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Short Communication

Nucleotide preparation from cells and determination of nucleotides by ion-pair high-performance liquid chromatography

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ABSTRACT

Procedures for the analysis of cellular purine and pyrimidine nucleotides are described. The commonly used perchloric acid and especially the trichloroacetic acid methods for nucleotide extraction interfere with ion-pair high-performance liquid chromatography, but we have developed such a system for the separation and determination of major cellular nucleotides in biological matrices, including tri-, di-, monophosphates, cAMP, cGMP, NAD, NADP, UDP-glucose and UDP-galactose. Compared with perchloric acid extraction, no degradation of the nucleotide standards used was observed with respect to triphosphates and other relatively unstable nucleotides. Cellular nucleotides were extracted by lysing cells in a hypotonic buffer containing an ion-pair reagent (tetrabutylammonium hydrogensulphate) to decrease enzymic degradation of nucleotides in combination with ultrafiltration of the cell lysate to remove compounds of higher molecular mass, for example enzymes. This method is a simple and reproducible procedure for investigating nucleotide pools in cells.

INTRODUCTION

The development and optimization of high-performance liquid chromatographic (HPLC) systems for the investigation of cellular nucleotide pools is a field of intensive research [1]. Nucleotides function as precursors in nucleic acid synthesis, as coenzymes or effectors of enzyme regulation in a broad spectrum of cellular reactions, and they are involved in cellular energy metabolism [1].

Ion-pair reversed-phase liquid chromatography (RPLC) has superseded ion-exchange chromatography, offering the possibility of simultaneous separation of substances of high and low polarities [2–4]. The commonly used procedures for extracting cellular nucleotides by perchloric acid (PCA) [5] and trichloroacetic acid (TCA) [6] interfere with ion-pair HPLC and, furthermore, have the disadvantage of degrading more labile triphosphates [7]. Reports on ion-pair RPLC of nucleotides mainly deal with the separation of standards [8]. Investigations on the ion pairing of nucleotides in biological matrices are limited, because the interference with other cellular compounds makes it difficult to separate and determine nucleotides in biological extracts [8].

We present an extraction procedure and an

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ion-pair RPLC separation for investigating nucleotides in cell matrices, which is an alternative to the commonly used methods.

EXPERIMENTAL

Chemicals

Reference standards of nucleotides (AMP, CMP, GMP, UMP, ADP, CDP, GDP, UDP, ATP, CTP, GTP, UTP, NAD, NADP, UDP-galactose, UDP-glucose, CDP-choline, cAMP and cGMP) of the highest analytical grade as well as potassium dihydrogenphosphate and tetrabutylammonium hydrogensulphate (TBAHS) were purchased from Sigma (Munich, Germany). Acetonitrile and potassium hydroxide were obtained from Merck (Darmstadt, Germany).

Extraction of cellular nucleotides

Rat mammary carcinoma cell line 1-C-2 was kindly provided by Dr. Scherf (Institute of Chemotherapy and Toxicology, German Cancer Research Center, Heidelberg, Germany). It was derived from a methylnitrosourea-induced mammary carcinoma growing in a female BD-VI rat at the stage of invasive growth. The 1-C-2 cell clone is characterized by 44 chromosomes and a doubling time of 14.7 h [9].

Cells were cultivated in 650-ml culture flasks to obtain a sufficient cell number ($3–4 \cdot 10^7$ cells). Cultivated cells were trypsinized, harvested by centrifugation and then treated according to one of the following two methods.

Method 1. The cell pellet was precipitated with 750 μl of ice-cold PCA (0.33 *M*) for 30 min (4°C), and after centrifugation (12 000 *g* for 3 min) the supernatant was neutralized with 250 μl of ice-cold K_2CO_3 (1 *M*). This solution was kept at 4°C for 20 min then centrifuged again (12 000 *g* for 10 min), and the supernatant was transferred into vials and stored at −80°C until analysis.

