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Determination of adenine nucleotides by fluorescence detection using high-performance liquid chromatography and post-column derivatisation with chloroacetaldehyde

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Abstract

A novel rapid method for the analysis of adenine nucleotides in cells and tissues using post-column derivatisation with chloroacetaldehyde (CAA) followed by fluorescence detection is described. The CAA is incorporated in the eluent, but only reacts post-column when the temperature is elevated to 100°C. Samples are chromatographed following neutralisation of acid extracts. Examples are given using both trichloroacetic acid extraction for cells in culture, and perchloric acid for murine liver.

1. Introduction

The determination of the relative concentrations of the adenine nucleotides adenosine mono-, di- and tri-phosphate, (AMP, ADP, and ATP, respectively) in tissues is of value in assessing the energy state of the cell. A measure of this is the "adenylate energy charge" as defined by Atkinson and Walton [1] as the ratio (ATP + 1/2ADP)/(ATP + ADP + AMP). This parameter has been employed as a measure of cellular viability in a wide range of experimental systems, including assessment of environmental stress in marine organisms [2,3], in transplant surgery and studies on reperfusion injury [4,5], and for the determination of energy status in tumours in experimental animals [6].

A large number of chromatographic techniques have been employed for the measurement of these nucleotides, including ion-exchange, reversed-phase and ion-pair reversed-phase methods, generally using UV detection at 254 or 260 nm, for example [7]. This use of a relatively non-specific detection wavelength frequently results in long analysis times in order to separate the compounds of interest from other nucleotides present in tissues, particularly to obtain an accurate estimate of the AMP which is present at only low concentrations.

Post-column detection of phosphate has been suggested as a means of specifically detecting nucleotides [8]. However, the reaction requires the use of two separate reagents, one of which contains 1.5 *M* sulphuric acid, and two reaction coils. Also, optimal detection was at 880 nm, a wavelength not available on most HPLC detec-

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tors. Adenine containing compounds will react relatively specifically with haloacetaldehydes to give highly fluorescent products [9], and both chloro- and bromo-acetaldehyde have been used as pre-column derivatisation reagents for the determination of adenine nucleotides [3,10–15]. However, the reaction is relatively slow with long incubation times or high temperatures required. Chloroacetaldehyde (CAA) typically requires 30 min at 100°C [10], or up to 24 h at lower temperatures [11]. Even the more reactive bromoacetaldehyde, which is not commercially available, takes 8 min at 100°C [16]. The nucleotides are not very stable at elevated temperatures, particularly at slightly acidic pH [13,16], so some degradation will inevitably occur. Hydrolysis of ATP yields ADP and AMP, so that a small decrease in ATP, which is present at a high concentration will result in a much larger increase in ADP and AMP, giving a low value for the energy charge.

This paper describes a method for the fluorescent detection of adenine nucleotides following post-column derivatisation with CAA, and illustrates the method with tissue samples analyzed after perchloric acid (PCA) extraction and neutralisation or cell culture samples extracted with trichloroacetic acid (TCA).

2. Experimental

2.1. Chemicals

CAA (50%) was from Aldrich (Poole, UK), nucleotides, enzymes and tetra-*n*-butyl ammonium hydrogen sulphate (TBA) were from Sigma (Poole, UK), acetonitrile and methanol were from Rathburn Chemicals (Walkerburn, UK). All other chemicals were from Merck (Lutterworth, UK).

