Journal of Chromatography, 619 (1993) 29–35
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 7005

Improved high-performance liquid chromatographic analysis of intracellular deoxyribonucleoside triphosphate levels

Ronald A. Rimerman, Gregory D. Prorok, Kaey L. Cordel, Ann M. Shahwan and William P. Vaughan*

Oncology/Hematology Section, Department of Internal Medicine, University of Nebraska Medical Center, Omaha, NE 68198 (USA)

(First received February 15th, 1993; revised manuscript received May 24th, 1993)

ABSTRACT

The ability to measure intracellular deoxyribonucleoside triphosphate (dNTP) pool sizes is important for understanding the intracellular metabolism of DNA synthesis and repair. We have developed an improved method for measuring intracellular dNTP pool size by high-performance liquid chromatography (HPLC). Previous methods have enabled accurate measurement of dNTPs only in concentrations greater than approximately 10 pmol per 10^6 cells due to the inability to partially purify cell extracts, to the inability to apply extracts from extremely large numbers of cells, to the lack of efficient columns, to the presence of incompatible solvents, and to the inability to inject large volumes. We have modified a low-pressure strong anion-exchange column pre-step developed by others to concentrate and partially purify oxidized cell extracts while at the same time eluting them in a more compatible solvent for HPLC injection. The HPLC column is a YMC ODS-AQ column operating in a combined hydrophobic-interaction chromatography–reversed-phase chromatography mode. The injection and elution solvents are both phosphate-based. Using this method it is possible to measure intracellular dNTP levels well below 0.5 pmol per 10^6 cells or at the sensitivity of the DNA polymerase assay.

INTRODUCTION

We have been interested in modeling the use of hydroxyurea and other ribonucleotide reductase inhibitors in combination and sequential chemotherapy for malignant diseases [1–4]. These drugs apparently exert cytotoxic antineoplastic effects at least in part by reducing intracellular deoxyribonucleoside triphosphate (dNTP) pools [5–8], thereby inhibiting DNA synthesis and possibly repair. The ability to measure low dNTP levels with greater accuracy would improve the utility of our *in vitro* and *in vivo* models. Consequently we set out to develop an improved high-performance liquid chromatographic (HPLC) procedure based upon recent technical improvements to permit rapid, simultaneous and reliable measurement of low levels of dNTPs.

The use of HPLC for determining dNTP pool sizes has proven to be the most simple and rapid method of measuring all of the dNTPs at the same time. However, the available HPLC methods for measuring low levels in cell extracts still have some limitations. Interference by ribonucleotides, which are present in much greater amounts than dNTPs, has been limited by removing ribonucleotides by periodate oxidation

* Corresponding author. Address for correspondence: Division of Hematology/Oncology, Department of Medicine, School of Medicine, University of Alabama at Birmingham, 513 Tinsley Harrison Tower, 1900 University Boulevard, Birmingham, AL 35294-0006, USA.

0378-4347/93/$06.00 © 1993 Elsevier Science Publishers B.V. All rights reserved

procedures to which dNTPs are resistant [9]. However, the determination of low dNTP levels in the oxidized extracts has been hampered by the lack of adequate procedures for the clean-up and concentration of the periodate-oxidized cell extract before it is injected onto the HPLC column. A method involving the use of a miniature low-pressure strong anion-exchange (SAX) column has partially solved this problem [10].

The use of SAX columns for the high-pressure analysis step, however, have significant limitations including low resolution using isocratic elution, baseline difficulties and long run times using buffer gradient elution, and poor separation of nucleotides from each other and from the void volume. Reversed-phase chromatography (RPC) has not solved these problems.