Method 2. The cell pellet was immediately frozen in liquid nitrogen to preserve the nucleotide pools of cells. After 10 min, a 1.5-ml volume of hypotonic buffer (15 m*M* TBAHS, pH 2.0) was added to lyse the frozen cells and to prevent enzymic degradation of nucleotides. The lysate was then centrifuged at 0°C through Centrisat tubes (Sartorius, Göttingen, Germany) at 2000 *g* to remove proteins and enzymes, transferred into vials and stored at −80°C until analysis.

High-performance liquid chromatography

The HPLC equipment consisted of a gradient system (Latek, Eppelheim, Germany) with a UV detector set at 260 nm, a Shimadzu CR 3A (Latek) combined with a refrigerated automatic injection system (Kontron, Munich, Germany). Two Hyperchrome columns, 250 mm × 4.6 mm I.D. (type NC04) and 53 mm × 46 mm I.D. (type SSC 04) (Bischoff, Leonberg, Germany), filled with 3-μm ODS Hypersil (C_{18}) (Shandon, UK), were used in series.

A gradient system was used. Buffer A consisted of 5 m*M* TBAHS and 20 m*M* KH_2PO_4, and buffer B of 5 m*M* TBAHS, 100 m*M* K_2HPO_4 and 10% (v/v) acetonitrile. The pH of both solutions was adjusted to 5.2 with KOH. The columns were equilibrated for 50 min at 0% buffer B before the separation started, and linear gradient from 0 to 100% buffer B in 120 min was used. After six to eight analyses, the columns were washed with 100 μl of dimethylsulphoxide (Merck), followed by four or five 100-μl volumes of acetonitrile and 100 μl of acetic acid (90%) to clean the columns and to prevent microbial growth.

RESULTS

The ion-pair HPLC elution profile of a standard mixture containing nineteen major cellular nucleotides is shown in Fig. 1. The concentrations were 5 n*M* for UDP-galactose and 2.5 n*M* for all other substances. This profile is characteristic of ion-pair HPLC, in that the nucleotides were separated in the following order: monophosphates, followed by diphosphates and finally triphosphates of cytosine, uracil, guanosine and adenosine. Furthermore, NAD, NADP, UDP-glucose, UDP-galactose and CDP-choline, as well as the second messengers cCMP and cAMP, were separated simultanously.

The recovery of a standard mixture of nucleotides exceeded 95% in the ultrafiltration method.

