Analytical Biochemistry **270,** 59–68 (1999)
Article ID abio.1999.4066, available online at http://www.idealibrary.com on IDEAL®

Simultaneous Determination of Deoxyribonucleoside in the Presence of Ribonucleoside Triphosphates in Human Carcinoma Cells by High-Performance Liquid Chromatography

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Received November 23, 1998

Simultaneous determination of ribonucleoside and deoxyribonucleoside triphosphates in cells by HPLC is an analytical challenge since the concentration of dNTP present in mammalian cells is several orders of magnitude lower than the corresponding NTP. Hence, the quantitation of dNTP in cells is generally performed after selective oxidation or removal of the major NTP. The procedures reported so far are lengthy and cumbersome and do not enable the simultaneous determination of NTP. We report the development of a simple, direct HPLC method for the simultaneous determination of dNTP and NTP in colon carcinoma WiDr cell extracts using a stepwise gradient elution ion-pairing HPLC with uv detection at 260 nm and with a minimal chemical manipulation of cells. Exponentially growing WiDr cells were harvested by centrifugation, rinsed with phosphate-buffered saline, and carefully counted. The pellets were suspended in a known volume of ice-cold water and deproteinized with an equal volume of 6% trichloroacetic acid. The acid cell extracts (corresponding to 2.5 × 10^6 cells/100 μl) were centrifuged at 13,000*g* for 10 min at 4°C. The resulting supernatants were stored at −80°C prior to analysis. Aliquots (100 μl) were neutralized with 4.3 μl saturated Na_2CO_3 solution prior the injection of 40 μl onto the HPLC column (injection speed 250 μl/min). Chromatographic separations were performed using two Symmetry C18 3.5-μm (2 × 3.9 × 150 mm) columns (Waters), connected in series equipped with a Sentry guard column (3.9 × 20 mm i.d.) filled with the same packing material. The HPLC columns were kept at 30°C. The mobile phase was delivered at a flow rate of 0.5 ml/min, with the following stepwise gradient elution program: % solvent A/solvent B, 100/0 at 0 min → 100/0 at 1 min → 36/64 at 5 min → 31/69 at 90 min → 31/69 at 105 min → 0/100 at 106 min → 0/100 at 120 min; 50/50 MeOH/solvent B from 121 to 130 min; 100% solvent A from 131 to 160 min. Solvent A contained 0.01 M KH_2PO_4, 0.01 M tetrabutylammonium chloride, and 0.25% MeOH and was adjusted to pH 7.0 (550 μl 10 N NaOH for 1 liter solvent A). Solvent B consisted of 0.1 M KH_2PO_4, 0.028 M tetrabutylammonium chloride, and 30% MeOH and was neutralized to pH 7.0 (1.4 ml 10 N NaOH for 1 liter solvent B). Even though dNTPs are minor components of cell extracts, satisfactory regression coefficients were obtained for their calibration curves (r^2 > 0.99) established with the addition–calibration methods up to 120 pmol/40-μl injection. The applicability of the method was demonstrated by *in vitro* studies of the modulation of NTP and dNTP pools in WiDr colon carcinoma cell lines exposed to various pharmacological concentrations of cytostatic drugs (i.e., FMdC, IUdR, gemcitabine). In conclusion, this optimized, simplified, analytical method enables the simultaneous quantitation of NTP and dNTP and may represent a valuable tool for the detection of minute alterations of cellular dNTP/NTP pools induced by anticancer/antiviral drugs and diseases. © 1999 Academic Press

Key Words: **deoxyribonucleoside triphosphate; ribonucleoside triphosphates; cell extraction; ion-pair HPLC.**

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0003-2697/99 $30.00

Many anticancer and antiviral drugs interfere with the synthesis of DNA and RNA and/or their precursors, notably dNTP[3] and NTP. An analytical method for the quantitative measurement of NTP and dNTP pools in cells is therefore required for studying the influence of pharmacologically active agents on DNA and RNA synthesis and regulation. Both the separation techniques and the extraction procedure dramatically influence the efficiency of the analysis (1, 2). In fact, determination of dNTP in cells and tissues is an analytical challenge since the concentration of dNTP present in mammalian cells is several orders of magnitude lower than the corresponding NTP (3), the latter being most likely to obscure the dNTP signals. To overcome this analytical limitation, attempts have been made at removing NTP from cell extracts prior to dNTP analysis. Selective degradation of the *cis*-diol NTP by periodate and methylamine (3, 4) has been proposed so as to oxidize NTP prior to high-performance liquid chromatography (HPLC) measurements of dNTP (5, 6). Though this method is effective, some interfering products from the periodate oxidation coeluted with the dNTP fractions with MeOH but not TCA extraction (2). However, the periodate oxidation procedure was reported to result in partial destruction of dGTP (5). *cis*-Diol compounds such as NTP can be selectively isolated by phenylboronate affinity cartridges (7). This elegant but lengthy procedure has been proposed for removing the *cis*-diol NTP from cell extracts (8). A similar approach, though cumbersome and tedious, implies removal of NTP by sequential boronate affinity chromatography of cell extracts prior to ion-exchange chromatography of dNTP eluted fractions (9).

Several attempts have therefore been made to apply HPLC and capillary electrophoresis (CE) to the separation and quantitation of NTP and dNTP in cell extracts. Various chromatographic approaches have been proposed, including zwitterion and anion-exchange chromatography by HPLC (9–12), a combination of techniques (13), or more recently CE (1, 14, 15). Ion-pair chromatography on reversed phase was quite successful at separating NTP (1, 16) or phosphorylated anticancer or antiviral nucleoside analogues (17–20).

