Journal of Chromatography, 188 (1980) 149–158
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CHROM. 12,381

DETERMINATION OF PYRIMIDINE RIBOTIDE AND DEOXYRIBOTIDE POOLS IN CULTURED CELLS AND MOUSE LIVER BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

JONATHAN MAYBAUM, FREDERICK K. KLEIN and WOLFGANG SADEE*

University of California, San Francisco, School of Pharmacy, Department of Pharmaceutical Chemistry, San Francisco, Calif. 94143 (U.S.A.)

(Received August 10th, 1979)

SUMMARY

High-performance liquid chromatographic (HPLC) assay for determining tissue pools of uridine, deoxyuridine, cytidine, deoxycytidine, and thymidine mono-, di-, and triphosphates is presented. The method utilizes anion-exchange and, after conversion of nucleotides to nucleosides by acid phosphatase, reversed-phase chromatography on a preparative column with UV detection at 254 and 280 nm. The yield of this procedure is $80 \pm 2\%$ with a sensitivity limit of 100 pmole nucleotide per sample. A sensitivity of 10 pmole can be achieved for each compound by rechromatographing appropriate nucleoside fractions on analytical columns. The recovery, including this step, is $66 \pm 7\%$. The assay is reproducible and highly selective, with a lower sensitivity limit of approximately 0.1 μM using 150–250 mg (wet weight) tissue samples. Nucleotide pools have been determined in Balb/c mouse liver and in mouse lymphoma (S-49) cell culture, the latter with and without addition of 5-fluorouracil (5-FUra) to the medium. Data obtained with this assay are similar to those using alternative methodologies. Observed depletion of dTXP pools and expansion of dUMP and dCXP pools after 5-FUra treatment are in agreement with published observations. Pools of dUDP and dUTP were not detectable (< 10 pmole/10^8 cells) in any tissue sample. These data illustrate the utility of the present method in studying actions of pyrimidine antimetabolites.

INTRODUCTION

Pyrimidine and purine mononucleotide metabolism consists of a complex network of biochemical pathways essential to the function and reproduction of cells. Perturbation of this tightly controlled substrate pattern by genetic alterations or antimetabolite treatment leads to changes in cell function and, potentially, to cell toxicity by largely unknown mechanisms[1]. Clearly, it is desirable to measure changes

* To whom correspondence should be addressed.

throughout the mononucleotide metabolism network in order to investigate the mechanisms leading to cell damage.

Numerous assay procedures have been developed for probing free pyrimidine and purine nucleotide tissue pools, including high-performance liquid chromatography (HPLC)[2,3]. However, none of these published methods is capable of detecting all of the major ribo- and deoxyribonucleotides at physiological concentrations. Failure to develop a comprehensive analytical approach has been due to the large number of naturally occurring ribo- and deoxyribonucleotides (≈ 30 major nucleotides), chemical similarity of the nucleotides, a broad range of physiological concentrations from mM to below μM levels, and the presence of chemically related interfering endogenous substrates. While chromatographic separation and detection of practically all major mononucleotides in one single step has been achieved using a mixture of pure standards[4], such techniques are not applicable to the assay of tissue samples due to interference by normal tissue constituents.

We report here an HPLC–UV assay system for the complete analysis of pyrimidine ribo- and deoxyribonucleotide pools in tissue samples with a sensitivity of approximately 0.1 μM, which is sufficient to detect physiological levels of pyrimidine deoxyribonucleotides. The assay (outline in Fig. 1) uses a combination of HPLC separations with enzymatic hydrolysis of nucleotides to nucleosides. Its applicability to biological samples has been demonstrated by measuring pyrimidine nucleotide pools in two divergent samples, *i.e.* in mouse liver and in cultured cells. Furthermore, we have observed pool-size changes in cells following exposure to 5-fluorouracil (5-FUra), thus establishing the utility of the assay for studying mechanisms of cytotoxicity of pyrimidine antimetabolites.

