy phosphate-containing metabolites in tissues of several origins [1–10]. Separation of nucleotides is carried out by both isocratic [1,6–8,11–13] and gradient [2–5,9,13,14] elution Isocratic analyses are rapid but not always reliable, as not all nucleotides are separated because of limitations in resolution. Therefore, samples with a complex composition are usually analysed by gradient elution The introduction of ion-pairing reagents in the isocratic liquid chromatography of nucleotides was of significant importance [4,6,7,12], and very useful for rapid separations of metabolites in samples with a relatively simple composition. With ion-pair chromatography the separation of nucleotides can be controlled by varying several factors such as pH, ionic strength and the concentrations of ion-pair reagents or modifiers, although the exact mechanisms are still unclear [10].

The extraction of nucleotides from different tissues can be carried out with either perchloric or trichloroacetic acid [1–9,11,12,15,16]. Perchloric acid is preferred, because the excess of acid can be removed as the potassium salt after neutralization with potassium-containing bases such as potassium carbonate or potassium hydroxide.

In this study we found that the use of perchloric acid as an extraction solvent

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0378-4347/89/$03 50

for nucleotides also has a disadvantage, namely that perchlorate ions can affect the retention behaviour of nucleotides during ion-pair liquid chromatography.

EXPERIMENTAL

Chemicals[b]

ATP, ADP, AMP, IMP, PC, NADH, NADP and the enzymes pyruvate kinase, lactate dehydrogenase, creatine kinase, hexokinase and glucose-6-phosphate dehydrogenase were obtained from Boehringer Mannheim (Mannheim, F R.G) All other chemicals were obtained from BDH (Poole, U K.). Potassium perchlorate was prepared by addition of equimolar amounts of potassium hydroxide to perchloric acid. The potassium perchlorate crystals were washed twice with water and freeze-dried overnight.

Muscle samples

Exercized and resting rat medial gastrocnemius muscles were freeze-clamped with a pair of tongs precooled in nitrogen The freeze-clamped muscles were pulverized with a mortar and pestle, while constantly adding liquid nitrogen in order to cool the muscle tissue, and subsequently freeze-dried overnight (Breda Scientific, Breda, The Netherlands) The freeze-dried muscle powder was stored at −30 °C until extraction.

Approximately 15 mg of freeze-dried muscle powder were mixed with 750 μl of 5% (v/v) cold (4 °C) perchloric acid using a tube mixer (Vortex, Etten-Leur, The Netherlands) After 15 min the homogenate was centrifuged (0 °C, 18 000 *g*, 15 min, MSE Mistral, Crawley, U.K.). The supernatant (525 μl) was neutralized in two steps, first with 5 *M* potassium carbonate solution (70 μl), followed by neutralization to pH 6.5–7.0 with 5 *M* potassium hydroxide solution (70 μl). pH Adjustments were made with 1 *M* hydrochloric acid Potassium perchlorate was removed by centrifugation after the samples had been frozen and thawed (10 000 *g*, 5 min, Eppendorf, Hamburg, F.R.G.). The neutralized extract was stored at −30 °C until the determination

High-performance liquid chromatography

For the separation and quantification of metabolites in standard solutions and samples, a Kratos (Ramsey, NJ, U.S.A.) HPLC system was used, composed of a Spectroflow 757 detector and a Spectroflow 400 pump. Metabolites were detected at 212 nm, at which wavelength both phosphocreatine and nucleotides can be detected.

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[b]Abbreviations AMP = adenosine-5′-monophosphate, ADP-adenosine-5′-diphosphate, ATP = adenosine-5′-triphosphate, PC = phosphocreatine, IMP-inosine-5′-monophosphate, NAD(P)H = nicotinamide dinucleotide (phosphate)

Chromatograms were processed using a Shimadzu (Kyoto, Japan) RC3A integrator. Further calculations were made with an Apple IIe microcomputer.

Elution was carried out under isocratic conditions, using ion-pair liquid chromatography and a high-speed reversed-phase 3-RP-18 column (Beckman, Fullerton, CA, U.S.A.; 70 mm×4.6 mm I.D.; particle size 3 μm) with a flow-rate of 1.5 ml/min. The eluent was 100 m*M* ammonium dihydrogenphosphate (pH 7.0) with 0.02% tetrabutylammonium hydroxide as ion-pair reagent and 2% acetonitrile as modifier; pH adjustments were made with 3 *M* ammonia solution. All solutions were filtered through a 0.45-μm filter (Schleicher & Schüll, Dassel, F.R.G.) and degassed with helium just before use. Solutions were prepared freshly every day in order to avoid bacterial growth or other contamination.