To the best of our knowledge however, only a few studies reporting the simultaneous determination of NTP and dNTP by HPLC have been published so far (21–23). One of the reported methods had little success in separating dGTP from ADP (21), and another was not very sensitive and required a tedious three-step extraction with ethylic ether for removal of TCA (22). A simplified method has been recently published for the simultaneous analysis of NTP and dNTP, along with other endogenous purine constituents from lymphocyte extracts (23). This was an important contribution, though the existing minute amount of dNTP could not be fully discriminated nor resolved from a relatively high baseline noise. Since the determinations of the early eluted bases and oxypurines were not of major interest to us, we focused our effort on improving the chromatograms specifically for NTP and dNTP. In our hands, the chromatographic profiles obtained with the published method did not meet the sensitivity and selectivity required for our pharmacological studies. The nonsymmetric shape of the last dATP peak—the only peak reported to be eluted after ATP—suggested that some other unknown interfering compound was eluted with a retention time very close to that of dATP. The reported extraction procedure for removing proteins from cell extracts with 1.2 M $HClO_4$ resulted in an erratic chromatographic profile. The method was cumbersome due to the prerequisite of organic extraction of lipids with $CHCl_3$ prior to injection onto the HPLC column and was not suitable for dealing with cell extracts due to the large volume needed (200 μl) and the risk of HPLC column overloading. Furthermore, relatively large solvent consumption by sample analysis was needed (150 ml, not including the rinsing and equilibration program). For all these reasons, a simplified method (sample preparation and HPLC analysis), amenable to automation and more frugal with laboratory expendables, was therefore highly desirable.

We report the adaptation, improvement, and optimization of the method of Di Pierro *et al.* (23) leading to adequate sensitivity (down to the picomole range with a 40-μl injection volume) and selectivity (separation from nearby interfering peaks) for the simultaneous separation and quantitation of dNTP and NTP.

This method was applied for elucidating the activity of the ribonucleoside reductase (RR) inhibitor FMdC (24–26) administered either alone or in combination with other nucleoside inhibitors, by determining its modulating effect on NTP/dNTP intracellular pool levels of exponentially growing colon carcinoma WiDr cells (24, 25).

EXPERIMENTAL

Chemicals

Uridine 5′-triphosphate (UTP), cytidine 5′-triphosphate (CTP), adenosine 5′-triphosphate (ATP), guanosine 5′-triphosphate (GTP), and tetrabutylammonium chloride were purchased from Sigma (Buchs, Switzerland). Solutions of ultrapure (99%) 100 mM standards of 2′-deoxythymidine 5′-triphosphate (TTP), 2′-deoxycytidine 5′-triphosphate (dCTP), 2′-deoxyadenosine 5′-triphosphate (dATP), and 2′-deoxyguanosine 5′-triphosphate (dGTP) were from Pharmacia Biotech (Dubendorf, Swit-

[3] Abbreviations used: dNTP, deoxyribonucleoside triphosphates; NTP, ribonucleoside triphosphates; TCA, trichloroacetic acid; CV, coefficient of variation.

zerland). Methanol (MeOH) for chromatography, LiChrosolv, trichloroacetic acid (TCA) (20% solution), and potassium dihydrogen phosphate GR were from E. Merck (Darmstadt, Germany). Sodium hydroxide puriss p.a. pellets and sodium carbonate were purchased from Fluka (Buchs, Switzerland). (E)-2′-Deoxy-2′-(fluoromethylene)-cytidine (FMdC, MDL101,731) was a gift from Hoechst Marion Roussel Inc. (Cincinnati, OH). All other chemicals were of analytical grade and used as received. Ultrapure water was obtained from a Milli-Q UF-Plus apparatus (Millipore).

Chromatographic System

The chromatographic system consisted of an HP 1050 isocratic/quaternary pump (Hewlett–Packard, Germany) connected to an HP 1050 autosampler and an HP 1050 multiwavelength detector set at uv 260 nm. Chromatographic separations were performed using two Symmetry C18 3.5-μm (3.9 × 150 mm) columns (Waters), connected in series equipped with a Sentry guard column.

First Approach: Gradient Elution Chromatography at +45°C

In a series of experiments, the columns were kept at 45°C in a column oven to reduce the high backpressure (210–320 bars).The mobile phase was delivered at a flow rate of 1 ml/min using the following stepwise gradient elution program (rinsing and reequilibration steps included): % solvent A/solvent B (for composition see below), 100/0 at 0 min → 52/48 at 5 min → 54/48 at 74 min → 54/48 at 80 min → 0/100 at 80.01 min → 0/100 at 83 min → 100/0 at 83.01 min → 100/0 at 90 min. Though very efficient, this procedure at 45°C in phosphate buffer, pH 7.0, resulted in a progressive deterioration of the shape of the signals (fronting peaks) after approximately 50 injections presumably due to the instability of the Symmetry packing at +45°C, despite the manufacturer's claims of column stability at temperatures up to +80°C. Moreover, the solvent consumption was relatively large. To preserve the stationary phase and prolong the HPLC column life span, chromatographic conditions were subsequently adjusted at 30°C and the flow rate was reduced to 0.5 ml/min. These changes enabled more than 200 injections without any decrease in the performance of the HPLC column. The last method was validated and applied throughout the pharmacological studies.