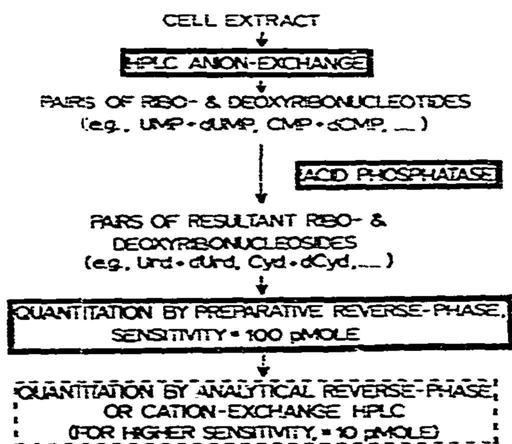

Fig. 1. Block diagram of the overall assay scheme.

MATERIALS AND METHODS

Reagents

Nucleotide and nucleoside standard samples, and all other reagents, were of analytical grade. Radioactive nucleotides, purchased as ammonium salts from

Amersham (Arlington, Ill., U.S.A.) were: [5-^{3}H]uridine 5'-monophosphate (^{3}H-UMP), sp.act. 11.2 Ci/mmol, 1 mCi/ml water–ethanol (1:1), and [5-^{3}H]cytidine 5'-monophosphate (^{3}H-CMP), sp.act. 13.0 Ci/mmol, 0.8 mCi/ml water–ethanol (1:1). Working solutions of these compounds were prepared by a 1000-fold dilution with water. The purity of ^{3}H-UMP and ^{3}H-CMP were verified prior to each experiment by chromatographic means. Acid phosphatase (orthophosphoric monoester phosphohydrolase; EC 3.1.3.2) type IV, from potatoes, was purchased as a lyophilized powder at an activity of 1.7 I.U./mg from Sigma (St. Louis, Mo., U.S.A.).

Chromatographic apparatus

HPLC analysis was performed on a liquid chromatograph (Waters Assoc., Milford, Mass., U.S.A.) equipped with a Model U6K injector, a Model 440 dual-wavelength (254/280 nm) UV absorbance detector, a Model 660 solvent programmer, and two Model M6000-A pumps.

HPLC columns

Aminex A-29 anion-exchange resin (styrene–divinyl benzene beads, 5–8 μm diameter) was obtained from Bio-Rad Labs. (Richmond, Calif., U.S.A.) and conditioned according to Khym[5]. This resin was slurry packed in pH 8.2, 25 mM sodium citrate at 8000 p.s.i., into a stainless-steel chromatographic column, 30 cm $\times$ 4 mm I.D. (prepared by Varian Aerograph, Walnut Creek, Calif., U.S.A.). The column was fitted with an outer jacket and connected to a circulating water bath for chromatography at elevated temperatures. The following HPLC columns were obtained prepacked: (1) an analytical μBondapak C_{18} reversed-phase column, 30 cm $\times$ 3.9 mm I.D. (Waters Assoc.); (2) a preparative LiChrosorb RP-18 reversed-phase column, 25 cm $\times$ 10 mm I.D. (Altex, Berkeley, Calif., U.S.A.), and a Partisil 10/25-SCX cation-exchange column, 25 cm $\times$ 4.6 mm I.D. (Whatman, Clifton, N.J., U.S.A.). The average particle diameter of each of these columns is 10 μm.

Chromatographic conditions

Aminex A-29 anion exchange. Solvent A: 25 mM sodium citrate, pH 8.2, 1.5 mM NaN_3; solvent B: 500 mM sodium citrate, pH 8.2, 1.5 mM NaN_3; flow program: isocratic elution with solvent A at 0.3 ml/min for initial 35 min followed by a linear gradient, maintaining total flow-rate of 0.3 ml/min, reaching 100% solvent B in 2 h; temperature: 50°.