A recently developed octadecyl silica (ODS) RPC column with hydrophilic end-capping groups (YMC) allows greater interaction of the ODS groups with the nucleotides in the aqueous mobile phase resulting in greater retardation of early eluting nucleotides. Further retardation permitting complete separation from the void peak may be able to be accomplished using hydrophobic-interaction chromatography (HIC) [11–17]. High salt-containing eluents have been used to retard nucleotides on RPC columns in an isocratic mode [18,19] but on these columns high salt causes the ODS side-chains to flatten out against the silica surface and not interact as well with the nucleotides. Thus the use of an RPC column with hydrophilic end-capping in a combined HIC–RPC mode should allow the development of a rapid sensitive analysis of low levels of dNTPs. A recently described low-pressure SAX column sample preparation step should allow significant concentration and decontamination of the samples, further increasing sensitivity and specificity [10,20].

EXPERIMENTAL

Chemicals

Nucleotides, potassium hydroxide, Freon (1,1,2-trichlorotrifluoroethane) and Histopaque 1077 were obtained from Sigma (St. Louis, MO, USA). Nucleotides were shipped on dry ice and solids and solutions were stored at −80°C. HPLC-grade monobasic potassium phosphate, HPLC-grade 85% phosphoric acid, 60% perchloric acid, and concentrated hydrochloric acid were from Fisher Scientific (Plano, TX, USA). Tri-*n*-octylamine was practical grade from Eastman Kodak (Rochester, NY, USA). Bond Elut SAX columns (100 mg packing, 1 ml reservoir) and phenylboronic acid columns (500 mg packing, 2.8 ml reservoir) were from Analytichem International (Harbor City, CA, USA). RPMI 1640 medium and Dulbecco's phosphate-buffered saline were from Gibco (Madison, WI, USA). Penicillin–streptomycin solution was obtained from Hazelton (JRH Biosciences, Lenexa, KA, USA). Bovine calf serum was from Hyclone (Logan, UT, USA).

Cells and extraction procedure

L1210 cells were grown as suspension cultures in RPMI 1640 supplemented with 15% bovine calf serum and 50 U/ml penicillin–streptomycin solution: $1 \cdot 10^5$ cells were initially seeded in 20 ml of the above medium and allowed to grow in a humidified 5% CO_2 atmosphere at 37°C. After three days the cells were spun at 900 *g* for 5 min. The old medium was aspirated, replaced with 5 ml of fresh medium, and the pellet resuspended. The cells were then counted and then seeded at densities of $1 \cdot 10^6$ and/or $10 \cdot 10^6$ cells/ml. After 6 h in the fresh medium cells were harvested and extracted. Final cell density was calculated as $1.27 \cdot 10^6$ cells/ml for exponentially growing cells and $10.3 \cdot 10^6$ cells/ml for cells approaching stationary or plateau phase. Preparing extracts from cells that have not been washed and concentrated eliminated the risk of artifactual reduction in dNTPs during preparation [21,22].

An approximately 50:50 mixture of human lymphocytes and monocytes was isolated from heparinized blood by the Histopaque method provided by Sigma. The mixture of lymphocytes and monocytes was washed according to the Sigma procedure with Dulbecco's phosphate-buffered saline.

The nucleotide extraction procedure was that

of Hunting and Henderson [19] with minor modifications. In a typical extraction, the pellet of 1–10 · 10^6 L1210 cells or 4 · 10^7 human lymphocytes and monocytes was resuspended in 0.5 ml of cold 0.4 *M* perchloric acid in a microfuge tube and kept on ice for 30 min. After centrifugation to remove the insoluble material the supernatant was removed to a second microfuge tube where the perchloric acid was neutralized by adding 0.5 ml of cold 0.5 *M* tri-*n*-octylamine in Freon (1,1,2-trichlorotrifluoroethane) and vortex-mixing for 10 s. Microfuge centrifugation for 3 min reseparated the mixture leaving the dNTPs in the neutralized aqueous upper layer.

Periodate oxidation of cell extract

The oxidation procedure of Garrett and Santi [9] was used to remove ribonucleoside triphosphates. Attempts to remove ribonucleotides by use of a phenylboronic acid column at pH 7 or 8 were not successful.

