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Note

Isocratic ion-pair reversed-phase high-performance liquid chromatographic analysis of ATP and ADP

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The critical role of ATP in nucleotide metabolism has been reviewed by Bridger and Henderson [1]. ATP concentrations and ATP-to-ADP ratio vary among species and cell types and may change depending on physiological conditions. The ATP-to-ADP ratio is indicative of the cell energy level and is particularly important for biochemical studies which involve phosphorylation of nucleosides and nucleotides because this reaction requires ATP. McKeag [2] and Perone [3] recently reviewed the high-performance liquid chromatographic (HPLC) assays of adenine nucleotides. ADP and ATP have been analyzed by ion-exchange, ion-pair reversed-phase (IP-RP) and zwitterion-pair HPLC [2,3]. The published IP-RP-HPLC assays used tetrabutylammonium (TBA) as the counter ion [2,3]. The analysis time ranged from 20 min by isocratic elution at a flow-rate of 3.5 ml/min to 35 min by gradient elution at 2 ml/min [2,3].

We previously described an IP-RP-HPLC assay for the ribose and deoxyribose nucleosides and nucleotides (mono-, di- and triphosphates) of 5-fluorouracil, using a combination of TBA and tetraethylammonium (TEA) ions [4]. Variation of the concentrations and ratios of these two counter ions provided excellent flexibility in the separation of the nucleotides. The present report describes the application of the same approach to the separation of ADP and ATP. This assay has a reduced analysis time than previously published procedures, uses isocratic elution at a relatively low flow-rate, and results in symmetrical nucleotide peaks. This method was used to quantify the concentrations of ADP and ATP in rat and human cells.

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EXPERIMENTAL

Chemicals

All HPLC solvents and reagents were of spectroquality or analytical grade and were purchased from Mallinkrodt (Paris, KY, U.S.A.). Disodium salts of ADP and ATP, and TEA hydroxide (20%, w/w) were purchased from Sigma (St. Louis, MO, U.S.A.) and TBA hydrogen sulfate from Aldrich (Milwaukee, WI, U.S.A.). The purity of ATP, analyzed by HPLC, was >99%, whereas ADP contained 7% of AMP as impurity. The TEA solution was filtered before use.

Apparatus

HPLC analysis was performed using an isocratic HPLC elution with UV absorbance detection. The equipment (Waters Assoc., Milford, MA, U.S.A.) was as described earlier [4]. Peaks were integrated using a HP3390 integrator (Hewlett-Packard, Menlo Park, CA, U.S.A.).

Cell procurement

Tissues were obtained from female Fischer rats, three to six months old (Charles River, Kingston, PA, U.S.A.), euthanized by cervical dislocation or diethyl ether. Femur and tibia were flushed with ice-cold saline to harvest bone marrow cells. The unwanted red blood cells were lysed by hypotonic shock, and the remaining cell pellet, obtained after centrifugation at 800 *g* at 4°C for 3 min, was resuspended in RPMI-1640 medium (Gibco). The method of Weiser [5] was modified and used to isolate the epithelial crypt cells from the small intestines. Cultured human Hep-2 cells were obtained from Dr. E. Woltering at the Department of Surgery of the Ohio State University. Cell viability was determined by the dye exclusion test using 0.02% Trypan blue dye solution.

HPLC analysis

The cellular macromolecules were precipitated with acetonitrile, and water was added to extract the cellular contents. Acetonitrile (300 μl) and water (200 μl) were used for $1 \cdot 10^6$–$5 \cdot 10^6$ cells. A 50- or 100-μl volume of the cell extract which contained the macromolecule-free nucleotides was placed under a stream of nitrogen for 5–10 min to remove the acetonitrile. The remaining aqueous fraction, about 20–50 μl, was injected into the HPLC system and analyzed for adenine nucleotides. The stationary phase was a Novapak C_{18} column pressurized in a Z-module compression chamber (Waters Assoc.). The aqueous mobile phase contained 20 m*M* TEA, 0.5 m*M* TBA, 20 m*M* sodium acetate, 5 m*M* sodium and potassium phosphate buffer of pH 7.38, and 10% methanol. The pH of the mobile phase was adjusted to 7.0 with acetic acid. The column was equilibrated with the mobile phase at a flow-rate of 4 ml/min for about 1 h. ATP and ADP were separated by isocratic elution at a flow-rate of 2 ml/min and monitored by their UV absorbances. The absorbance ratios at 254/280 nm were 5.6, 5.1 and 4.5 for AMP, ADP and ATP, respectively, and 2.0 and 1.8 for UTP and GTP, respectively, which coeluted just prior to ADP. These ratios were used to identify the eluted peaks. Standard curves were obtained from the UV absorbance peak areas after

injections of known amounts of ADP and ATP and were used to determine the concentrations of ADP and ATP in cell extracts.