Preparative RP-18 reversed phase. Solvent C: Methanol–water (0.1:99.9); solvent D: acetonitrile–water (2.0:98.0); flow-rate: 7.0 ml/min; temperature: ambient.

Analytical C_{18} reversed phase. Solvent E: 10 mM NaH_2PO_4, pH 3.0–acetonitrile (99.5:0.5); solvent F: 10 mM sodium acetate, pH 4.7–acetonitrile (97.5:2.5); flow-rate: 2.5 ml/min; temperature: ambient.

Analytical cation exchange. Solvent G: 5 mM NaH_2PO_4, pH 3.0; flow-rate: 1.0 ml/min; temperature: ambient.

Cell culture sample preparation

Wild type S-49 mouse fibroblast cells were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum from an initial density of 10^5 cells/ml.

Cells were incubated in a total volume of 100 ml culture medium for 72 h at 37° in the absence or presence of 0.67 μM 5-FUra which inhibits cell growth by 50% under these conditions. 5-FUra disappearance from the medium was found to be negligible over the incubation period. After the cell cultures were centrifuged, the pellet, containing $1-3 \cdot 10^8$ cells, was washed with ice-cold phosphate buffered saline, and the suspension centrifuged again. The resultant pellet was homogenized in six volumes of ice-cold 0.4 N $HClO_4$ for 15 sec. Precipitates and cell debris were removed by centrifugation at 1000 g for 5 min at 4° and the resultant supernatant was extracted with an equal volume of a cold freshly prepared solution of 0.5 M tri-n-octylamine in Freon TF (1,1,2-trichloro-1,2,2-trifluoroethane), to remove $HClO_4$ (ref. 5). Extraction of a wide variety of nucleotides from biological specimens was shown to be quantitative by this method[6]. The aqueous layer (pH 4–6) was removed and lyophilized. Just prior to injection onto the A-29 column, the entire sample was reconstituted with 300 μl water.

Liver sample preparation

Male Balb/c mice, Berkeley strain, ($\approx$ 20 g) were sacrificed by cervical disarticulation and the entire liver ($\approx$ 1.2 g) immediately (<20 sec) excised and frozen in liquid nitrogen. The frozen liver was ground into a coarse gravel and homogenized in six volumes of ice-cold 0.4 N $HClO_4$. This homogenate was extracted and lyophilized analogously to the S-49 homogenate. The dried extract was reconstituted with water to give a solution corresponding to 1 mg liver (wet weight)/μl ($\approx$ 1.2 ml), including 100 μl of each of ^{3}H-UMP and ^{3}H-CMP working solutions. A 250-μl aliquot of this solution, equivalent to 250 mg liver, was used for each analysis.

Aminex A-29 chromatography

Samples were injected onto the column and eluted using the gradient system described earlier. This procedure represents a minor modification of an HPLC analysis of nucleotides on Aminex A-28 suggested by Khym[4] in which we employ an iso-pH elution and use a resin of smaller particle size. Fractions were collected as shown in Fig. 2, using the position of AMP as a marker to adjust for small variations in retention times between runs. These fractions were lyophilized in preparation for phosphatase treatment.

Separation of pyrimidine nucleoside monophosphates from pyrimidine nucleosides

This procedure is necessary only when samples contain appreciable amounts of endogenous nucleosides. Fractions which may include endogenous pyrimidine nucleosides (A-29-1 and A-29-2) were reconstituted with 1 ml water and injected onto the preparative reversed-phase column, using solvent C as the eluent. Effluent was collected between 1 and 3 min after injection, which included all of the nucleotides of interest and excluded the retained nucleosides. The nucleotide-containing fractions were concentrated to 1 ml on a hot plate under a stream of nitrogen, not exceeding a temperature of 65°, and subsequently treated in the same way as the other A-29 fractions.

