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Note

High-performance liquid chromatographic determination of adenine nucleotides in biological materials

Improvements and adaptations to routine analysis

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A variety of similar methods for the determination of adenine nucleotides in biological materials by high-performance liquid chromatography (HPLC) has already been published (see ref. 1–6 and references cited therein). However, important information concerning factors such as the day-to-day precision of the assay, the stability and lifetime of the HPLC column is generally lacking. This information becomes particularly important when the method is to be used routinely for analysis of a large number of samples.

A suitable method for performing serial assays ought to be as simple as possible, especially with regard to the sample work-up procedure and the analytical instrumentation. In addition, for reasons of capacity and sensitivity, the analysis time should be as short as possible. It is therefore necessary to avoid, if possible, any solvent gradient.

With this in mind I have adapted a procedure described in the literature[1] for the determination of ATP, ADP and AMP in rat heart and brain. The tissue was homogenized in nine volumes of 0.3 *M* perchloric acid and centrifuged; 1 volume of supernatant was added to 19 volumes of HPLC column buffer (pH 6.5) [10.5 g potassium dihydrogen phosphate, 5.6 g potassium hydroxide, 8.0 g citric acid dihydrate, 850 mg tetrabutylammonium phosphate (Sigma, St. Louis, MO, U.S.A.) and 1000 g water]. A 20-μl aliquot of the resulting solution [perchloric acid–column buffer (1:19, v/v)] was injected. Flow-rate: 1 ml/min. HPLC column: LiChrosorb RP-8, 7 μm (E. Merck, Darmstadt), 4.6 × 150 mm. Temperature: 40°C. Under these conditions the adenine nucleotides of interest are reasonably separated (retention times, min: AMP, 4.0; ADP, 4.8; ATP, 6.6) and detected by UV spectroscopy at 254 nm.

Two problems, however, made this procedure unsuitable as a routine assay. First, after a short period of time, independent of the number of samples injected, the three peaks of interest became distorted and each ultimately split into two peaks. Secondly, with increasing number of samples injected, the retention times gradually decreased so that the column had to be replaced after approximately 50 sample injections.

Both problems could be solved. The split-peak phenomenon seemed to be related to the amount of column buffer which had been pumped through the column.

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I noticed that when the peaks became split, the top of the column was discoloured. Since this occurred independently of the number of samples injected, I hypothesized that some impurity contained in the column buffer (probably from the ion-pairing agent) might be retained by the column and accumulate at its top, causing (for unknown reasons) the split peaks. This was confirmed when a column (4.0 × 4.6 mm), packed with the same material (RP-8, 7 μm; or RP-2, 7 μm, see below) and connected between the pump and the injector completely prevented the occurrence of any split peaks. This "pre-column" was replaced every 500 injections. Since its function is that of an on-line filter and the samples injected do not pass through it, the quality of packing is irrelevant and can easily be carried out without any special equipment.

The second problem is quite common in HPLC. The shortening of the retention times as a function of the number of injections can in general be corrected by decreasing the flow-rate and/or the column temperature. These measures, however, have their limits. An almost unlimited possibility for adjusting retention times is available in this system by increasing the concentration of ion-pairing agent (tetrabutylammonium phosphate) in the mobile phase. As shown in Fig. 1A and B, a satisfactory separation of adenine nucleotides with the same column can still be achieved even after the injection of approximately 500 tissue samples, simply by increasing the concentration of the ion-pairing agent to 450 mg/l, while with the low

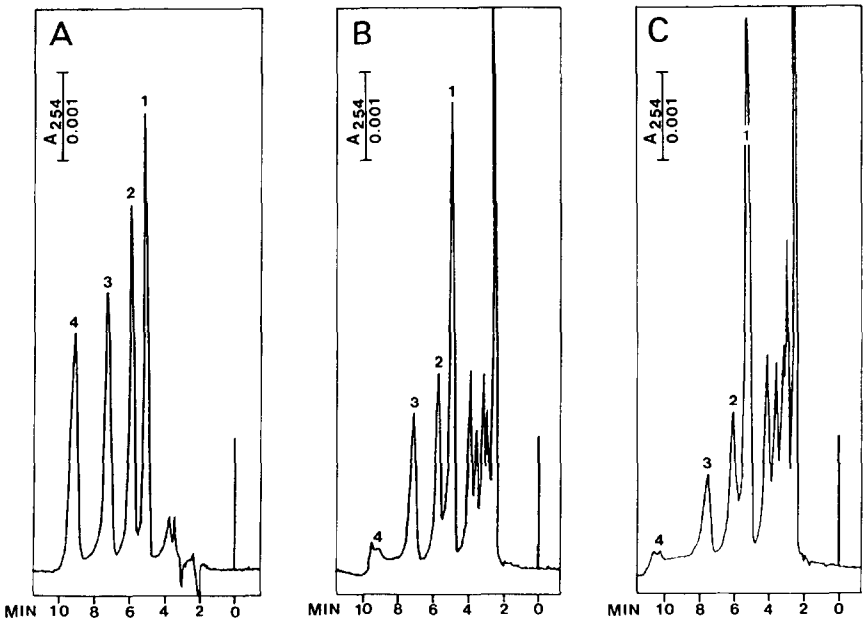

Fig. 1. Separation of adenine nucleotides on RP-2. Peaks: 1 = AMP; 2 = ADP; 3 = ATP; 4 = adenosine. Column: LiChrosorb RP-2, 7 μm (E. Merck), 150 × 4.6 mm; temperature 30°C. Flow-rate: 0.9 ml/min. Mobile phase: 10.5 g potassium dihydrogen phosphate, 5.6 g potassium hydroxide, 8.0 g citric acid dihydrate, 1000 g water, 450 mg (A and B) or 250 mg (C). tetrabutylammonium phosphate. Detection: UV, 254 nm. A, Injection of standards, 1–4 (0.1 nmol each), on a used column with high ion-pairing agent concentration (450 mg/l). B, Injection of rat brain extract, corresponding to 0.1 mg wet weight, on a used column with high ion-pairing agent concentration (450 mg/l). C, Injection of rat brain extract, corresponding to 0.1 mg wet weight, on a new column with low ion-pairing agent concentration (250 mg/l).

starting concentration of ion-pairing agent (250 mg/l, Fig. 1C) the peaks would have fused completely. In order to achieve an adequate separation of adenine nucleotides on a new RP-8 or RP-18 column, 3–6 times more ion-pairing agent is needed than on a RP-2 column. Consequently, on RP-2 the retention times can be corrected more easily by addition of less ion-pairing agent than on other RP materials. Therefore I now use LiChrosorb RP-2, 7 μm, for routine analysis of adenine nucleotides.

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