Standard solutions

For the calculation of the concentration of ATP, ADP, AMP, IMP and PC in samples, standard solutions of these components in the mobile phase were used. A stock solution was prepared in 100 m*M* ammonium dihydrogenphosphate (pH 7.0) containing 1.0 m*M* ATP, 0.25 m*M* ADP, 0.05 m*M* AMP, 0.083 m*M* IMP and 3.0 m*M* PC. The relative concentrations of these compounds were similar to those present in muscle extracts.

Calibration graphs were obtained by injection of dilute standard solutions, prepared by diluting the standard solution either 10-, 20-, 30-, 40-, 60-, 80-, 160- or 240-fold with the mobile phase. Calibration data were plotted and linear regression lines calculated using the least-squares method.

The influence of potassium perchlorate on the chromatography of the above nucleotides and phosphocreatine was studied with a 10-fold diluted stock solution saturated with 10 mg/ml potassium perchlorate. This standard was injected either undiluted or diluted 2- or 6-fold for analysis. In order to investigate the effect of pH on the chromatography of these standards, which were partially saturated with potassium perchlorate, the eluent was used at different pH values (5.5, 6.25, 6 7 and 7.0).

Perchloric acid extracts of muscle powder

All muscle samples were analysed with an eluent of pH 7.0 and diluted 10-fold with the mobile phase just before injection. Mobile phase was used rather than other buffers in order to obtain better chromatograms Significant breakdown of nucleotides occurred within 1 h after dilution of the samples in the mobile phase However, the original samples which were stored with ice–water cooling during the assay were stable during the period of analysis.

Enzymatic determination of ATP, ADP and PC

In order to compare the results obtained with HPLC with another method as a reference, ATP, ADP and PC concentrations were also determined enzy-

matically according to Bergmeyer [17]. Enzymatic reactions were coupled with NAD(P)H usage or formation and determined with a double-beam spectrophotometer (Shimadzu UV190).

RESULTS

Chromatography of standards

Calibration graphs were obtained for PC, ATP, ADP, AMP and IMP. Least-squares regression lines for all metabolites had intercepts near zero with correlation coefficients higher than 0.999. A typical chromatogram of a standard solution is shown in Fig. 1A, which shows that a good separation was obtained under the conditions used. Good reproducibility of the measurements of the high-energy phosphates in the standard solution was obtained, as shown in Table I. A higher reproducibility was obtained with peak height as the parameter rather than the more commonly used peak area.

Chromatography of muscle samples

Typical chromatograms of a resting and an exercized muscle sample are shown in Fig. 1. The IMP and AMP concentrations were very low in the resting muscle sample. As a result, the reproducibility of these nucleotides in resting muscle samples is also low (Table I). The concentrations of PC, ATP and ADP obtained by HPLC were compared with those obtained by enzymatic determinations (Table II). Small differences were noted, predominantly for the exercized muscle samples, in which the PC and ATP concentrations were low. Whereas there was a difference of approximately 5% when peak height was used in the calculations, a difference of 20% was obtained when using peak

TABLE I

REPRODUCIBILITY OF MEASUREMENTS OF HIGH-ENERGY PHOSPHATES EXPRESSED AS COEFFICIENTS OF VARIATION (%)

One standard solution and two muscle samples (one resting and one exercized) were each injected ten times. Quantification was based on peak-height (PH) and peak-area (PA) measurements.

Phosphate	Standard		Muscle samples			
	PH	PA	Resting		Exercized	
			PH	PA	PH	PA
PC	1.7	2.2	2.0	2.3	1.7	3.2
IMP	2.4	5.6	17.5	19.7	2.1	6.3
AMP	1.4	2.0	4.0	7.2	3.0	3.8
ADP	1.7	2.4	2.2	2.2	1.7	1.9
ATP	1.7	2.3	2.0	2.0	2.2	1.8