Phosphatase conversion of nucleotides to nucleosides

Dried nucleotide fractions were reconstituted with 1 ml water, except for

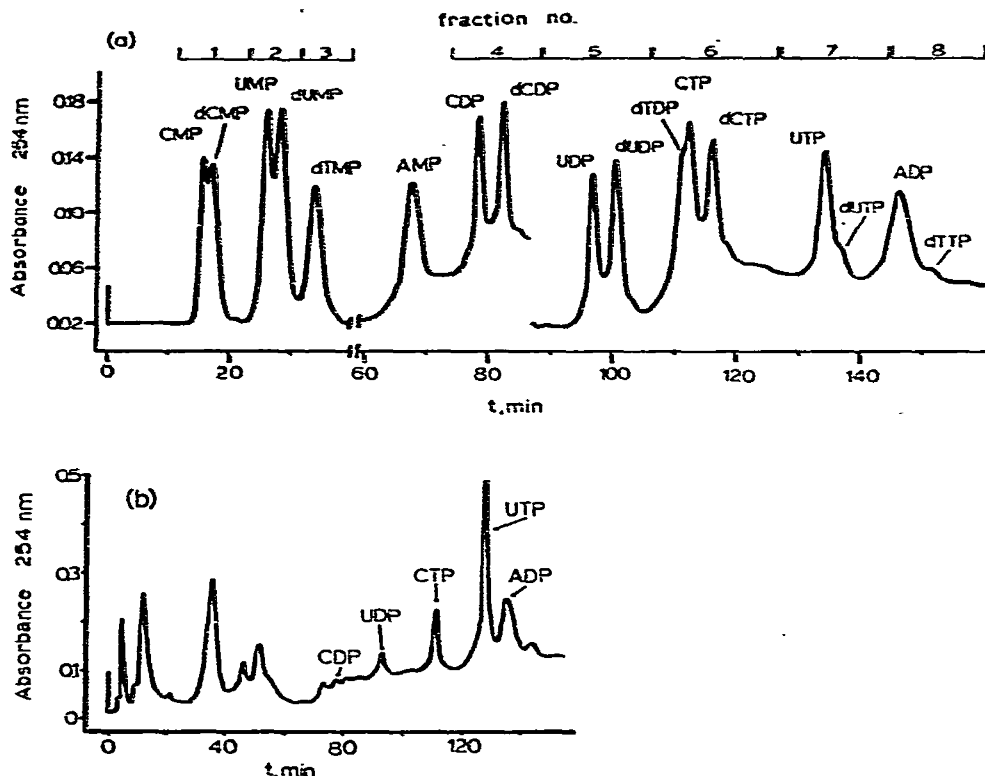

Fig. 2. (a) Aminex A-29 chromatogram of nucleotide standards (approx. 10 nmole each, except for dUTP and dTTP which were substantially degraded during storage), detected by UV absorbance at 254 nm. The eight fractions collected for phosphatase treatment are indicated at the top of the figure. (b) Aminex A-29 chromatogram of S-49 (untreated) cell extract.

fraction 6 from Aminex A-29, which was brought to 2 ml and then split into two equal portions for separate analysis of Thy- and Cyt-containing nucleotides. To each fraction was added 25 μl glacial acetic acid and 250 μl of a solution of acid phosphatase (10 I.U./ml water). The tubes were sealed, mixed and incubated at 37° for 30 min, after which they were immersed in a 95° sand bath for 15 min. Precipitated proteins were removed by centrifugation.

Analysis of resulting ribonucleosides and deoxyribonucleosides by preparative reversed-phase chromatography

A preparative-size column was needed because of the high salt concentration of the samples, which saturates analytical size columns. Prior to injection of the entire supernatant obtained at the end of the phosphastase step, 50 μl of 58% NH_4OH was added to each of the Cyt-containing solutions, in order to stabilize retention times of the nucleosides. The mobile phase used for eluting Ura- and Cyt-containing nucleosides was solvent C; solvent D was used for elution of thymidine. The separated ribo- and deoxyribonucleosides were monitored by UV absorbance at 254 and 280 nm, with a sensitivity of 100 pmole per injection. If higher sensitivity was needed,

individual nucleoside fractions were collected and dried on a hot plate under a stream of nitrogen, without exceeding temperature of 65°.

