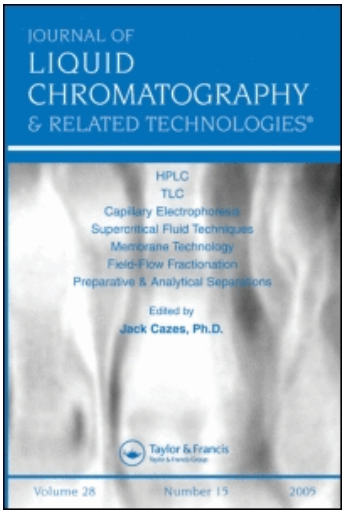

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REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) OF NUCLEOSIDES IN CELL EXTRACTS WITH SPECIAL REFERENCE TO DEOXYTHYMIDINE

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ABSTRACT

A reversed-phase high performance liquid chromatography (HPLC) system was designed to assay the nucleoside concentrations, especially deoxythymidine, of tissue cultured cells. Concentration and purification of the acid extracted cell samples were achieved by utilizing a Sep-Pak C_{18} cartridge. After adsorption of the sample, the cartridge was washed with 0.5 ml of water followed by 2 x 0.5 ml of 2.5% methanol. The compounds of interest were subsequently eluted in 2 x 0.5 ml of methanol. The cartridge procedure was found to be fast, inexpensive and showed good recoveries for most tested nucleosides. The nucleosides uridine, deoxythymidine and adenosine could be detected in green monkey kidney cells. Ribonucleotides and deoxyribonucleotides could be separated from each other with the HPLC system used.

INTRODUCTION

High performance liquid chromatography (HPLC) had made it possible to analyse nucleotide and nucleoside concentrations in biological materials. Information about intracellular concentrations of nucleotides and nucleosides is important for our understanding of

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the metabolic state of cells. Altered levels of these compounds in biological materials may indicate changes in nucleic acid metabolism in tissues with certain diseases or in conjunction with drug responses. There have been many reports on mono-, di- and triphosphate nucleotide pools of cells (see 1, 2, 3) and a good deal of work has been published on nucleoside concentrations in urine, serum and plasma (4, 5, 6, 7). Only a few reports have appeared on the unphosphorylated nucleoside concentration in animal cells (8, 9, 10, 11, 12). The present paper describes a method for determining nucleoside pools in cell extracts from cell cultures. Thymidine pools were studied in detail.

Using a combination of Sep-Pak C_{18} cartridges and a reversed-phase HPLC system, a fast, reproducible, sensitive and inexpensive method has been developed for nucleoside determination in cells.

MATERIALS AND METHODS

Reagents

Nucleotide and nucleoside standard samples of analytical grade were purchased from Sigma (St. Louis, MD., USA). Water, methanol and acetonitril were for chromatographic use (Merck, Darmstadt, Germany).

Chromatographic Apparatus

HPLC analysis was performed on a liquid chromatograph (Waters Assoc., Milford, Mass., USA), consisting of a Model 6000 A pumps, a Model U6K manual injector, a Model 440 dual wavelength (254, 280 nm) UV absorbance detector and a Model 730 integrator.

HPLC Columns

Analytical 10 μ Rad-Pak C_{18} columns (8 mm Ø x 100 mm) and Sep-Pak C_{18} cartridges were purchased from Waters Assoc., Milford, Mass., USA.

Chromatographic Conditions

Analytical C_{18} reversed phase; 10 mM sodium acetate in water, pH 4.7: acetonitril (95.0:5.0); flow-rate 2.5 ml/min., temperature: ambient; detector sensitivity: 0.01 a.u.f.s.

Quantification Procedure

All nucleosides were quantified through area calculation and comparison to areas obtained with known amounts of deoxythymidine. This could be done since equal amounts of the tested nucleosides gave roughly similar areas to that of deoxythymidine.

Cell Culture Sample Preparation

African green monkey kidney (GMK) cells were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum or calf serum to confluence. Thereafter, medium contained 2% fetal calf or calf serum. The cells were stationary for at least 7 days before analysis. The cells were tested routinely for mycoplasma with the Hoechst 33258 method (13). After the medium had been removed, appropriate amounts of ice-cold 0.5 M perchloric acid ($HClO_4$ or PCA), were added without any prior wash. The cells were then scraped from the surface. Approximately 2 x 10^8 cells in PCA were pooled and centrifuged and the supernatant was collected and neutralized with ice-cold 4 M KOH with 0.4 M KH_2PO_4. Remaining cell debris and precipitates were removed by additional centrifugation.