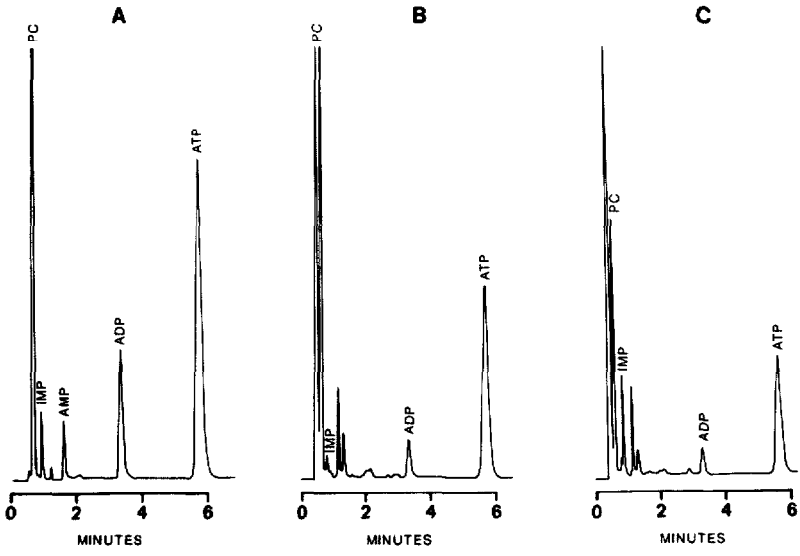

Fig 1 Separation of PC and nucleotides by ion-pair HPLC Typical chromatograms are shown for (60-fold diluted) standard solution (A), resting muscle (B) and exercized muscle (C) For elution conditions, see Experimental

TABLE II

COMPARISON OF CONCENTRATIONS OF ATP, ADP AND PC IN RESTING AND EXERCIZED MUSCLES AS DETERMINED BY HPLC AND ENZYMATICALLY

Method	Concentration[a] (mean ± S D) (μmol/g dry weight)		
	PC	ATP	ADP
Resting muscles (n=8)			
HPLC	106 1±5 5	35 7±1 3	3 2±0 3
Enzymatic	105 8±7 6	36 6±1 6	3 0±0 3
HPLC/enzymatic (%)	100 6±3 4	97 4[b]±1 9	106 1±9 7
Exercised muscles (n=8)			
HPLC	28 1±5 6	25 8±2 8	4 0±0 7
Enzymatic	30 3±7 2	27 6±2 9	3 8±0 7
HPLC/enzymatic (%)	93 5[b]±6 6	93 4[b]±5 2	105 0[b]±5 0
All muscles (n=16)			
HPLC/enzymatic (%)	97 1±6 2	95 4[b]±4 2	105 6[b]±7 5

[a]HPLC concentrations are based on peak height
[b]Significantly different from 100% ($p < 0$ 05, Student's t-test)

area. The lower calculated ATP concentrations given by HPLC might be caused by peak broadening by perchlorate present in the muscle samples.

Influence of perchlorate and pH on the separation of nucleotides

After about 150 injections of muscle samples, the pressure of the system rose to 30% above normal and the ATP peak was split into two components These effects could be removed by regeneration of the analytical column by rinsing with 50 ml of water followed by 100 ml of acetonitrile. As these effects only occurred when several samples were injected, it was investigated whether perchlorate present in the muscle samples could have caused these disturbances. As we increased the pH of the eluent to 7.0 in order to obtain a better separation of the nucleotides and PC, we also investigated whether a possible effect of perchlorate was pH-dependent.

Typical examples of chromatograms of standard solutions saturated with perchlorate are shown in Fig. 2. With increasing perchlorate content the retention times of ATP and to a lesser extent those of ADP and AMP decreased, whereas those of PC and IMP remained unchanged (Table III) Table III also shows that the retention times increased with lower pH values of the eluent, as would be expected from the influence of pH on the interaction between the phosphate groups of the nucleotides and the tetrabutylammonium ions. The effects of perchlorate were also enhanced at lower pH values (Table III). Splitting of the ATP peak into two components occurred at all pH values, but was more pronounced at lower pH.

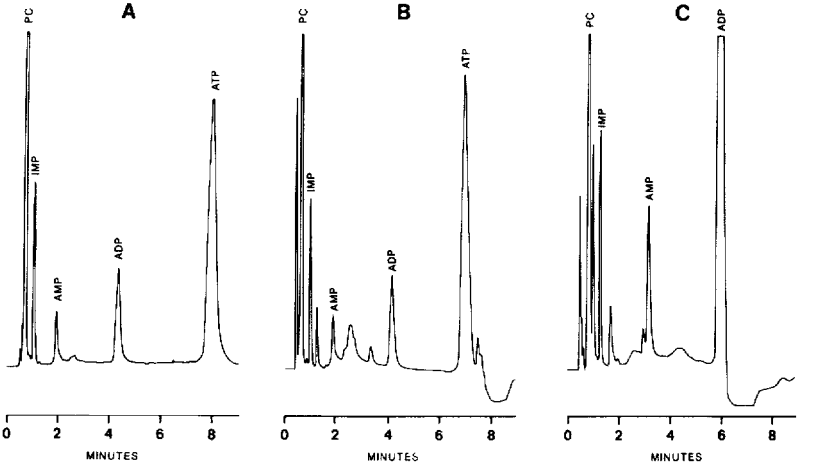

Fig 2 Influence of pH and perchlorate concentration on ion-pair HPLC Typical chromatograms are shown for a standard solution (A) and a perchlorate-saturated standard solution eluted at pH 7 0 (B) and pH 5 5 (C) The standard solutions were diluted 60-fold before injection For elution conditions, see Experimental

