Journal of Chromatography, 385 (1987) 119–124
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 964

SEPARATION AND QUANTITATION OF BACTERIAL RIBONUCLEOSIDE TRIPHOSPHATES EXTRACTED WITH TRIFLUOROACETIC ACID, BY ANION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

P. K. DUTTA AND G. A. O'DONOVAN*
Department of Biological Sciences, North Texas State University, Denton, TX 76203-5218 (U.S.A.)

SUMMARY

Endogenous ribonucleoside triphosphates were determined in *Escherichia coli* and *Pseudomonas aeruginosa* by using anion-exchange high-performance liquid chromatography after extraction with trifluoroacetic acid (TFA). Results indicate that in *E. coli* this extraction method is more sensitive and reliable than trichloroacetic acid (TCA) and formic acid extraction. However, whereas in *P. aeruginosa* best yields of adenosine 5′-triphosphate and guanosine 5′-triphosphate are obtained following extraction with TFA, the yields of uridine 5′-triphosphate and cytidine 5′-triphosphate can be increased if extraction is performed with TCA.

INTRODUCTION

For the past twenty years, purine and pyrimidine metabolism in microorganisms has been a subject of intense research[1,2]. Since intracellular purine and pyrimidine nucleotide pools control the individual biosynthetic pathways producing the substrates for RNA (UTP, uridine 5′-triphosphate; CTP, cytidine 5′-triphosphate; ATP, adenosine 5′-triphosphate, and GTP, guanosine 5′-triphosphate) and DNA (dTTP, 2′-deoxythymidine 5′-triphosphate; dCTP, 2′-deoxycytidine 5′-triphosphate; dATP, 2′-deoxyadenosine 5′-triphosphate; and dGTP, 2′-deoxyguanosine 5′-triphosphate), accurate measurement of endogenous nucleoside triphosphates could serve as a diagnostic tool for the estimation of microbial growth rate. Moreover, GTP/ATP ratios[3] might also be a means of assessing growth rates in organisms that do not form colonies or readily lend themselves to conventional growth measurements.

Measurement of endogenous ribo- and deoxyribonucleotide pools has been revolutionized by the development of high-performance liquid chromatography (HPLC). One of the major emphases so far has been on refining HPLC procedures for nucleotide separation while very little effort has been made in developing extraction procedures for the detection and quantitation of low levels of nucleotide pools in microorganisms. During primary work in our laboratory involving quantitation of nucleosides and nucleotides in bacterial extracts, we devised a novel extraction procedure using trifluoroacetic acid (TFA) as solvent instead of the more conventional extraction solvents such as trichloroacetic acid (TCA) and formic acid.

0021-9673/87/$03.50

TFA is a clear, colorless volatile liquid with a very low UV cut-off point. TFA has been used as a solvent system for the purification and analysis of peptides by reversed-phase HPLC[4,5]. The present study was to compare TFA extraction of ribonucleoside triphosphates with known extraction by TCA and formic acid. Extraction with TFA was found to permit detection of nanomolar concentrations of ribonucleoside triphosphates, notably ATP and GTP in microbial samples.

EXPERIMENTAL

Chemicals and reagents

Nucleotides, TFA, TCA and tri-*n*-octylamine were purchased from Sigma (St. Louis, MO, U.S.A.); monobasic ammonium phosphate from Mallinckrodt (Paris, KY, U.S.A.), and 1,1,2-trichloro-1,2,2-trifluoroethane (Freon) from Eastman-Kodak (Rochester, NY, U.S.A.). All other chemicals were of analytical grade and were purchased from Fisher Scientific (Fair Lawn, NJ, U.S.A.).

Bacterial strains

Wild-type *Escherichia coli* Luria strain B and *Pseudomonas aeruginosa* were used in these studies.

Extraction of nucleotides from bacterial cultures

Bacterial cells were grown in M9 minimal medium with 0.2% (w/v) glucose as carbon source. The turbidity was measured with a photoelectric Klett-Summerson colorimeter, using a green filter No. 54. Growth was measured at 37°C and recorded as Klett Units (KU), where 1 KU = $1 \cdot 10^7$ cells/ml. Volumes of 50 ml of bacterial culture with a density of 100 KU were harvested and centrifuged at 4°C at 12 000 *g* for 2 min. The supernatant was decanted, and the cell pellet was used for nucleotide extraction.