Final Optimized Version at +30°C

Chromatographic separations were performed using two Symmetry C18 3.5-μm (2 × 3.9 × 150 mm) columns (Waters), connected in series equipped with a Sentry guard column (3.9 × 20 mm i.d.) filled with the same packing material. The HPLC columns were kept at 30°C in a column oven (Bio-Rad column heater). The mobile phase was delivered at a flow rate of 0.5 ml/min, during the analysis (0–105 min), rinsing (105–130 min), and reequilibration (130–160 min) steps using the following stepwise gradient elution program: % solvent A/solvent B, 100/0 at 0 min → 100/0 at 1 min → 36/64 at 5 min → 31/69 at 90 min → 31/69 at 105 min → 0/100 at 106 min → 0/100 at 120 min; 50/50 MeOH/solvent B from 121 to 130 min; 100% solvent A from 131 to 160 min.

Detection was done at uv absorption at 260 nm (for maximum detection of the minor deoxypurine triphosphates such as dATP and dGTP) with a peak width of 0.053 min and a time response of 0.25 min. HPChemStation A.02.05 software loaded on an HP Vectra 486/33N was used to pilot the HPLC instrument and to process the data (area integration, calculation, and plotting of chromatograms) throughout the method validation and sample analyses. Baselines were visually inspected and were manually adjusted using peak start and end features of the HPChemStation software.

Mobile-Phase Solutions

Solution A contained 0.01 M KH_2PO_4, 0.01 M tetrabutylammonium chloride, and 0.25% MeOH and the pH was adjusted to 7.0 with 10 N NaOH (550 μl 10 N NaOH for 1 liter solvent A). Solvent B had the following composition: 0.1 M KH_2PO_4, 0.028 M tetrabutylammonium chloride, and 30% MeOH and the pH was adjusted to 7.0 with 10 N NaOH (1.4 ml 10 N NaOH for 1 liter solvent B). Solution A was at high risk of microbial contamination and was therefore passed through a 0.22-μm filter. Both solutions were prepared regularly and stored in the dark at +4°C prior to use. Both solvents were degassed by sparging with helium.

Injection Volume

The volume of cell extract injected onto the HPLC column was 40 μl in our experiments but could conveniently be decreased down to 25 μl which resulted in an improved resolution of dNTP (notably dGTP) from their nearby peaks in control cell extracts, without affecting notably the sensitivity of the detection of the other dNTP. The drawing speed—from the sample HPLC vial—and the injection speed of the sample onto the HPLC column were found to be critical and were set at 200 and 250 μl/min, respectively.

Cell Culture

Media, supplements, and fetal calf serum (FCS) were purchased from Gibco BRL (Basel, Switzerland). The WiDr cell line (a human primary adenocarcinoma of the rectosigmoid colon) was purchased from American

TABLE 1

Calibration Samples Used for the Addition–Calibration Analysis of dNTP

Calibration level	Volume neutralized cell extract pool [μl]	Volume solution 50 μM dNTP [μl]	Volume bidist water [μl]	Amount dNTP added in a 40-μl injection [pmol]
1	180	0	20	0
2	180	4	16	40
3	180	8	12	80
4	180	12	8	120

Type Culture Collection (ATCC, Rockville, MD) and grown in media specified by ATCC. WiDr cells were grown on minimum essential medium (MEM) with 0.85 g/liter $NaHCO_3$, supplemented with 10% FCS, 1% nonessential amino acids, and 2 mM L-glutamine. WiDr cultures (5×10^6 cells) were maintained as monolayers in a 150-cm^3 flask (FCT-150, TPP, Millian, Geneva, Swizerland) at 37°C in a humidified atmosphere containing 5% CO_2.

NTP/dNTP Extraction

Cells were trypsinized and washed three times with ice-cold PBS buffer excess. After centrifugation at 300*g* the pellet was suspended in a known volume of ice-cold ultrapure water (dilution according to cell count) and deproteinized with the same volume of 6% TCA (final applied TCA concentration: 3%). Acid cell extracts (corresponding to 2.5×10^6 cells/100 μl) were centrifuged at 13,000*g* for 10 min at 4°C. The resulting supernatants were stored at −80°C prior to analysis. Before HPLC assay, samples were thawed and aliquots of 100 μl were neutralized with 4.3 μl saturated (room temperature) Na_2CO_3 solution. The supernatant was introduced into a 200-μl HPLC microvial (Hewlett–Packard), and 40 μl of this solution was injected onto the HPLC column. All experiments were done in triplicate with the triplication process starting at the cell culture step to detect variability associated with the culture growth conditions.

Calibration Standards

Stock solutions of dNTP were prepared by diluting the commercially available 100 mM dNTP stock solution with bidistilled water down to a working solution of 50 μM dNTP. On each day of operation, serial volumes of the 50 μM dNTP working solution were added to a previously prepared reference WiDr cell extract pool (see Table 1) in accordance with the recommendations on bioanalytical method validation (27) (total volume added was ≤10% of the biological sample volume).