Preparation of the oxidized cell extract for HPLC analysis

This procedure is a modification of methods of Harmenberg *et al.* [10] and Bodell and Rasmussen [20]. Briefly, the SAX column was equilibrated in 0.01 *M* KH_2PO_4–K_2HPO_4 pH 6, approximately 4–6 ml of the diluted oxidized cell extract (diluted ten-fold with water to bring the concentration of dissolved salts down to 0.02 *M*) was applied at a rate of about 1.0 ml/min, the column was washed at about 1.0 ml/min with 0.8 ml of 0.01 *M* KH_2PO_4–K_2HPO_4 pH 6, and 0.8 ml of 0.1 *M* KH_2PO_4–K_2HPO_4 pH 6, then the dNTPs were released by elution with 0.4 ml of 0.5 *M* KH_2PO_4–K_2HPO_4 pH 6 by gravity flow. This gave a dNTP fraction volume of about 0.4 ml with quantitative recovery of dNTPs. Elution at higher rates with either 0.5 or 1.0 *M* potassium phosphate pH 6 did not completely remove the dNTPs.

Chromatography

The HPLC system was a Hewlett-Packard HP 1090 Series M equipped with a HP 9000 Series 300 computer and 9153C disc drive, PV5 solvent delivery system, temperature-controlled autosampler, autoinjector, heated thermostatically controlled column compartment, HP 79880A diode array detector module with a November 1, 1989, firmware update for more sensitivity and a ThinkJet printer. The computer software was update 5.1, November 1, 1989.

The column used was a 250 mm × 4.6 mm I.D., 5-μm ODS-AQ reversed-phase column (YMC, Morris Plains, NJ, USA) utilized in a combined HIC–RPC mode with a high concentration of phosphate in the elution buffer. The number of theoretical plates using naphthalene as a reference peak was 20 192 when new and used for these studies. The column temperature was 40°C.

A linear gradient from 0 to 30% methanol was used. The total run time was 16 min with a post run time of 13 min for reequilibration. The potassium phosphate, pH 6.0, concentration was 0.200 *M*. The increase in methanol concentration caused a moderate increase in ultraviolet baseline absorption. The flow-rate was 1.0 ml/min. Solvent bottle A contained 0.200 *M* potassium phosphate pH 6, made from 0.400 *M* phosphate pH 6.00. Solvent bottle B contained a mixture of 0.200 *M* potassium phosphate, made from 0.400 *M* potassium phosphate pH 6.00–30% methanol so that the apparent pH is 6–7. Solvent bottle C contained 30% methanol. The gradient went linearly from 100% A to 100% B over 15 min and then linearly back to 100% A over 1 min. Extending the run time of solvent B or running the gradient to 40% methanol may improve column cleaning between runs. At the end of the day the column and pump were washed at 1.0 ml/min from bottle C and then overnight at 0.05 ml/min.

Levels of the four dNTPs of DNA were determined by the external standard method. dIMP and dAMP were added to the 0.5 *M* SAX column fraction of dNTPs as reference peaks. Cordycepin-5′-triphosphate (3′-deoxy-ATP) should prove useful as an internal standard and reference peak since its retention time is toward the end of the chromatogram and is different from that of the nucleotides studied here or many other nucleotides such as some of those that are

methylated or the tetraphosphates of deoxyadenosine and adenosine; however, the material we used contained an impurity that had the same retention time as dGTP, which prevented us from using it as an internal standard. It should be stable to periodate oxidation since it lacks a 3′-hydroxyl group.

RESULTS AND DISCUSSION

Analysis of low levels of nucleotides

A chromatogram of low levels of dNTPs and NTPs is shown in Fig. 1. The five dNTPs and four NTPs are all resolved except for GTP which remained incompletely resolved from dUTP. Based upon the assumption of detection at a signal-to-noise ratio of 2:1, detection limits for dCTP, dTTP, dGTP, and dATP are 1.4, 1.3, 1.3, and 0.5 pmol, respectively, at the wavelength of maximum absorption for each dNTP. Since an extract from more than 10^7 cells can be reduced to a 100-μl injection volume using the SAX column concentration and purification step, the lower limit of detection of dNTPs in cells is well under 0.5 pmol per 10^6 cells or at the detection limit of the DNA polymerase assay [22]. Use of a 0.200 *M* phosphate elution buffer rather than an even higher concentration appeared to be optimum because the dCTP peak was retarded and resolved sufficiently and GTP was almost resolved from dUTP. Use of a 0.260 *M* phosphate buffer at pH 6.0 improved separation of the GTP and dUTP peaks but moved ATP and dGTP closer together.