RESULTS AND DISCUSSION

HPLC analysis of ADP and ATP

The elution volumes of ADP and ATP were 24 and 50 ml, respectively. Fig. 1 shows the HPLC profiles of extracts of freshly isolated cells and cells boiled for 15 min. The boiled rat intestinal epithelial cells did not contain adenine nucleotides, while the freshly isolated cells contained ADP and ATP. AMP, UTP and GTP were also detected, but UTP and GTP were not separated. The elution volumes of AMP, UTP and GTP were 16, 20 and 21 ml, respectively. Standard curves of ADP and ATP were obtained using their UV-absorbing peak areas after injection of known amounts of ADP and ATP. The peak areas were proportional to the amounts injected. The coefficients of determination (r^2) of the regression lines were 0.994 for ADP and 0.990 for ATP. The assay precision at a concentration range of 100–1000 ng per injection, expressed as coefficients of variation from results of four to five injections, were between 1 and 2% for ADP and 1 and 5% for ATP.

Under constant concentrations of TBA (0.5 m*M*) and buffers, addition of TEA reduced the retention of ADP and ATP. For example, the retention volumes of ADP were reduced from 26.0 to 23.2 and 19.7 ml, and those of ATP were reduced from 59.7 to 45.1 and 31.9 ml, when the TEA concentrations were increased from 10 to 15 and 25 m*M*. The reduced retention of ADP and ATP by addition of TEA to TBA-containing mobile phase is consistent with our previous contention of ion-pair formation between the elute and the counter ions in the mobile phase, and that TEA competes with TBA for the nucleotides [4]. An optimal mixing of the two counter ions with different chain lengths gave a baseline separation of ADP and ATP by isocration elution with a short analysis time (within 30 min).

Reduced elution times of both compounds were observed when unfiltered TEA

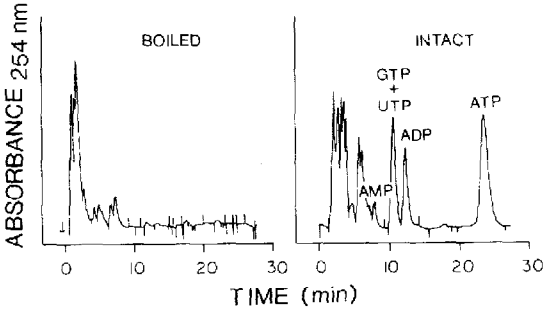

Fig. 1. HPLC profiles of (left) an extract of intestinal crypt cells boiled for 15 min and (right) an extract of freshly isolated intestinal crypt cells. The stationary phase was Novapak C_{18}, pressurized in a Z-module compression unit. The mobile phase was an aqueous mixture of 20 m*M* TEA, 0.5 m*M* TBA, 20 m*M* acetate, 5 m*M* phosphate and 10% methanol. The pH was adjusted to 6.0 with glacial acetic acid. The flow-rate of the isocratic elution was 2 ml/min.

was used to prepare the mobile phase. The commercially available 20% (w/w) solution of TEA upon storage formed a precipitate, which dissolved when added to the mobile phase. It was suggested by the manufacturer (Aldrich) that this precipitate was the carbonate salt of TEA. The anionic carbonate could compete with the phosphate groups of the nucleotides for the ion-pairing reagent and hence reduce the retention of the ionized nucleotides. Consistent with this speculation, we found that addition of 10–20 μM sodium bicarbonate to the mobile phase reduced the retention of ADP and ATP.

Cellular ATP and ADP levels

Table I summarizes the amounts of ADP and ATP in rat and human cells. Data were normalized per 10^6 cells. The cultured human Hep-2 cells contained the highest ADP and ATP concentrations and showed the least variability between experiments. Of the freshly isolated rat cells, intestinal crypt cells had higher ADP and ATP concentrations, but bone marrow cells had the higher ATP-to-ADP ratio. The ATP-to-ADP ratio in the intestinal cells was highly dependent on the method of isolation (unpublished results). The ATP-to-ADP ratio in intestinal cells diminished from 6 immediately after isolation to 4 after 4 h incubation at 37 °C; this ratio in Hep-2 cells remained about 7 ($n=5$) after a 60-min incubation. Compared to the previously reported ATP-to-ADP ratios [1], the ratio in Hep-2 cells is similar to other cultured cells, while the ratios in rat bone marrow and intestinal cells were significantly higher than those reported in other freshly isolated mammalian cells [1].

TABLE I

CONCENTRATIONS OF ADP AND ATP IN FRESHLY ISOLATED RAT CELLS AND CULTURED HUMAN CELLS

Cells were extracted with acetonitrile and water, and analyzed by HPLC. Mean ± S.D. of five experiments.

Sample	Concentration (pmol per 10^6 cells)		ATP-to-ADP ratio
	ADP	ATP	
Bone Marrow			
Mean ± S.D.	88.7 ± 80.6	866 ± 476	14.3 ± 7.8
Range	23–217	296–1414	6.5–25.7
Intestinal crypt			
Mean ± S.D.	284 ± 77	1842 ± 1366	6.4 ± 3.7
Range	166–347	834–4172	2.4–12.3
Hep-2			
Mean ± S.D.	309 ± 60.3	2159 ± 434.3	7.22 ± 1.86
Range	209–358	1679–2798	4.73–8.96

CONCLUSION

The described method is rapid and sensitive. Further, column equilibration could be accomplished in the relatively short period of 1 h. This method is useful in biochemical studies which require routine analysis of ATP-to-ADP ratio.

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