Calibration Curves

Since the retention time of NTP/dNTP in the HPLC column was affected by the presence of the biological matrix, it was necessary to prepare matrix-matched calibration samples. In the absence of dNTP-free cellular matrix extract, quantitation of dNTP was performed using the addition–calibration method. On each day of analysis, serial quantities of dNTP (40, 80, and 120 pmol) were added to a previously prepared reference WiDr cell extract pool (Table 1). Calibration curves were obtained by unweighted least-squares linear regression analysis of the peak area of the respective dNTP versus the amount of dNTP added to the cell pool extract in each standard solution. The parameters [slope and offset of the calibration curves of the four dNTPs (dCTP, dGTP, TTP, and dATP)] were used for quantitative analysis of dNTP in all subsequent samples (absolute values in picomoles in each sample), using Excel 7.0 for Windows 95 (Microsoft). In our pharmacological studies (carcinoma cells treated with various pharmacological agents), relative variations of NTP pool levels, as assessed by the measurement of peak area, were expressed as percentage variations from the peak area of NTP in untreated cells.

Analytical Method Validation

The validation of the method was based on the recommendations published as a Conference Report of the Washington Conference on Analytical Methods Validation: Bioavailability, Bioequivalence, and Pharmacokinetic Studies (27).

A series of four pool extracts (one blank and three cell extracts onto which known quantities of dNTP had been added) were analyzed and used for the determination of the variability associated with the HPLC analysis. The precision was calculated as the coefficient of variation (CV%) of a series of measurements within a single run (intraassay) which could last as much as 48 h in a row.

In the absence of dNTP free cell extract, the recovery of dNTP reflects the accuracy of the method. A selected cell extract pool (with known values of dNTP concentrations) was spiked with 40, 80, and 120 pmol dNTP. Calibration curves were established with the addition–calibration method and each individual fortified sample was quantitated, enabling the recovery determination of the amount of dNTP added, i.e., the increase in the measured concentration compared to the basal endogenous dNTP levels. Each measurement was done in triplicate.

The precision and accuracy of the NTP measurements were not considered in this study, since there are no analytical limitations to measure by HPLC the high (at the μM range) concentrations of NTP in cell

extracts. Moreover, analysis of NTP has been reported in detail in previous studies (14–16).

The stability of dNTP/NTP in cell extracts at room temperature—i.e., left in the autosampler—was established during the precision assessments. Processed extract pool samples (i.e., neutralized with Na_2CO_3) were stored in the autosampler rack at room temperature during the duration of the analysis (typically 48 h). The areas of the signals of dNTP and NTP were therefore determined in samples left at room temperature for up to 48 h and compared with those measured immediately after sample preparation.

Modulation of dNTP/NTP by Various Anticancer Agents

The detailed pharmacological method will be published elsewhere (24, 25). Briefly, exponentially growing WiDr cells were exposed to FMdC (MDL101,731) at concentrations ranging from 0 nM (untreated control cells) to 300 nM for 24 and 48 h. Similarly, WiDr cells were also incubated with IUdR at concentrations ranging from 0.1 to 3 μM. Other pharmacological protocols associating various drugs are underway.

RESULTS

Cell Extract Preparations

The precipitation of proteins of cell extracts by 1.2 M $HClO_4$ (23) was not satisfactory, producing erratic HPLC profiles of supernatants even after careful neutralization. Diluted (3%) TCA was found to be suitable for adequately removing proteins from extracts which were centrifuged and neutralized with Na_2CO_3 just prior to HPLC analysis. The lipid extraction step with $CHCl_3$ (21) did not increase the chromatographic performance and was therefore omitted. Most importantly, no prior removal of NTP was performed, neither by periodate oxidation which increases the risk of producing spurious results (5) nor by boronate affinity column, a very cumbersome procedure generating large volume (up to 1.95 ml) of diluted sample to be injected onto the HPLC column (9).

Chromatograms

Trying to replicate Di Pierro's report, we encountered high backpressure (up to 350 bar) which was first circumvented by decreasing the flow rate of the mobile phase at 1 ml/min and placing the HPLC columns into an oven at +45°C. The Symmetry column packing was unstable at this temperature, which was subsequently reduced to +30°C with concomitant reduction of the flow rate down to 0.5 ml/min. These changes resulted in a decreased backpressure (140–250 bar) and a satisfactory life span of the columns.

Our approach provides a simple procedure to measure simultaneously the eight NTPs and dNTPs in cell extracts in a single run. Figure 1 shows the chromatogram of a WiDr colon carcinoma cell extract either blank, with scale adjusted to the highest peak (ATP) (Fig. 1a) or at lower scale (Fig. 1b) or spiked with 80 pmol of each dNTP (Fig. 1c). The identity of each NTP peak has also been assessed by coinjecting NTP standard substances (data not shown). With the gradient program used at +30°C, the retention times for CTP, dCTP, GTP, UTP, dGTP, TTP, ATP, and dATP were 47.5, 50.5, 52, 54, 60, 72, 80, and 95 min respectively.

Careful control of pH, composition, and gradient elution program of the mobile phase was mandatory for standardizing peak shape and elution order of dNTP and NTP, with satisfactory separation from nearby peaks. Separation was found optimal if the HPLC columns were equilibrated with 100% solvent A at a reduced flow rate (typically 0.2 ml/min for 2 h). It should nevertheless be anticipated that the first injection of a series of analyses may not be fully resolved, presumably due to incomplete equilibration of the HPLC column. The chromatographic profiles of all subsequent runs were found to be highly satisfactory. The beginning of the elution program was run for 1 min with solvent A only; otherwise, the major NTP peaks tended to appear as doubled signals.