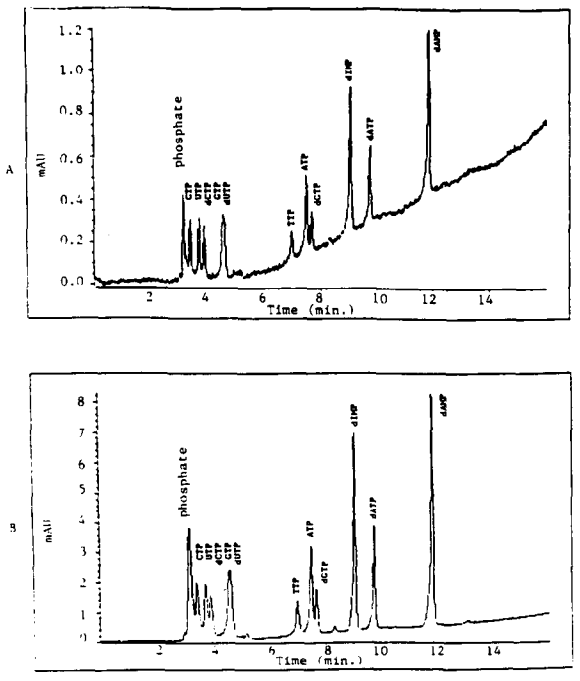

Fig. 1. HPLC gradient separation of (from left) phosphate buffer (P) from injected sample, CTP, UTP, dCTP, GTP and dUTP (merged), TTP, ATP, dGTP, dIMP (reference peak), dATP, and dAMP (reference peak); 5 pmol of each dNTP and NTP were injected in 10 μl (A) or 50 pmol in 100 μl (B) of approximately 0.36 *M* K_2HPO_4–KH_2PO_4 pH 6. UV absorption at 252 nm is plotted.

Enhanced resolution of dCTP peak using high phosphate concentration

When large volumes (greater than 10 μl) of nucleotides were injected on the HPLC column using water as a solvent, peak broadening or, at larger volumes, splitting of one peak into two or three peaks occurred. This problem was overcome by injection in phosphate buffer of pH 6.0. This broadening and splitting apparently results because the nucleotides exist in a mixture of ionic species in the water solution which differs from that in the phosphate buffer and injection of large volumes of water solutions does not allow adequate mixing with the phosphate elution buffer. Even in phosphate buffer injection of a large volume caused considerable broadening of the dCTP peak. Increasing the concentration of phosphate in the sample sharpened this peak presumably by inducing hydrophobic interactions between the nucleotide and the ODS groups on the silica packing of the HPLC column, thereby causing binding and concentration of the peak (Fig. 2). Thus larger volumes can be injected. Using a higher concentration of phosphate in the HPLC elution buffer may cause a similar effect.

Concentration and partial purification of dNTPs in oxidized cell extracts

Monophosphates, some diphosphates, and oxidation products were removed by the SAX column. On the basis of integration of the HPLC