HPLC conditions for highly sensitive analysis of individual nucleosides

Individual nucleoside fractions were quantitated using analytical columns coupled with dual wavelength UV absorbance detection at 254 and 280 nm. Dried fractions from the preparative step were dissolved in 250 μl of solvent E (for Cyd, Urd and dUrd) or solvent F (for dThd), injected onto a μBondapak C_{18} analytical column, and eluted with the same solvent as used for reconstitution. Deoxycytidine was analyzed on a cation-exchange HPLC column, using solvent G for reconstitution and elution from the column due to interferences on the reversed-phase column at low deoxycytidine levels. Absorbance at 254 and 280 nm was monitored and the 254–280 ratio of each peak of interest was compared to that of authentic material to confirm the identity and homogeneity of these peaks. Representative examples of these data are given in Fig. 4. The amount of nucleoside recovered was determined by measuring the peak height and using an external standard curve. Absorbance at 280 nm was used for quantitation of Cyt nucleosides and at 254 nm for Ura and Thy nucleosides.

Determination of nucleotide analysis yields

Known amounts of all pyrimidine nucleotides were carried through the entire analytical procedure and recoveries determined by comparing amounts of nucleosides recovered to amounts of corresponding nucleotides present at the start of analysis. Due to the varying degree of purity of the nucleotide standards, recovery measurements were performed with ^{3}H-UMP and ^{3}H-CMP as tracers added to dried liver extracts. Peaks containing Cyd and Urd derived from CMP and UMP were collected and concentrated to 1 ml. To each of these was added 10 ml Aquasol® and the samples were analyzed for tritium on a Searle Mark III liquid scintillation counter. After correction for quenching the amounts of ^{3}H-Urd and ^{3}H-Cyd derived from ^{3}H-UMP and ^{3}H-CMP were determined for each run. The average recovery was $80 \pm 2\%$ for nucleosides eluting from the reversed-phase preparative column. Nucleoside recovery including the analytical step for high sensitivity was $66 \pm 7\%$.

RESULTS AND DISCUSSION

Description of method

In this paper we present a method for determining levels of all endogenous pyrimidine ribo- and deoxyribonucleoside mono-, di-, and triphosphates, in biological samples. The acid-soluble cell extract is first applied to an Aminex A-29 anion-exchange column, and eight fractions are collected as shown in Fig. 2a. Each of these fractions usually contains one ribonucleotide and the corresponding deoxyribonucleotide. The fractions are then treated with acid phosphatase, and the resulting ribo- and deoxyribonucleosides are separated and quantitated by a preparative reversed-phase column with dual-wavelength (254/280 nm) UV detection (see Fig. 3). The preparative column is required to eliminate the large quantities of salt collected in A-29 fractions, which overload smaller analytical columns. A minimum of 100 pmole of nucleoside is measurable in this way.

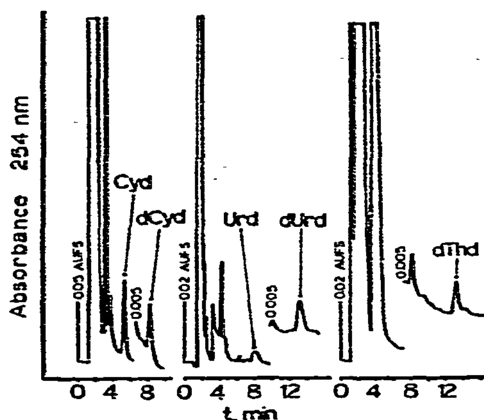

Fig. 3. Preparative reversed-phase chromatogram of nucleosides derived from nucleotides in S-49 cell extract. Amounts of nucleoside shown are: Urd, 0.700 nmole (from UMP), dUrd, 0.572 nmole (from dUMP), Cyd, 3.9 nmole (from CDP), dCyd, 0.293 nmole (from dCDP) and dThd, 0.376 nmole (from dTTP).