The separation of dGTP and dATP from their nearby peaks proved difficult. dGTP [RT (retention time) = 60 min] could be separated from its early (RT = 57.5 min) or late (RT = 61 min) neighbor peaks (both of them unknown) by fine tuning the gradient elution program to %A/B 36/64 → 31/69 within 85 min. This was of particular importance, since the peak eluted at 61 min was markedly increased in FMdC-treated cells, tending to obscure the dGTP signal in cells treated at FMdC concentrations >100 nM (Fig. 1e). The dATP signal (RT = 95 min) coalesced with its late nearby peak (RT = 99.5 min) when using isocratic %A/B 60/40. However, a slightly different composition, such as an isocratic elution with %A/B 65/35 mixture, resulted in an excellent separation of dATP from its two nearby peaks (RT = 90 and 99.5 min, respectively). This efficient separation was also obtained with the proposed gradient elution program (i.e., %A/B 36/64 → 31:69 over 85 min).

In all analyses, no detectable interfering peaks were observed at the retention times of dCTP, TTP, and dATP, as shown in the cell extract, either blank or spiked with 80 pmol of dNTP (Figs. 1b and 1c). In a few cell extracts however, a baseline separation between dGTP (RT = 60 min) and its nearby peak (RT = 61 min) could not be achieved, without precluding however an acceptable determination of dGTP levels. Signals of NTP were never obscured by

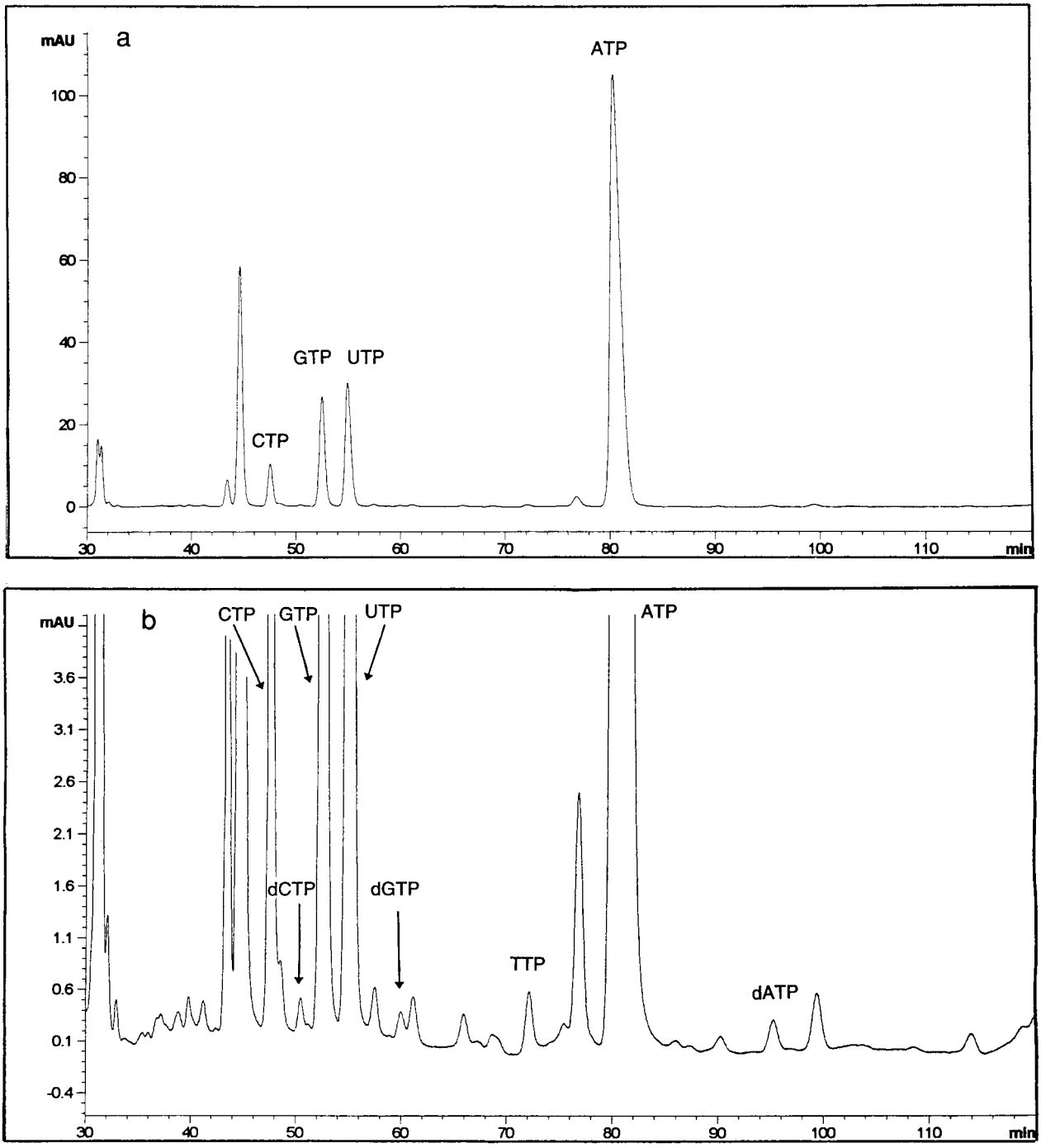

FIG. 1. Chromatograms of NTP/dNTP pools in human colon carcinoma WiDr cell extracts (mAU, milliabsorbance units). (a) Untreated (control) WiDr cells with the scale adjusted for the highest peak (ATP). (b) Untreated (control) WiDr cells at a lower scale. (c) Untreated cells spiked with 80 pM each of four dNTPs. (d) WiDr cells treated with 30 nM FMdC (24 h incubation). (e) WiDr cells treated with 300 nM FMdC.

other compounds. Using a gradient elution containing less MeOH (i.e., of longer duration than the proposed one), a minor additional peak was observed just before the large ATP signal. The intensity and overall contribution of this unknown component were negligible relative to ATP. The 25-min rinsing program with pure solvent B followed by a 50/50 mixture of solvent B/MeOH—and a reequilibration of long enough duration (i.e., approximately 30 min) with 100% solvent A—was mandatory for avoiding the perturbation of the next analysis by late-eluting peaks arising from the current HPLC run.