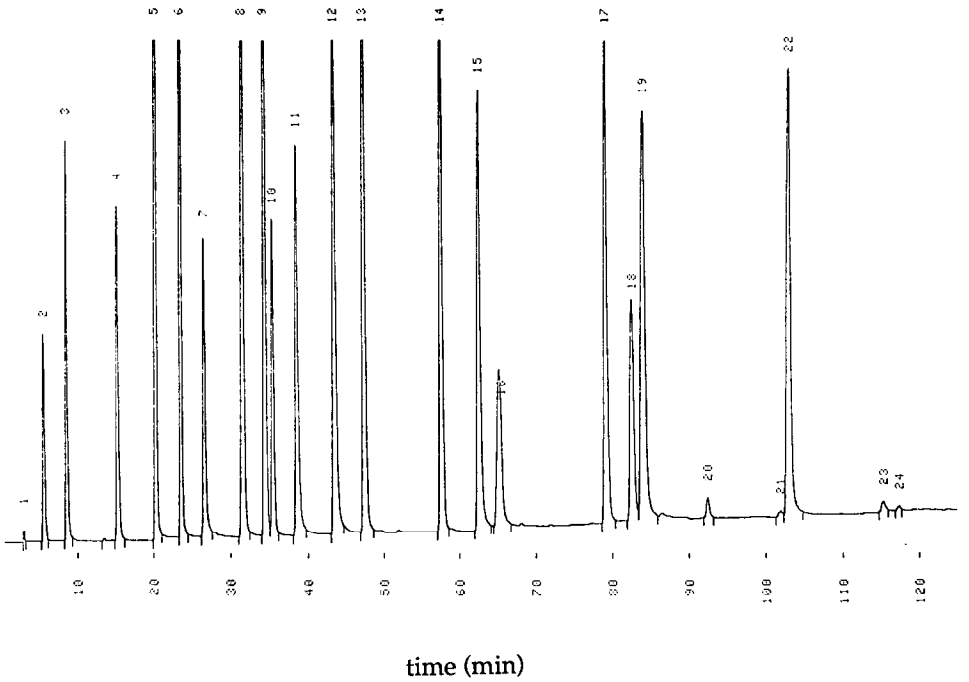

Fig. 1. HPLC–UV profile of a standard mixture containing nineteen nucleotides (5 n*M* UDP-galactose and 2.5 n*M* of each of the others): injection volume, 100 µl; detection wavelength, 260 nm; a.u.f.s., 0.005. Peaks: 1 = injection; 2 = CDP-choline; 3 = CMP; 4 = UMP; 5 = GMP; 6 = NAD; 7 = CDP; 8 = AMP; 9 = UDP-galactose; 10 = UDP-glucose; 11 = UDP; 12 = GDP; 13 = NADP; 14 = cGMP; 15 = ADP; 16 = CTP; 17 = cAMP; 18 = UTP; 19 = GTP; 22 = ATP; 20, 21, 23 and 24 = impurities of the standard solutions.

Following extraction by TCA no nucleotide measurement was possible because of interference in ion-pair RPLC. The nucleotide recovery of triphosphates and cyclic nucleotides obtained with PCA extraction method is shown in Table I.

TABLE I

RECOVERY OF TRIPHOSPHATES AND CYCLIC NUCLEOTIDES AFTER PRECIPITATION AND EXTRACTION WITH PCA COMPARED WITH THE STANDARD MIXTURE (100%)

Nucleotide	Recovery of the standard mixture (n = 2) (%)
ATP	63
GTP	96
UTP	92
CTP	61
cAMP	90
cGMP	89

ATP and CTP yielded recoveries following PCA precipitation that were distinctly lower than those after ultrafiltration. This suggests a sensitivity towards strong acids.

Table II shows a comparison of the determination of cellular nucleotides by the two methods. PCA extraction yielded higher recoveries of GTP and UTP, whereas ultrafiltration was superior to PCA extraction with regard to CTP, ADP, GDP and UDP. The recovery of ATP was similar with both methods.

The separation of the nucleotides from 1-C-2 rat mammary carcinoma cell lysate is shown in Fig. 2. The nucleotide peaks in the chromatogram of biological matrices were characterized by comparing the retention times with those of authentic nucleotides. The retention times of cyclic nucleotides were slightly different when cell lysates or standard mixtures were injected. Therefore, several cell lysates were spiked with

TABLE II

DETERMINATION OF CELLULAR TRIPHOSPHATE AND DIPHOSPHATE NUCLEOTIDES AFTER PRECIPITATION AND EXTRACTION WITH PCA COMPARED WITH THE ULTRAFILTRATION METHOD (100%)

Values are in nmol per $2 \cdot 10^6$ cells.

Nucleotide	Ultrafiltration (n = 2)	PCA extraction (n = 2)	Percentage of ultrafiltration
ATP	21.8 ± 2.4	21.4 ± 0.8	98
GTP	3.1 ± 0.3	3.5 ± 0.2	113
UTP	5.9 ± 1.1	7.7 ± 0.2	130
CTP	2.4 ± 0.4	2.1 ± 0.1	84
ADP	8.2 ± 0.5	6.2 ± 0.1	76
GDP	1.7 ± 0.1	1.5 ± 0.1	88
UDP	4.7 ± 0.2	4.0 ± 0.2	85
CDP	N.D.[a]	N.D.	

[a] N.D. = not determined.

authentic cyclic monophosphates. These additional substances coeluted with the corresponding cyclic nucleotide peaks of the cell lysate.

Depending on the molar absorptivities [10], the detection limit varied from *ca.* 30 p*M* for cytosine nucleotides to *ca.* 15 p*M* for adenosine nucleotides, using UV absorbance detection at 260 nm.