Preparative Sep-Pak C18 Cartridge Procedure

Cell samples or water solutions of standard nucleosides and nucleotides were loaded onto the cartridge. Subsequently 0.5 ml of water was pushed into the cartridge as a primary wash. The Sep-Pak C_{18} was washed again twice with 0.5 ml of 2.5% methanol in water to eliminate compounds that might interfere with HPLC. Finally, the

nucleosides were removed from the cartridge by rinsing twice with 0.5 ml 100% methanol. The solutions were concentrated by drying under reduced pressure and then frozen at -70°C. Prior to injection into the HPLC apparatus, the samples were redissolved in the HPLC buffer.

Medium Sample Procedure

Medium samples were treated in the same manner as the cell samples. As the PCA extraction was not used, the samples were loaded directly onto the Sep-Pak cartridge.

RESULTS

The Analytical Reversed-Phase HPLC Procedure

The system was optimized for deoxythymidine detection, utilizing 5% acetonitril (Figure 1). With the procedure used, cytidine (Cyd) and deoxyuridine (dUrd) did not separate well from each other. Good separation was obtained for uridine (Urd), guanosine (Guo), deoxyguanosine (dGuo), deoxythymidine (dThyd), adenosine (Ado) and deoxyadenosine (dAdo).

The lower sensitivity limit for this system was 3 pmoles for standard samples. The peaks were identified with the procedure recommended by Brown et al. (5). Identification includes retention times, standard addition and absorbance ratios between 254 nm and 280 nm. The dThyd peak was further identified with gas chromatography mass spectrometry.

The Preparative Pre-Column Procedure

Figure 2A shows a chromatogram of GMK cells extracted by the traditional PCA procedure (cells collected by centrifugation before addition of PCA) without using pre-column purification. Interfering substances preclude any analysis during the first 10 minutes, as

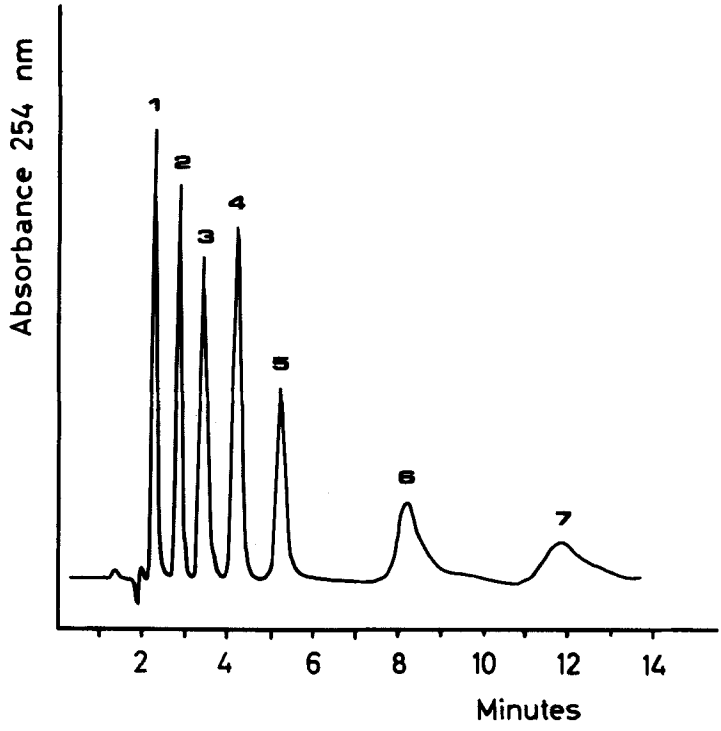

FIGURE 1

Chromatography profile of 7 standard samples of nucleosides. Nucleoside concentration: 10 µM. Injected volume: 25 µl. Conditions as in Materials and Methods. The peaks were the following: 1: Urd; 2: dUrd; 3: Guo; 4: dGuo; 5: dThyd; 6: Ado; 7: dAdo.

also reported by other workers (14). To overcome this problem, the sample was cleaned with a preparative pre-column Sep-Pak C_{18} (Figure 2B), which removes interfering substances.

For Guo, dGuo and dThyd, the overall recovery of aqueous solutions of nucleosides with the Sep-Pak procedure ranged between 93 and 103% in all experiments (Table 1). Even when the methanol concentration used for washing was raised from 0 to 3.5%, the losses of these nucleosides were 0% for Guo and dGuo and 0-3% for dThyd (Table 2). Washing with 5% methanol increased the losses.