If a 10-fold increase in sensitivity is needed, the nucleosides eluted from the preparative reversed-phase column may be collected, concentrated, and reinjected onto an analytical reversed-phase column (see Fig. 4). Low levels of deoxycytidine were measured on a cation-exchange column due to an interference on reversed-phase columns. The minimum detectable amount of nucleoside at this step is 10 pmole.

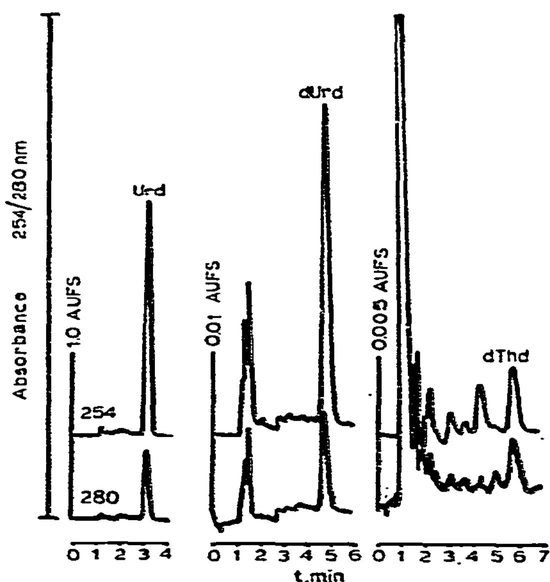

Fig. 4. Analytical reversed-phase chromatogram of nucleosides derived from nucleotides in S-49 cell extracts. Amounts of nucleosides shown are: Urd, 60.1 nmole (from UTP), dUrd, 1.08 nmole (from dUMP), and dThd, 0.109 nmole (from dTDP). Absorbance ratios (254/280 nm) for these peaks are 3.33, 3.15 and 1.41, while those of corresponding standards are 3.07, 3.00 and 1.48, respectively.

All of the data presented here were obtained using the high-sensitivity step, although, in routine practice, the preparative reversed-phase chromatography is the final step, if nucleotide levels are above 100 pmole per sample.

Precision and specificity of the method

Table I shows the results of the analysis of three identical aliquots of the acid-soluble extract of one Balb/c mouse liver. We present these data as a demonstration of the precision of the present analytical method, and not as estimates of the *in vivo* nucleotide pools in mouse liver, since it is probable that some conversion of triphosphates to monophosphates occurred during excision of the liver[7]. The standard deviation for the determination of any given nucleotide ranges from 3% to 15%, the average being 7.3%.

Peaks representing resultant nucleosides were identified by their retention volume, and their homogeneity was confirmed by comparing the 254–280 nm absorption ratio to that of an appropriate standard. Using these criteria, the assay was specific for all pyrimidine nucleotides. Examples of these data are given in Fig. 4.

TABLE I

REPRODUCIBILITY STUDY ON THREE EQUAL ALIQUOTS OF AN EXTRACT OF ONE BALB/C MOUSE LIVER

Concentrations of nucleotides are given in μM (per wet weight).

Nucleotide	*Av.*	*S.D.*	*S.D. (%)*	*Nucleotide*	*Av.*	*S.D.*	*S.D. (%)*
UMP	301	18	6	dUMP	0.17	0.026	15
UDP	50	1.6	3	dUDP	<0.1	—	—
UTP	29	0.80	3	dUTP	<0.1	—	—
CMP	13.6	0.84	6	dCMP	0.16	0.022	14
CDP	4.08	*	—	dCDP	<0.1	—	—
CTP	3.64	0.12	3	dCTP	<0.1	—	—
				dTMP	0.47	0.024	5
				dTDP	0.55	0.084	15
				dTTP	0.23	0.008	3

* One of three samples lost.