TABLE III

INFLUENCE OF pH ON THE RETENTION TIMES OF ATP, ADP, AMP, IMP AND PC AT DIFFERENT PERCHLORATE CONCENTRATIONS

For elution conditions, see Experimental

pH	Perchlorate concentration (mg ml^{-1})	Retention time (min)				
		ATP	ADP	AMP	IMP	PC
7 00	0 00	8 17	4 67	2 19	1 16	0 81
	1 66	8 00	4 64	2 18	1 16	0 81
	5 00	7 60	4 59	2 18	1 16	0 81
	10 00	6 93	4 45	2 15	1 16	0 80
6 70	0 00	9 52	5 13	2 34	1 22	0 75
	1 66	8 87	5 10	2 31	1 19	0 75
	5 00	8 36	4 99	2 30	1 19	0 74
	10 00	7 72	4 92	2 29	1 19	0 74
6 35	0 00	11 68	5 68	2 63	1 35	0 81
	1 66	10 01	5 62	2 63	1 33	0 81
	5 00	9 34	5 58	2 63	1 34	0 81
	10 00	8 05	5 50	2 63	1 34	0 80
5 50	0 00	N D [a]	8 45	3 86	1 72	1 04
	1 66	N D	8 45	3 86	1 73	1 05
	5 00	N D	8 40	3 81	1 73	1 03
	10 00	N D	8 36	3 77	1 71	1 03

[a] N D = not detectable

DISCUSSION

The determination of nucleotides and other high-energy phosphates in perchloric acid extracts of muscle tissues is often achieved with isocratic ion-pair HPLC. Chromatographic parameters such as pH, ionic strength and the concentrations of ion-pair reagent and modifier have been optimized for several compositions of nucleotides in muscle tissues of different origins [1–16]. Perchloric acid is a preferred extraction solvent, because it has good extraction properties and can be removed as the potassium salt after neutralization. However, despite its poor solubility, potassium perchlorate is soluble in water to a certain extent (10 mg/ml) and therefore some perchlorate will remain in the muscle extract. A knowledge of the influence of perchlorate on ion-pair liquid chromatography is very limited, and there are only a few indications in the literature about the negative effect of perchlorate on the quality of nucleotide separations.

From our results, it can be concluded that perchlorate disturbs the chro-

matography of nucleotides in more than one way. First, the retention time of ATP and to a lesser extent those of ADP and AMP decreased in the presence of perchlorate in the standard solution This effect is greater at lower pH. Second, after several injections with perchlorate-containing solutions, the ATP peak split in two components. This effect was also more pronounced at lower pH. Moreover, this effect seemed to occur faster when the perchlorate concentration in the sample solution was higher. Tomlinson et al. [10] described the ion-pairing properties of perchlorate with organic ammonium ions. Both tetrabutylammonium ions and adenosine-containing nucleotides have a high affinity for perchlorate ions The changes in the retention times of ATP, ADP and AMP are therefore probably caused by the ion-pairing capacity of perchlorate. Splitting of the ATP peak occurred together with an increase in the pressure of the analytical column and seemed to be related to the amount of perchlorate injected into the column. Probably the analytical column was progressively loaded with perchlorate, which was not removed during elution with the normal eluent This loading of perchlorate caused an increase in the pressure on the system to unacceptable levels and further analysis was impossible. Since at this stage the determination of nucleotides is impossible, the analytical column has to be regenerated before further applications can take place

The mechanism of interaction between perchlorate and the tetrabutylammonium complexes of ATP and ADP is unclear. UV spectra of ATP in the eluent in the presence and absence of perchlorate were not significantly different However, it is likely that a number of tetrabutylammonium ions are involved in the interactions. Moreover, such an interaction would be pH-dependent (Table III). Clearly, it is necessary to minimize the amount of perchlorate in the muscle extracts that have to be analysed by chromatography. An excess of potassium ions and precipitation of potassium perchlorate directly after freezing and thawing in a cooled centrifuge limit the amount of perchlorate left in muscle extracts If possible (depending on the detection limits and the amount of available muscle tissue), the muscle extracts should be diluted to decrease the perchlorate concentration further. By using a relatively high pH of the eluent (6.5–7 0), the contamination of the column proceeds fairly slowly. It is further advisable that the column be regenerated regularly to prevent a decrease in accuracy of the analyses Although we were not able to prevent the influence of perchlorate completely, we are able to obtain good analyses of muscle extracts by isocratic ion-pair liquid chromatography

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