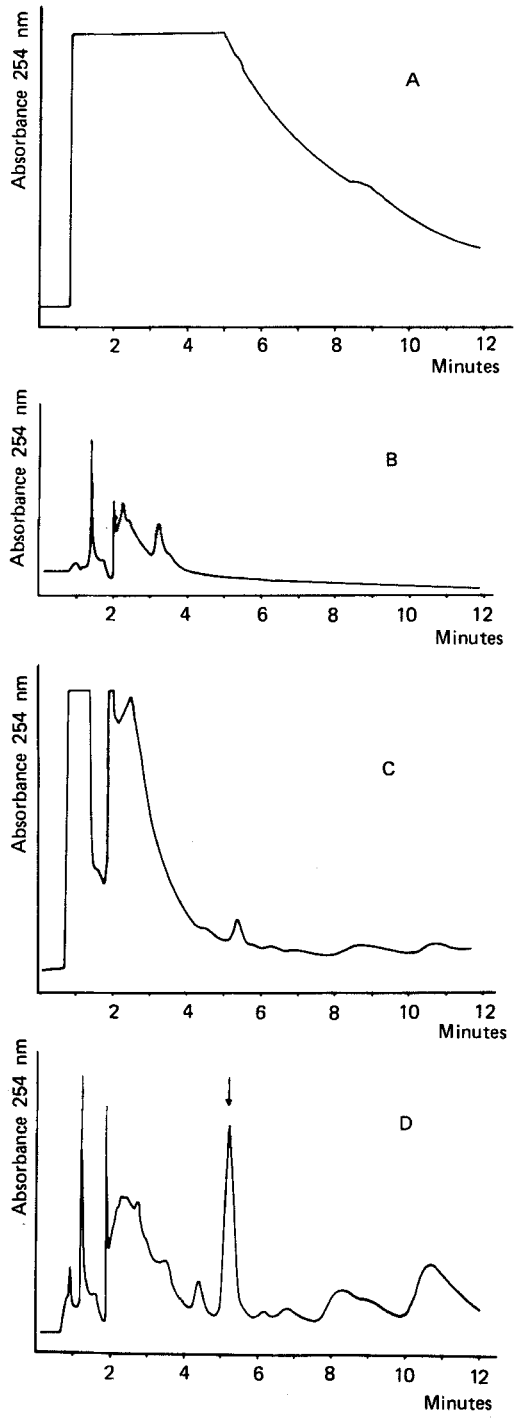

TABLE 1

% Recovery of Nucleosides from the Pre-Column Sep-Pak Cartridge.

	Percentage methanol used in the Sep-Pak C_{18} wash.				
	0%	0.5%	1.5%	2.5%	3.5%
Urd	88	90	89	90	91
dUrd + Cyd	89	92	94	92	96
Guo	100	95	100	95	103
dGuo	97	98	98	94	100
dThyd	98	96	102	93	101
Ado	91	83	91	71	96

TABLE 2

Percentage of Nucleosides Found in the Sep-Pak wash, Determined as Stated in the Text.

	Percentage methanol in the Sep-Pak C_{18} wash					
	0%	0.5%	1.5%	2.5%	3.5%	5.0%
Urd	11	13	10	10	9	39
dUrd + Cyd	11	13	10	11	11	34
Guo	0	0	0	0	0	-
dGuo	0	0	0	0	0	-
dThyd	2	2	1	1	3	7
Ado	0	0	0	0	0	-
dAdo	0	0	0	0	0	-

FIGURE 2

Chromatographic profiles of 1.5 x 10^8 GMK cells. Injected volume: 50 µl. Conditions as in Materials and Methods. The cell extracts were dissolved and injected in methanol.

Profile after extensive washing of the cells but before the Sep-Pak procedure (A). Profile after extensive washing of the cells and after the Sep-Pak procedure (B). Profile after the Sep-Pak procedure and after a short (one second) wash (C). Profile after Sep-Pak and without any previous wash (D). The arrow indicate the position of the thymidine peak.

The reason for the seemingly variable recovery of late eluting Ado (between 71-96%) probably lies in difficulties with exact quantitative measurement, since Ado did not give distinct peaks in the present system. To exclude the possibility that some Ado remained in the cartridge after the 100% methanol elution step, the cartridge was subsequently eluted with acetonitril followed by ethanol. The eluting portions were analysed, but no Ado was detected (data not shown). The nucleosides eluting early (Urd, dUrd + Cyd) show a recovery around or above 90% in all experiments. Washing accounted for these losses. 2.5% methanol was chosen for the Sep-Pak wash. This concentration proved to eliminate the early eluting interfering substances associated with the PCA extraction procedure.

Cell Culture Preparation Procedure

Analysis of triphosphate nucleotide pools of cells often involves centrifugation of the cells and resuspension in PBS before the final centrifugation and PCA addition (1, 2, 3), although the reverse has also been published (15). In the case of nucleosides, the traditional extensive washing produces the result shown in Figure 2B. A wash for only one second with +4°C PBS to remove remaining medium improved the detection of nucleoside pools (Figure 2C). Addition of PCA without washing yielded much higher nucleoside amounts (Figure 2D). The latter procedure should therefore be used.