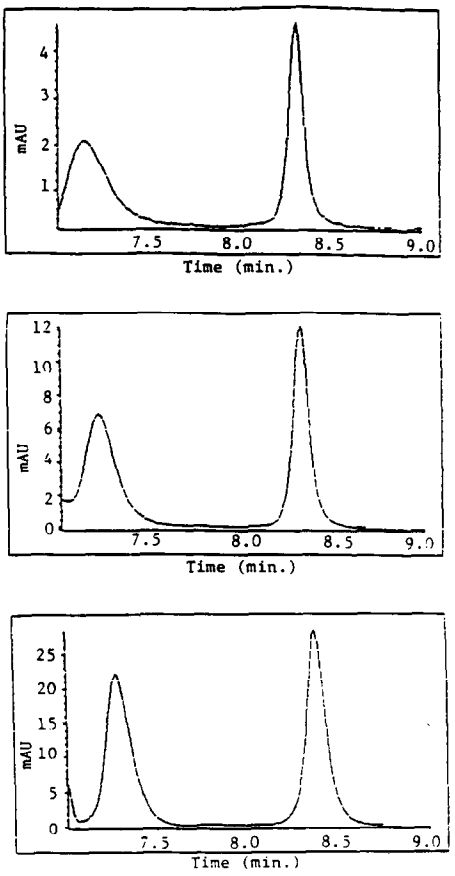

Fig. 2. Effect of increasing potassium phosphate concentration in the sample injected on peak widths during HPLC gradient separation of (from left) dCTP and dUTP. A 100-μl aliquot of sample solution was injected. (A) 0.500 *M*, (B) 0.985 *M*, and (C) 1.92 *M* potassium phosphate, pH 6. UV absorption at 267 nm is plotted.

elution peaks a 500- to 1500-fold purification was obtained. Using deoxyribonucleotide standards, recovery of the triphosphates was greater than 95%. Use of 1 *M* hydrochloric acid to elute the dNTPs as was done by Harmenberg *et al.* [10] could not be used for it caused the dATP peak to disappear over a period of 2 h and formation of a large void peak. Utilization of the SAX column permitted efficient extraction of large numbers of cells (greater than 10^7), since the resultant extract is concentrated on the SAX column and the dNTPs eluted in a small volume. Measurement of ribonucleoside triphosphate levels should be possible by chromatographing the unoxidized cell extract on the SAX column under the same conditions used for the dNTPs [20].

dNTP levels in cell extracts

Fig. 3 shows a chromatogram of the dNTPs in a cell extract of exponentially growing and nearly stationary phase L1210 tumor cells that contain low to high levels of dNTPs. All of the dNTPs that are components of DNA appear to be completely resolved. The dNTPs were identified by their retention times and by the wavelengths dependence of the heights of their chromatographic peaks. NTPs appear to be absent. More conclusive identification of the dNTPs would require spiking of the extracts with dNTP standards or use of HPLC–mass spectrometry. At least 100 μl of the 0.5 *M* SAX column fraction can be injected, producing fairly sharp peaks. dNTP levels

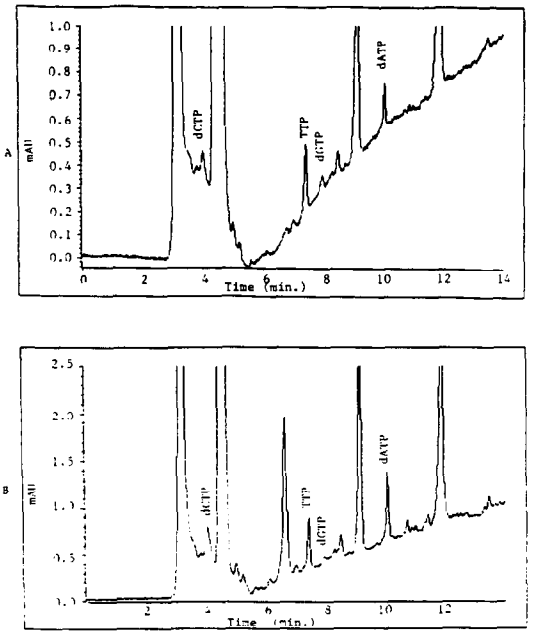

Fig. 3. HPLC gradient separation of dNTPs in periodate oxidized and SAX column pre-purified extracts of (A) $1.27 \cdot 10^6$ exponentially growing or (B) $10.3 \cdot 10^6$ plateau phase L1210 cells. UV absorption at 252 nm is plotted. A 100-μl aliquot of the 0.38 ml of 0.5 *M* potassium phosphate pH 6.0, strong anion-exchange (SAX) column fractions was injected. Thus the injection volumes contained the entire extract of $3.3 \cdot 10^5$ or $2.7 \cdot 10^6$ cells for exponential and plateau cultures, respectively.