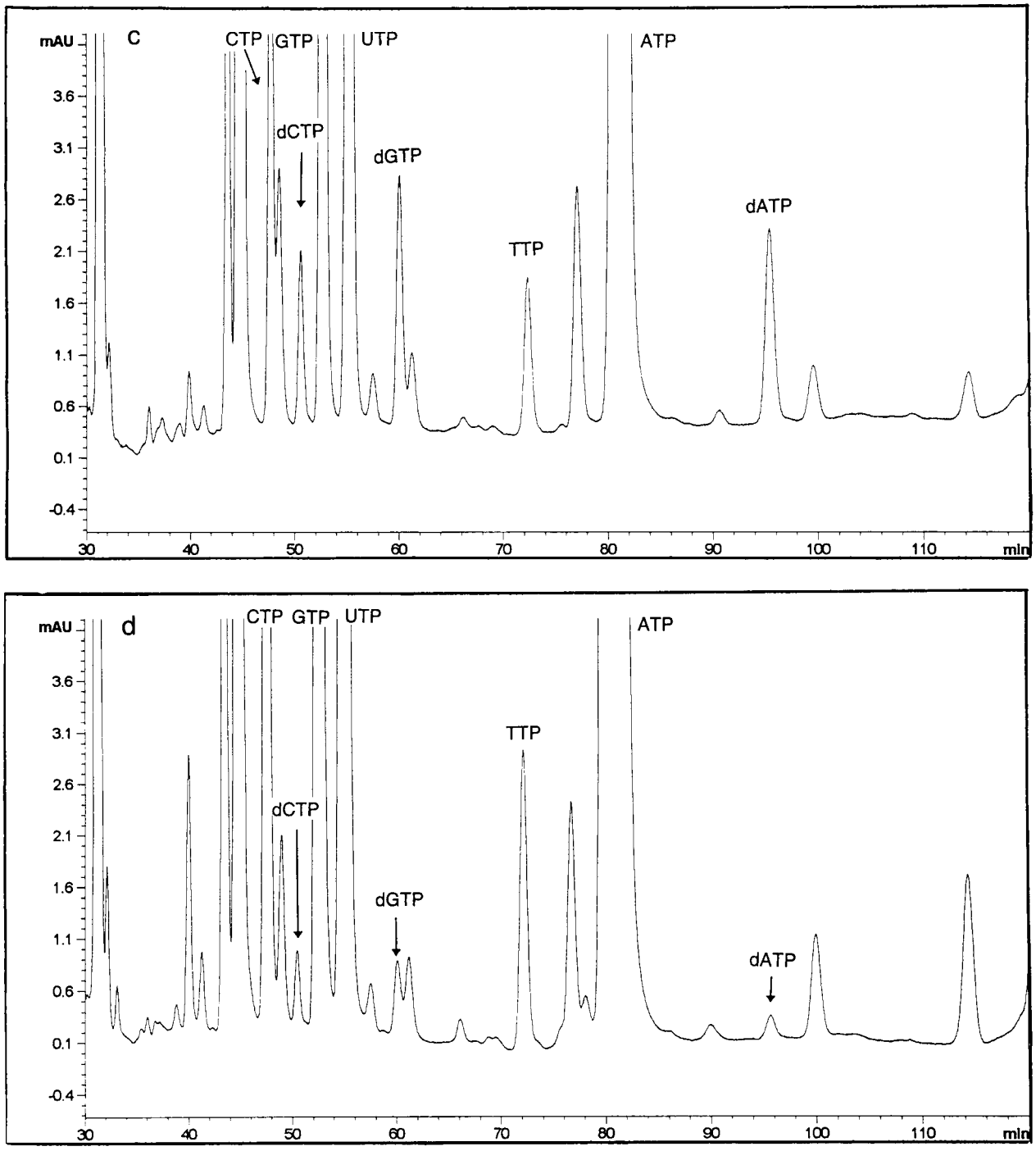

FIG. 1—*Continued*

Calibration Curves

The standard curves for dCTP, dGTP, TTP, and dATP were satisfactorily described by unweighted least-squares linear regression analysis. The average slope of the addition–calibrations ($n = 7$) established for the dNTPs were 0.66 (±6.2%), 1.22 (±5.7%), 0.78 (±2.8%), and 1.36 (±3.9%) for dCTP, dGTP, TTP, and dATP, respectively. Over the range 40–120 pmol dNTP, the regression coefficients r^2 of the calibration curves for the four dNTPs were good and always >0.997.