2.2. Chromatography

Chromatography was carried out using a Waters 820 system which included two 510 pumps, a WISP 712 autosampler, a 486 variable-wavelength detector set at 260 nm (Millipore, Watford, UK), and an LS40 fluorimetric detector (Perkin-Elmer, Beaconsfield, UK), using 230 nm and 430 nm as the excitation and emission wavelengths, respectively. Separation was achieved using gradient elution at a flow-rate of 1.6 ml/min, using ion pairing on a Hypersil 5ODS column (125 × 4.6 mm I.D., particle size 5 μm), with a Hypersil guard cartridge (10 × 2 mm, particle size 5 μm) (Hichrom, Reading, UK). The solvents used for tissues were: (A) 100 m*M* potassium phosphate, 8 m*M* TBA, pH 6.0, 15 m*M* CAA; (B) 75% acetonitrile, 25% water, with a linear gradient from 3 to 35% B in 6 min, returning to initial conditions in 1 min. For cells in culture, the conditions were: (A) 38 m*M* citric acid, 62 m*M* dipotassium hydrogen orthophosphate, 8 m*M* TBA, pH 4.5, 15 m*M* CAA; (B) as above, with a linear gradient from 2–20% B in 5 min. The post-column reaction was carried out at 100°C using a home-made 2-ml (1.25 min) knitted reaction coil made from 10 m PTFE tubing (0.5 mm I.D., 1.6 mm O.D.) [17] folded into an aluminium tube filled with a thermally-conductive resin (Devcon, RS Components, Corby, UK), to fit a Kratos URA100 post-column reactor (Applied Biosciences, Warrington, UK). The reaction coil was placed between the UV detector and the fluorescence detector.

2.3. Sample preparation

Tissue samples were freeze-clamped between two aluminium plates pre-cooled in liquid nitrogen, ground to a powder under liquid nitrogen, weighed and frozen at −70°C until analysis. The frozen powdered tissues were stable for several weeks. For analysis, the tissues were homogenised in 9 ml of 2.5% PCA/1 g tissue. Aqueous standard solutions of ATP, ADP and AMP were diluted in the PCA. A 200-μl sample was neutralised with 2 *M* KOH–0.2 *M* K_2HPO_4 on ice, and the potassium perchlorate spun off (9500 *g*, 1 min) at 4°C.

Aliquots of cells ($\sim 0.5 \cdot 10^6$) grown in suspension were spun down, and the supernatant discarded. A 250-μl volume of 5% TCA was added to the pellet, the tube vortex-mixed, spun, and a 200-μl aliquot of the supernatant neutralised with 2 *M* KOH–0.2 *M* K_2HPO_4. Samples could

be stored at this stage for several weeks at −20°C, without loss of ATP.

Peak purity was checked by enzymic degradation of the nucleotides with hexokinase, myokinase and 5′-adenylate deaminase [18].

3. Results

Fig. 1 shows the fluorescence chromatograms resulting from the injection of 25 μl of a standard containing 50 nmol/ml ATP, ADP and AMP, which illustrates that with a reaction time of 1.25 min, fluorescent CAA adducts of the nucleotides are formed.

Fig. 2a,b shows the UV and fluorescence chromatograms of a mouse liver extract, illustrating the reduced level of interfering peaks using post-column derivatisation with fluorescence detection. The absence of underlying interfering peaks in the fluorescence trace was demonstrated by enzyme preincubation of the sample with hexokinase which removes ATP, myokinase (ADP) and 5′-adenylate deaminase (AMP) (Fig. 3a,b).

Fig. 4 illustrates the technique applied to an extract of EMT6 tumour cells grown in culture, and the large interfering peak which necessitated

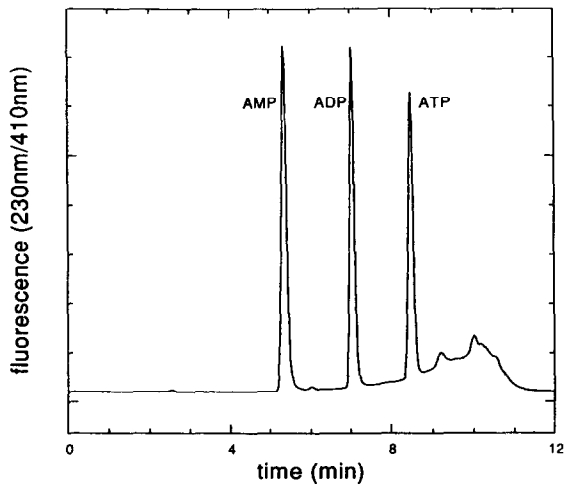

Fig. 1. Chromatogram of a neutralised adenine nucleotide standard prepared in PCA (50 μM AMP, ADP, ATP). Chromatographic conditions were as described for tissues in the Experimental section.