TABLE I

dNTP POOLS IN L1210 CELLS AND IN HUMAN PERIPHERAL BLOOD LYMPHOCYTES

Nucleotide	dNTP content (pmol/10^6 cells)				
	L1210		Human peripheral blood lymphocytes		
	Log phase[a]	Plateau phase[b]	Present study	Literature values [22,23]	
				Non-stimulated	Stimulated
dCTP	25.5	9.0		0.07–0.2	3–4
TTP	56.7	14.2		0.01–0.06	7–11
dGTP	12.1	2.1	0.7–1.0	0.05–0.13	1.5–2.3
dATP	14.1	6.6	0.46	0.2–0.3	3–5

[a] $1.27 \cdot 10^6$ cells/ml in culture.
[b] $10.3 \cdot 10^6$ cells/ml in culture.

are shown in Table I. The reduction in dNTP pool size when the cells approach stationary phase is as expected.

Fig. 4 displays a chromatogram of the dNTPs in a cell extract of human lymphocytes, which contain extremely low levels of dNTPs when the percentage of cells in S phase is very low [23,24]. Only dGTP and dATP could be resolved and identified by retention time and wavelength dependence of their chromatographic peak heights due to the presence of other compounds having peaks at the same retention times as dCTP and TTP. The peak very close to and at a slightly longer retention time than dATP also exhibited a wavelength dependence characteristic of dATP, but observation of the complete absorption spectrum using the diode-array detector and accurate comparison to the absorption spectrum of standard dATP was not possible due to the small heights of the peaks.

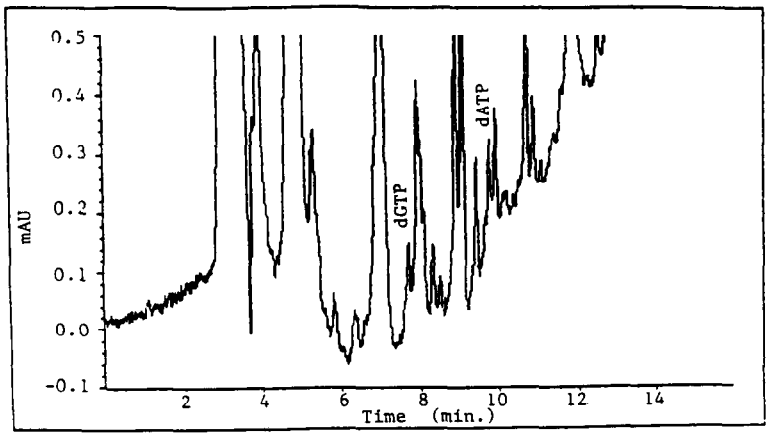

Fig. 4. HPLC gradient separation of dNTPs in a periodate oxidized and SAX column pre-purified extract of an approximately 50:50 mixture of $3.8 \cdot 10^7$ human lymphocytes and monocytes. UV absorption at 252 nm is plotted. A 100-μl aliquot of the 0.39 ml of 0.5 *M* potassium phosphate pH 6.0, strong anion-exchange (SAX) column fraction was injected. Thus the injection volume contained the entire extract of $1.0 \cdot 10^7$ cells.

The pool sizes of dGTP and dATP found are shown in Table I and compared with levels found in phytohemagglutinin-stimulated and non-stimulated human lymphocytes using the DNA polymerase method [23,24]. It should be noted that the value for dATP that we found agrees fairly well with the value for non-stimulated cells from those studies. The fact that the dATP peak in Fig. 4 is much larger than the baseline noise shows that this HPLC assay is as sensitive as the DNA polymerase assay, which has a detection limit of 0.1 pmol per 10^6 cells [21]. Because some of the cell extraction procedures used for the DNA polymerase assay extract some kinases and phosphatases and a nuclease, this method can overestimate or underestimate the levels of the dNTPs.