Pool size changes in S-49 cells following treatment with FUra

Pyrimidine nucleotide pool sizes are given in Table II for S-49 cells grown with or without 0.67 μM FUra. These data represent the average of two control and two experimental cell culture flasks. While all of the ribonucleotide pools were unchanged with FUra treatment, there were considerable changes in the deoxyribonucleotide levels. Thymidine nucleotides were depressed by about 50%, while deoxycytidine nucleotides and dUMP were elevated sharply. dUDP and dUTP were not detectable (<10 pmole/10^8 cells) in either case. The rise in dUMP and fall in thymidine nucleotide pools are consistent with thymidylate synthetase (TS) inhibition, although the failure of dThd to prevent growth inhibition under these conditions[8] indicates that TS blockade is not the growth limiting event. The sharp rise in deoxycytidine nucleotides has been observed previously in a similar cell line[9], although the causes and significance of this change are not clear.

TABLE II

NUCLEOTIDE POOLS IN WILD-TYPE S-49 CELLS, WITH AND WITHOUT 5-FUra TREATMENT

Concentrations are given in nmole/10^8 cells. The figures in parentheses are 1/2 of the range of duplicate experiments.

Nucleotide	*Control*	*FUra*	*Nucleotide*	*Control*	*FUra*
UMP	1.52 (0.30)	1.39 (0.84)	dUMP	<0.01	0.80 (0.02)
UDP	10.8 (7.4)	8.0 (0.4)	dUDP	<0.01	<0.01
UTP	56 (2)	56 (14)	dUTP	<0.01	<0.01
CMP	0.84 (0.25)	1.17 (0.54)	dCMP	0.034 (0.010)	0.16 (0.02)
CDP	2.09 (1.32)	2.08 (0.26)	dCDP	0.17 (0.09)	0.74 (0.15)
CTP	16.6 (3.5)	18.1 (4.0)	dCTP	1.81 (0.5)	7.2 (0.5)
			dTMP	0.052 (0.035)	0.015 (0.007)
			dTDP	0.34 (0.25)	0.078 (0.046)
			dTTP	1.99 (0.20)	1.09 (0.40)

Comparison of the present method with existing methods

The present method gives results which are quite consistent with those obtained using other techniques (Table III). The primary advantage of the present assay is its flexible and comprehensive scope, allowing simultaneous determination of any or all endogenous nucleotides*, as well as nucleotide derivatives of administered drugs. For example, we project the use of the method for studying nucleotide pool size changes following various treatments with fluoropyrimidine antimetabolites, simultaneously monitoring levels of ribonucleoside triphosphates, deoxyribonucleoside triphosphates, dUMP, FdUMP and FUTP. Using currently available techniques, this study would entail the application of four or five different methods.

TABLE III

A COMPARISON OF RESULTS IN THIS STUDY WITH LITERATURE VALUES OBTAINED BY OTHER METHODOLOGIES

Nucleotide	*nmole per 10^8 S-49 cells*		*Type of method*	*Reference*
	This study	*Previous study*		
UTP	56	69.3	Anion exchange + UV absorbance	10
dCTP	1.81	1.58	DNA Template	11
dTTP	1.99	1.87	DNA Template	11

ACKNOWLEDGEMENTS

The authors wish to thank Dr. Buddy Ullman, Department of Medicine, UCSF, for his assistance in the S-49 cell studies. We acknowledge the assistance of Dr. Seth Abbott of Varian Associates in the preparation of Aminex A-29 columns. This work was supported by a Public Health Service Research Grant No. GM-16496.

* The approach presented here has been adapted to produce a comprehensive purine nucleotide assay, with only minor modifications[12].

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