Validation of the HPLC Method: Precision and Accuracy

The precision (CV%) levels achieved by analyzing a blank cell extract pool of control samples and three pools spiked with 40, 80, and 120 pmol dNTP is given

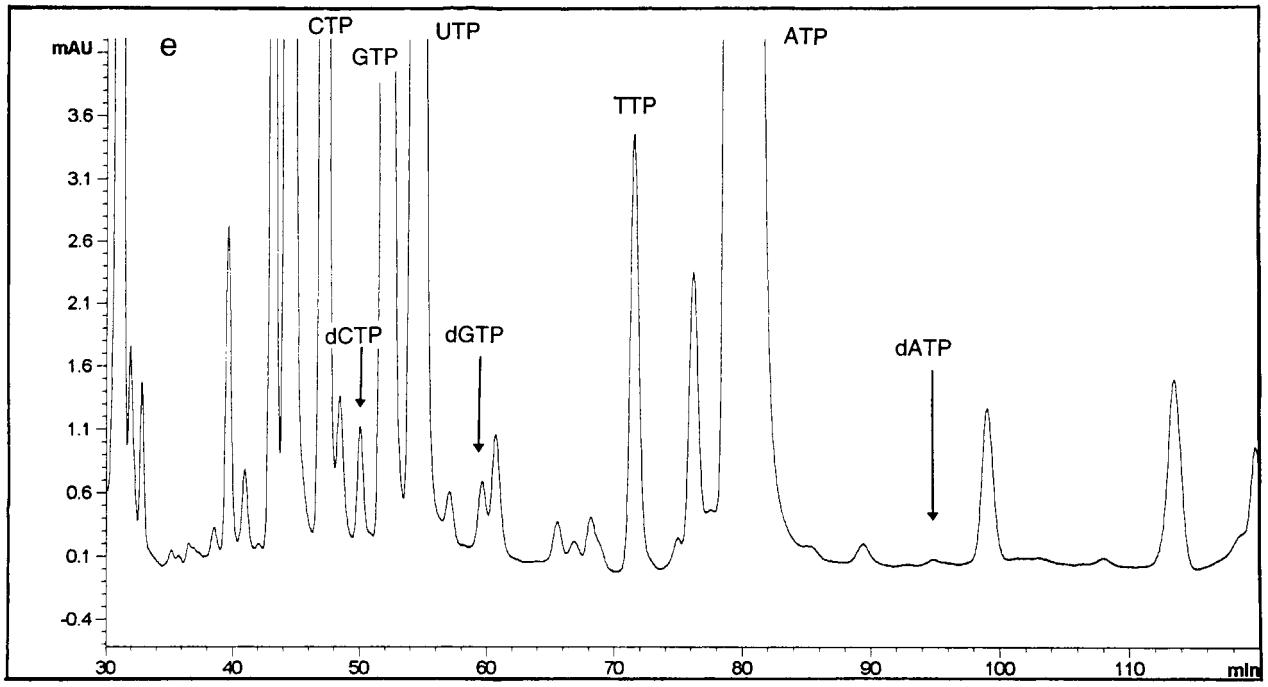

FIG. 1—*Continued*

in Table 2. The amounts of dNTP added were selected to encompass the range of concentrations presumably present in cell extracts treated with various pharmacological agents (see below). Within these concentration ranges, the mean intraassay precisions were 2.6, 2.9, 2.2, and 1.5% for dCTP, dGTP, TTP, and dATP, respectively. The low variability from a series of analyses performed during a prolonged period of time (over 48 h) indicates that neutralized samples are stable at room temperature.

To assess the accuracy of the method, a selected cell extract pool (with known values of dNTP concentrations) was spiked with 40, 80, and 120 pmol dNTP. The determination of the amount of dNTP added, i.e., the increase in the measured concentration compared to the basal endogenous dNTP levels, is shown in Table 3. The levels measured in the spiked cell extracts never differed by more than 4% from those expected (i.e., endogenous dNTP levels plus added amounts).

Sample Stability

In our series of experiments, unneutralized control cell extracts stored at −80°C were found to be stable for at least 2 months, taking into account the experimental variability, in accordance with previously reported studies (4, 23). However, after prolonged storage at −80°C (up to 8 months), additional peaks—presumably from decomposition products formed on prolonged storage at −80°C—were eluted just before dATP.

The stability of dNTP in processed (i.e., containing Na_2CO_3) samples submitted to HPLC analysis was checked. After the removal of cellular proteins by TCA and subsequent neutralization, the variation over time

TABLE 2

Variability of the dNTP/NTP Levels in Neutralized Cell Extract Samples Left at Room Temperature (CV%) (n = 4)

	CPT	dCPT	GPT	UTP	dGTP	TTP	ATP	dATP
Neutralized pool extract[a]	0.8	4.5	0.6	1.4	6.6	1.9	1.0	2.9
Neutralized pool extract + 40 pM dNTP	0.6	1.8	0.5	0.4	1.5	1.7	0.6	1.6
Neutralized pool extract + 80 pM dNTP	0.2	2.4	1.5	1.9	2.0	0.4	1.6	0.7
Neutralized pool extract + 120 pM dNTP	0.9	1.6	1.0	1.0	1.3	4.6	1.1	0.8

[a] Left at room temperature over 48 h in the autosampler rack.