TFA, TCA and formic acid extraction procedures

One ml of ice-cold 0.5 *M* TFA or 6% (w/v) TCA or 1.0 *M* formic acid was added to the cell pellet, which was then vortex-mixed for 2 min and allowed to stand at 4°C for 30 min before further centrifugation at 12 000 *g* for 10 min. The clear supernatant was then neutralized with ice-cold Freon amine[6]. The Freon amine–sample mixture was vortex-mixed for 2 min and then allowed to separate for 15 min at 4°C. The top aqueous layer, which contains the nucleotide extract, was removed, filtered through a 0.45-μm ACRO LC13 filter (Gelman Sciences, Ann Arbor, MI, U.S.A.) and frozen at −20°C until analyzed.

Chromatographic apparatus and conditions

The HPLC equipment (Waters Assoc., Milford, MA, U.S.A.) consisted of two Model 510 pumps, a Model 680 automated gradient controller, a U6K injector, and a Model 481 LC spectrophotometer. Nucleotides were detected by monitoring the column effluent at 254 nm with a sensitivity fixed at 0.05 absorbance units full scale (a.u.f.s.). Separations were performed on a Waters Radial-Pak Partisil SAX Cartridge (10 cm × 0.8 cm) using a Waters radial compression Z-module system. The elution buffer system used consisted of eluent A, 7 m*M* $NH_4H_2PO_4$ (pH = 3.8), and

eluent B, 250 mM $NH_4H_2PO_4$ (pH = 4.5) with 500 mM potassium chloride[7].

Nucleotide samples (100 μl) obtained from bacteria were injected into the column. A linear gradient of eluent A to eluent B was applied for 20 min followed by an isocratic period of 10 min with eluent B buffer. The column was regenerated by washing with 30 ml of eluent A (pH = 3.8) buffer. The flow-rate was maintained at 4 ml/min; analyses were performed at ambient temperature. Peaks were integrated either manually on a Houston Instrument (Austin, TX, U.S.A.) OmniScribe strip-chart recorder or with an Apple IIe computer system, using an ADALAB dual-slope integrating A/D converter and Chromatochart software (Interactive Microware, State College, PA, U.S.A.).

CALCULATIONS

The mole per gram dry weight for all nucleotides was computed as follows:

$$\frac{Sa}{St} \times C \times \frac{V}{Vi} \times \frac{1}{Dw}$$

where Sa = peak height of sample, St = peak height of standard, C = g compound in standard/molecular weight of compound, V = total volume of extract, Vi = volume of extract injected and Dw = dry weight.

RESULTS AND DISCUSSION

The 8-mm diameter of the radial compression column provided greater than six times the surface area of conventional steel columns. Consequently, the speed of separation was enhanced more than three times that originally reported[8] without any appreciable loss in resolution.

The procedure for initial sample preparation is a very critical step in the quantitation of bacterial ribonucleoside triphosphates for avoiding potential sources of error. The positions and heights or areas of the peaks of the ribonucleoside triphosphates are not reproducible. Therefore, the following precautions are recommended: (a) ice-cold Freon amine must be used for neutralization; (b) the cold Freon amine extract must be vortex-mixed after neutralization for 2 min and thereafter allowed

TABLE I

YIELD OF NUCLEOSIDE TRIPHOSPHATES FROM *E. COLI* CELLS (μmole/g DRY WEIGHT) WITH DIFFERENT EXTRACTION SOLVENTS

	TCA (6% w/v)	*Formic acid (1.0 M)*	*TFA (0.5 M)*
UTP	2.04	1.76	4.6
CTP	1.01	1.21	1.84
ATP	3.04	3.32	5.28
GTP	1.48	1.20*	2.96

* Derived by manual integration from a separate run.

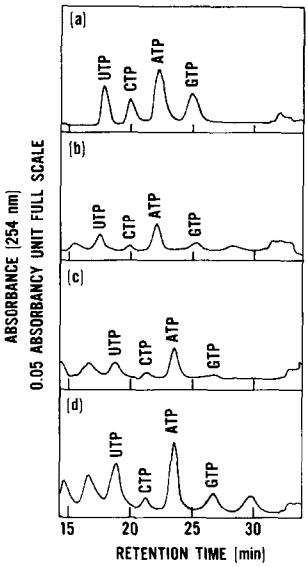

Fig. 1. Ion-exchange HPLC chromatograms of ribonucleoside triphosphates on a Radial-Pak Partisil SAX cartridge. (a) Standard ribonucleoside triphosphate mixture (100 μl) consisting of 10^{-5} *M* of each nucleotide. (b)–(d) Sample (100 μl) from *E. coli* cells after extraction with 6% (w/v) TCA, 1.0 *M* formic acid, and 0.5 *M* TFA, respectively.

to settle at 4°C for at least 15 min; (c) the clear, aqueous layer must be gently and carefully removed without disturbing the interphase. Often, the aqueous layer looks clear, even though the interphase is mixed with it. This interphase layer, which is composed of micellar aggregates, produces anomalous peaks.