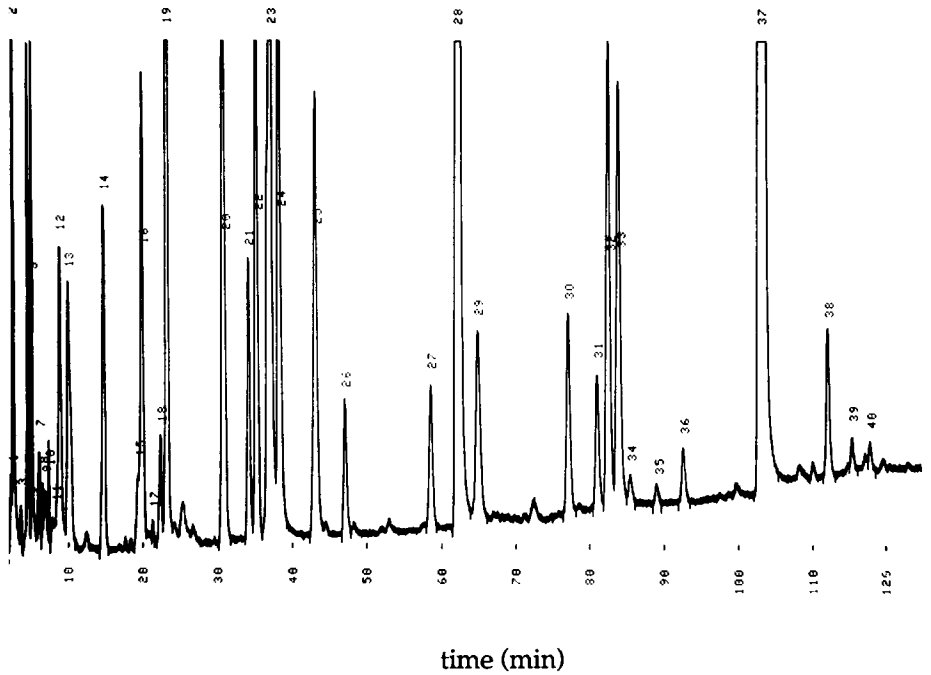

Fig. 2. HPLC–UV profile of the 1-C-2 cell lysate: injection, $2 \cdot 10^6$ cell equivalent; injection volume, 100 μl; detection wavelength, 260 nm; a.u.f.s., 0.005. Peaks: 12 = CMP; 14 = UMP; 16 = GMP; 19 = NAD; 20 = AMP; 21 = UDP-galactose; 22 = UDP-glucose; 24 = UDP; 25 = GDP; 26 = NADP; 27 = cGMP; 28 = ADP; 29 = CTP; 31 = cAMP; 32 = UTP; 33 = GTP; 37 = ATP; CDP not detected; other peaks were not identified.

DISCUSSION

Nucleotides fulfil many essential functions in biological systems. They are precursors in nucleic acid metabolism, in energy metabolism, and they have donor and regulatory functions in many cellular processes [1]. In preparing biological samples for nucleotide investigations, proteins and other macromolecules must be removed, because they can cause serious problems by clogging the columns and interfering with the analysis of nucleotides. For this purpose, strong acids are widely used for sample preparation, but they have the disadvantage of breaking down triphosphates to diphosphates [7,11,12].

Other methods, such as protein precipitation with salt, do not completely remove proteins, and extraction with organic solvents, such as methanol, is not useful because nucleotides have only a low solubility in organic solvents [11]. In the method described here, we combined ultrafiltration with lysis by a hypotonic ion-pair reagent buffer. The ion-pair reagent was added to reduce enzymatic degradation of sensitive nucleotides, such as triphosphates and cyclic monophosphates.

The advantages of ultrafiltration are that (1) no compounds that might interfere with HPLC analysis are added to the sample, (2) proteins and macromolecules are removed, and (3) the method is rapid and efficient.

A comparison of both methods for cellular nucleotide extractions shows that PCA has advantages with regard to GTP and UTP, which seem to be less sensitive towards strong acids, whereas ultrafiltration is superior for CTP. Determination of ATP levels was similar with both methods. Further reduction of enzymic nucleotide breakdown will allow full exploitation of the theoretical advantage of the ultrafiltation method, expected from the recovery of standards.

In summary, the procedure described for extracting nucleotides from cells and tissues in combination with the ion-pair HPLC, in which substances of high and low polarity can be separated simultaneously, is sufficiently simple and reproducible for the investigation of cellular nucleotide pools.

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