TABLE 3

Recovery of dNTP in Neutralized Cell Extract Pools[a]

	Cell extract 180 μl + 20 μl H_2O (±SD)	Cell extract 180 μl + 40 pmol dNTP in 20 μl H_2O			Cell extract 180 μl + 80 pmol dNTP in 20 μl H_2O			Cell extract 180 μl + 120 pmol dNTP in 20 μl H_2O		
	Experimental amount (in 40-μl injection volume)	Theoretical amount (pmol)	Experimental amount (pmol) (±SD)	Deviation (%)[b]	Theoretical amount (pmol)	Experimental amount (pmol) (±SD)	Deviation (%)[b]	Theoretical amount (pmol)	Experimental amount (pmol) (±SD)	Deviation (%)[b]
dCTP	14.02 ± 1.95	54.02	54.22 ± 1.03	0.38	94.02	96.53 ± 1.75	2.68	134.02	139.32 ± 1.81	3.96
dGTP	7.66 ± 0.87	47.66	49.61 ± 0.91	4.10	87.66	90.68 ± 1.41	3.44	127.66	132.40 ± 3.37	3.72
TTP	29.29 ± 0.9	69.29	69.30 ± 0.68	0.01	109.29	110.56 ± 0.55	1.16	149.29	150.48 ± 3.19	0.8
dATP	14.21 ± 0.55	54.21	54.73 ± 0.21	0.96	94.21	94.57 ± 0.77	0.37	134.21	135.31 ± 2.4	0.82

[a] Analysis done in triplicate with neutralized samples left at room temperature over 40 h (40-μl injection volume).
[b] Accuracy = (experimental-theoretical)/theoretical.

of NTP and dNTP, expressed in percentage of the starting level of the four dNTPs, was always below 6.6% in samples left at room temperature for 48 h (Table 2).

DNTP Concentrations in the WiDr Human Colon Carcinoma Cell Line

Using this method, the concentrations of dCTP, dGTP, TTP, and dATP in WiDr human colon carcinoma cells were found to be 14.6 ± 2.0, 8.0 ± 0.9, 30.5 ± 0.9, and 14.8 ± 0.6 pmol/10^6 cells, respectively. With the exception of the higher levels of TTP found, these values are in good correspondence with the concentrations of 10.4 ± 1.3, 7.4 ± 0.9, 6.1 ± 0.8, and 11.4 ± 2.0 pmol/10^6 cells for dCTP, dGTP, TTP, and dATP, respectively, reported in resting human lymphocytes (23). For comparison, the dNTP levels measured in the WiDr colon carcinoma cells are lower than the reported 22.5 ± 1.0, 9.7 ± 0.8, 45.1 ± 1.6, and 30.4 ± 0.9 pmol/10^6 cells measured for dCTP, dGTP, TTP, and dATP, respectively, in Ehrlich ascites tumor cells using the method of Tanaka *et al.* (6).

Application of the Method to Pharmacological Studies

Figure 1 shows the chromatographic profile of NTP/dNTP pools in WiDr cell extracts (corresponding to 2.5×10^6 cells/100 μl) either untreated (Figs. 1a and 1b) or exposed for 24 h to 30 and 300 nM FMdC (Figs. 1d and 1e). The disappearance of dATP shown in Fig. 1e is accompanied by a marked increase in TTP, a less pronounced rise of dCTP and dGTP, and a significant overall increase in all four NTPs. A detailed discussion of these findings is reported elsewhere (24, 25).

DISCUSSION AND CONCLUSION

Significant simplifications were carried out in the extraction procedure resulting in a minimal chemical manipulation of cells, except protein precipitation with diluted TCA and neutralization with Na_2CO_3. This optimized HPLC method provides a simple procedure for determining simultaneously in a single run the eight NTPs and dNTPs in cell extracts from the human carcinoma cell line. Sodium tetrabutylammonium chloride was chosen as ion-pairing agent to retain NTP/dNTP on the reverse-phase column long enough to achieve an efficient separation. As expected with charged molecules, careful control of the mobile phase at pH 7.0 was crucial for obtaining reproducible retention times for the analytes of interest. After a series of analyses, rinsing of the column with pure water followed by MeOH enabled adequate cleaning of the HPLC column from adsorbed contaminants, but increased the difficulty for achieving good separation in the first series of subsequent analyses, presumably due to incomplete equilibrium between A/B buffering solvents and column-packing material. Indeed, stable baseline signals and efficient separations of NTPs/dNTPs were achieved only after a sufficient volume of the solvent A/B mixture has been pumped through the column (typically 150 ml).

Good sensitivity was achieved with an injection volume as low as 40 μl (which could be even decreased down to 25 μl in some experiments), a volume markedly lower than in previously reported HPLC methods (200 μl–2 ml) (9, 23), making this method suitable for semi-microanalysis of low-yield cell extract samples.

Both precision and accuracy of the presented method were always $<\pm 15\%$ in accordance with published recommendations (27).

The HPLC run may be considered somewhat lengthy (more than 2 h for analysis of the eight NTPs/dNTPs), but since the neutralized cell extract samples are very stable at room temperature—in the autosampler rack—the assay is fully automatable and does not require any tedious technical supervision. In our experiments, samples could be analyzed in a row over 48 h without any problems.

The applicability of the method was demonstrated by *in vitro* studies on the modulation of NTP and dNTP pools in the WiDr colon carcinoma cell line exposed to various pharmacological concentrations of cytostatic drugs (i.e., FMdC, IUdR, gemcitabine).

In conclusion this simplified analytical method enables the simultaneous quantitation of NTP and dNTP and represents a valuable tool for the detection of minute alterations of cellular dNTP/NTP pools induced by anticancer/antiviral drugs and diseases.

ACKNOWLEDGMENTS

The Fondation Radiobiologie 2000, the Stiftung zur Krebsbekämpfung/Fondation pour la Lutte contre le Cancer, and DebioChine provided support for this research.

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