Typically, TCA is used in our laboratoiry for extracting bacterial nucleotides. Since the use of this extraction procedure proved inadequate for estimating ribonucleoside triphosphates, especially ATP and GTP, different extraction solvents were tested. Initial results of repeated analyses following TFA extraction of ribonucleotides from *E. coli* produced a much greater increase in yields than TCA or formic

TABLE II

YIELD OF NUCLEOSIDE TRIPHOSPHATES FROM *P. AERUGINOSA* CELLS (μmole/g DRY WEIGHT) WITH DIFFERENT EXTRACTION SOLVENTS

The *P. aeruginosa* cells were grown at 37°C in glucose minimal medium and did not reach the density of 10^9 cells/ml. This is due to the fact that *P. aeruginosa* does not use glucose as a source of carbon very well. The pool levels are accordingly, slightly low.

	TCA (6% w/v)	*Formic acid (1.0 M)*	*TFA (0.5 M)*
UTP	2.6	2.1	1.64
CTP	1.04	0.96	0.52
ATP	4.2	4.1	4.84
GTP	2.68	1.9	2.96

TABLE III

EFFECT OF VARIOUS CONCENTRATIONS OF TFA ON RECOVERY (μmole/g DRY WEIGHT) OF RIBONUCLEOSIDE TRIPHOSPHATES IN *E. COLI* AND *P. AERUGINOSA*

For *P. aeruginosa*, see comment Table II. All values are the average of two separate analyses.

	E. coli				*P. aeruginosa*			
	0.25 M	*0.50 M*	*0.75 M*	*1.0 M*	*0.25 M*	*0.50 M*	*0.75 M*	*1.0 M*
UTP	4.52	4.6	4.32	4.56	2.08	1.64	1.68	2.1
CTP	1.04	1.84	1.0	1.32	0.76	0.52	0.55	0.68
ATP	4.68	5.28	4.08	4.72	6.16	4.84	4.96	6.04
GTP	2.76	2.96	2.68	3.16	2.6	2.96	2.64	3.4

acid extraction procedures (Table I). TFA extraction procedures resulted in sharper peak heights (Fig. 1), a major criterion for estimating yields accurately. The same extraction procedures were used with TFA as the extracting solvent for *P. aeruginosa*. Results (Table II) showed that yields of ATP and GTP similarly increased following TFA extraction procedure, although UTP and CTP can be best extracted following initial treatment with TCA.

Different concentrations of TFA were next tested to ascertain the optimal extraction concentration. In principle, the lowest concentration of TFA should be used to avoid losses of nucleotides. As Table III shows, for *E. coli*, 0.5 *M* TFA gave the best yields of UTP, CTP, and ATP, while the best yield of GTP was obtained with 1.0 *M* TFA. However, for *P. aeruginosa*, the best yields of ATP and GTP were obtained following extraction with 0.25 *M* and 1.0 *M* TFA, respectively. These data, therefore, clearly illustrate the importance of extraction procedures for individual ribonucleoside triphosphates, a point that has often been overlooked.

Another study[9] to evaluate the optimal extraction concentration of TCA showed that there was a twofold higher recovery of cytosine, guanine, and adenine nucleoside monophosphates from erythrocytes when 15% TCA was used instead of 6%. No such correlation was evident in our studies with TFA.

The manner in which TFA interacts with bacterial nucleotides to maintain their solubility is not known. TFA is a weak acid and thus degrades triphosphates to a lesser extent than TCA or formic acid. It is also possible that Freon amine neutralization of TFA is complete, whereas this is probably not the case for TCA or formic acid. Finally, TFA met all the basic extraction criteria such as (a) sensitivity, (b) stability, (c) ease of sample recovery because of its volatility, (d) it is clear and colorless and, (e) easily removable by Freon amine solutions. We have developed this procedure primarily to measure ATP and GTP in bacterial strains. However, this method should prove useful for investigating many other aspects of purine and pyrimidine metabolism in bacteria and other organisms.

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