In summary, we have defined a rapid and reliable HPLC method for measurement of dNTP (and other nucleotide) pool sizes which is as sensitive as the polymerase method. With very low levels of dNTPs in the cell extracts, other compounds present in the extracts interfere with the observation of some of the dNTPs even though we use a SAX column pre-step and an efficient ODS column. Varying HPLC assay conditions such as mobile phase composition, pH, and column temperature change the sensitivity and resolution of the column for specific nucleotides, which may allow even lower limits of detection or greater resolution of single compounds. Adding electrochemical detection may increase the sensitivity further for purines [25,26]. Current work in our laboratory will extend these data to antineoplastic drug-treated cells, other cell lines and cells collected from *in vivo* models.

ACKNOWLEDGEMENTS

This work was supported by NIH Grant R01 CA455329. We would like to acknowledge Tom Veys for technical assistance and Dr. Ray Smith for helpful discussions. We would like to thank the Ladies Auxiliary of the VFW for partial support in obtaining the HPLC system.

REFERENCES

1 P. J. Burke, W. P. Vaughan and J. E. Karp, *Blood*, 55 (1980) 960.
2 W. P. Vaughan, J. E. Karp and P. J. Burke, *Cancer*, 45 (1980) 859.
3 W. P. Vaughan, J. E. Karp and P. J. Burke, *Blood*, 64 (1984) 975.
4 W. P. Vaughan and P. J. Burke, *Cancer Res.*, 43 (1983) 2005.
5 R. D. Snyder, *Biochem. Pharmacol.*, 33 (1984) 1515.
6 T. Lindahl, P. Karran, B. Demple, B. Sedgwick and A. Harris, *Biochimie*, 64 (1983) 581.
7 A. Cohen, *Adv. Exp. Biol. Med.*, 195 (Part B) (1986) 201.
8 T. M. Plucinski, R. S. Fager and G. P. V. Reddy, *Mol. Pharmacol.*, 38 (1990) 114.
9 C. Garrett and D. V. Santi, *Anal. Biochem.*, 99 (1979) 268.
10 J. Harmenberg, H. J. Karlsson and G. Gilljam, *Anal. Biochem.*, 161 (1987) 26.
11 S. Hjerten, *J. Chromatogr.*, 87 (1973) 325.
12 J. Porath, L. Sundberg, N. Fornstedt and I. Olsson, *Nature*, 245 (1973) 465.
13 R. A. Rimerman and G. W. Hatfield, *Science*, 182 (1973) 1268.
14 R. A. Rimerman and G. W. Hatfield, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, 33 (1974) 1502.
15 W. M. Holmes, R. E. Hurd, B. R. Reid, R. A. Rimerman and G. W. Hatfield, *Proc. Natl. Acad. Sci. U.S.A.*, 72 (1975) 1068.
16 H. Bussey, R. A. Rimerman and G. W. Hatfield, *Anal. Biochem.*, 64 (1975) 380.
17 S. Pahlman, in O. Hoffman-Ostenhof, M. Breitenbach, F. Koller, D. Kraft and O. Scheiner (Editors), *Affinity Chromatography*, Pergamon Press, New York, 1978, p. 161.
18 C. Horvath, W. Melander and I. Molnar, *Anal. Chem.*, 49 (1977) 142.
19 J. K. Christman, *Anal. Biochem.*, 119 (1982) 38.
20 W. J. Bodell and J. Rasmussen, *Anal. Biochem.*, 142 (1984) 525.
21 D. Hunting and J. F. Henderson, *Methods Cancer Res.*, 20 (1982) 245.
22 G. Trysted, *Exp. Cell Res.*, 91 (1975) 429.
23 B. Munch-Petersen, G. Tyrsted and B. Dupont, *Exp. Cell Res.*, 79 (1973) 249.
24 G. Tyrsted, *Exp. Cell Res.*, 91 (1975) 429.
25 R. J. Henderson and C. A. Griffin, *J. Chromatogr.*, 298 (1984) 231.
26 J. B. Kafil, H.-Y. Cheng and T. A. Last, *Anal. Chem.*, 58 (1986) 285.