Extraction of a wide variety of nucleotides from biological specimens was shown to be quantitative by the PCA procedure (16). The first four minutes of the chromatogram were not reproducible. The methanol dissolvent appeared to cause disturbances. The samples were therefore concentrated to dryness and redissolved in HPLC buffer. This procedure yielded the final chromatogram (Figure 3).

The possibility existed that the nucleosides might derive from medium remaining between the cells at the time of PCA addition. The nucleoside concentration of discharged medium was therefore analysed, too (Table 3).

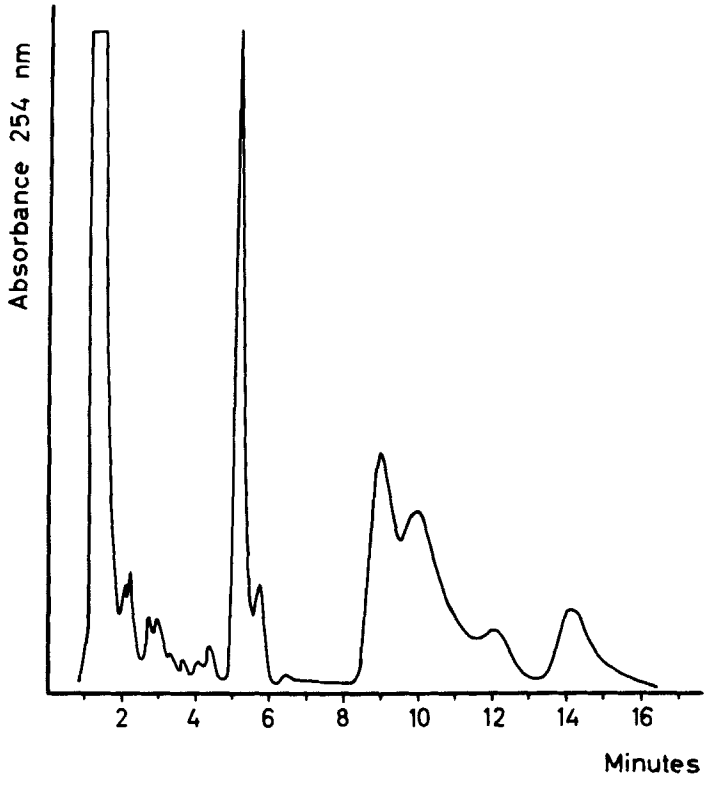

FIGURE 3

Chromatographic profile of 2 x 10^8 extracted GMK cells, after the Sep-Pak procedure and without any primary wash. The methanol extract was concentrated to dryness under reduced pressure. The sample was dissolved in 0.3 ml HPLC buffer before injecting. Conditions as in Materials and Methods. Injected volume: 10 µl.

The concentration of nucleosides from the cellular fraction compared to the used medium (Table 3) showed that the nucleosides derived from the cells. Deoxythymidine seemed to be secreted into the medium from GMK cells, since dThyd was only present in the fresh medium in minute amounts (0.03 µM). The Urd peak of the medium was hidden under the peak of the amino acid tyrosine.

TABLE 3

Concentrations of nucleosides from GMK cells and from medium discharged from the GMK cells.

	GMK cells pmol/10^6 cells	Medium µM
Urd	26	not evaluable
dUrd	2	<0.1
Cyd	<1	<0.1
dCyd	<1	<0.1
Guo	2.8	<0.1
dGuo	<1	<0.1
dThyd	185	12
Ado	250	<0.1
dAdo	100	not evaluable

DISCUSSION

The present method was shown to be fast, efficient and inexpensive. Utilizing the Sep-Pak C_{18} cartridge has proved to be a way of minimizing the interference due to the PCA extraction procedure and to yield acceptable chromatograms. The disadvantage that not all medium is washed away, can be offset in part by analysing the nucleoside concentration of the discharged medium. The method has been designed for the analysis of nucleoside pools of tissue biopsies, where this problem is not encountered. Indeed this method seems to work even better for analysing nucleosides (especially deoxythymidine) in biopsy samples from humans and animals (Harmenberg, to be published). The reason may be that a smaller volume of PCA is used with biopsies than with cell culture monolayers. It might be possible to improve the resolution of early eluting nucleosides still further using gradient elution.

There are several potential areas of application for our procedure. Major and modified nucleosides excreted in the urine have

been studied in cancer patients (4). With the present method these nucleosides might also be studied in the tumor cells themselves. Furthermore, deoxythymidine pools in tissue need to be measured in order to evaluate the antiviral action of nucleoside analogues utilizing thymidine kinase for their activation (17).

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