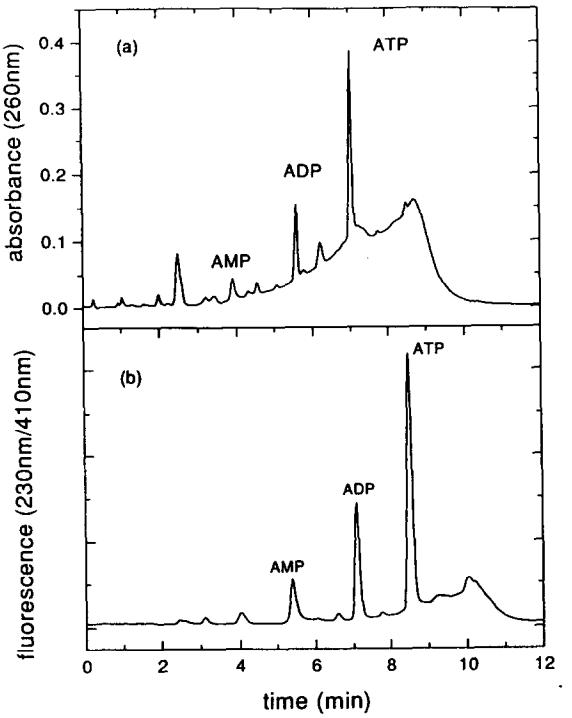

Fig. 2. Chromatograms of a neutralised PCA freeze-clamped liver extract with (a) absorbance and (b) fluorescence detection. Chromatographic conditions were as described for tissues in the Experimental section.

the different eluent for cells can clearly be seen eluting just before the ATP. Using the tissue eluent, this peak elutes very close to the AMP. Comparing the UV and fluorescence traces also highlights the specificity of the derivatisation; the labelled peaks for AMP and ADP on the UV chromatogram correspond to the elution times of the standard, but clearly from their relative size contain coeluting peaks.

Calibration curves were constructed in PCA for the three components from 0.1 to 100 nmol/ml which were found to be linear using a computer-based linear least-squares program (correlation coefficients, $r > 0.9998$). Intra- and inter-assay precision and accuracy is shown in Table 1 while Table 2 shows data for replicate analyses of a liver extract. The detection limit was around 10 pmol on column injection (signal-to-noise ratio = 5). Recovery of the compounds relative to an aqueous standard was high (101% for ATP,

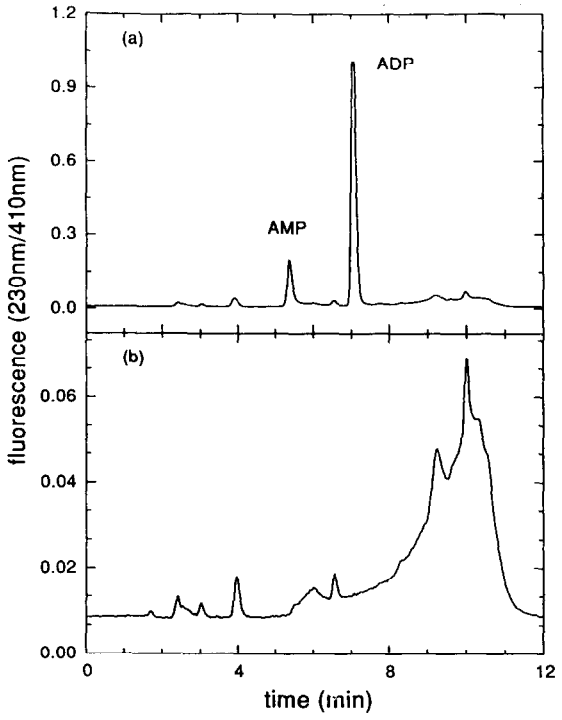

Fig. 3. Chromatograms of a liver extract following enzymatic digestion with (a) hexokinase, and (b) hexokinase, myokinase and 5'-adenylate deaminase. Chromatographic conditions were as described for tissues in the Experimental section.

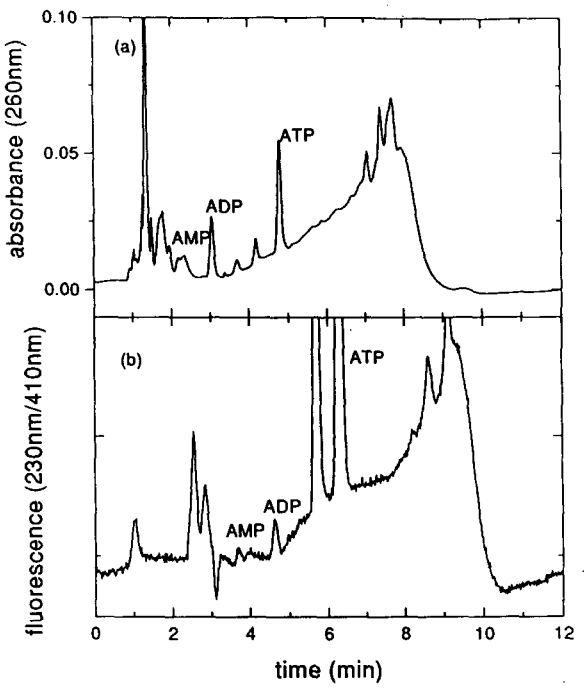

Fig. 4. Chromatogram of a neutralised TCA EMT6 cell extract with (a) absorbance and (b) fluorescence detection. Chromatographic conditions were as described for cells in culture in the Experimental section.

109% for ADP, and 104% for AMP). Very similar results were obtained for TCA (data not shown).

4. Discussion

The data presented show that with a simple gradient on a short column, it is possible, using the specificity conferred by the post-column reaction with CAA, to determine the concentrations of the three adenine containing nucleotides in only 11 or 12 min. The technique is very robust because of the lack of interfering peaks, and indeed it may be possible to significantly shorten the analysis. Our experience with the use of reversed-phase systems for nucleotide separations, with or without ion-pairing, is that it is often difficult to maintain a separation over prolonged periods. This method is simple because the reagent can be included in the eluent because of its low reactivity, so there is no requirement for an additional pump to add the reagent post-column. Because derivatisation is incomplete during the post-column reaction however, it will be important to maintain good flow and temperature control to obtain reproducible results. The linearity of the calibration and the reproducibility data in the tables would indicate that this can be achieved. The CAA is also inexpensive, which is an important consideration for any post-column derivatisation technique. One possible complication with the CAA is its potential reactivity with certain polymeric materials. We began this study using a commercially available reaction coil constructed from Tefzel (Kratos), which is reported to be susceptible to attack by some chlorinated chemicals; after some weeks there was a gradual rise in back-pressure which eventually resulted in rupture of the coil.

Table 1
Intra- and inter-assay precision and accuracy of the HPLC procedure for adenine nucleotides

Component	Intra-assay		Inter-assay	
	R.S.D (%)	Accuracy (%)	R.S.D. (%)	Accuracy (%)
AMP	0.67	100.3	1.34	102.4
ADP	0.41	99.8	0.34	99.5
ATP	0.22	100.2	0.31	100.3

We subsequently knitted our own coil from PTFE which has been used without problems.

For the measurement of tissue nucleotides, the method as described gives more than adequate sensitivity, and because the CAA is completely non-fluorescent, the background fluorescence signal is extremely low, contributing to the sensitivity of the technique. The method is also sufficiently sensitive to allow the measurement of nucleotides in extracts of cells grown in culture. In this case the cell medium contained a peak which interfered with the AMP peak. Use of a citrate/phosphate eluent with a lower pH obviated this problem.

Use of trichloroacetic acid as extractant also proved satisfactory, although the recoveries obtained with PCA did not indicate any problem with the loss of nucleotides which has been reported to occur on precipitation of the potassium perchlorate [19]. Nor did we find any evidence for accelerated loss of ATP in PCA-extracted samples due to residual ATPase activity, as suggested by a recent report with cultured human cells [20]. However, we did find that it was very important to remove as much potassium perchlorate as possible by keeping the samples chilled; if this was not done, we experienced chromatographic problems with residual perchlorate, as has been reported by other workers [21].

Table 2
Mean and standard deviation of replicate analyses of a liver extract

Component	Concentration (mean ± S.D.) (m*M*)
AMP	0.205 ± 0.013
ADP	0.819 ± 0.020
ATP	2.274 ± 0.020

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