Journal of Chromatography, 415 (1987) 53–63
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3492

COMPARISON OF HIGH-PERFORMANCE ION-EXCHANGE AND ION-PAIR LIQUID CHROMATOGRAPHIC METHODS FOR THE SEPARATION OF TUMOUR CELL NUCLEOTIDES

T. KREMMER*, M. BOLDIZSÁR and L. HOLCZINGER

National Oncological Institute, Research Institute of Oncopathology, Department of Biochemistry, H-1122 Budapest (Hungary)

(First received August 11th, 1986; revised manuscript received October 31st, 1986)

SUMMARY

Ion-exchange liquid chromatography (IELC) on a novel anion- exchanger, Polyanion SI HR 5/5, and the ion-pair technique (IPLC) using Hypersil ODS and/or MinoRPC reversed phases with tetrabutylammonium phosphate as pairing agent were compared for the separation of nucleotides. Modifications to the concentration gradient in IELC in the range 0.01–0.3 *M* ammonium phosphate resulted in the simultaneous separation of twelve to fourteen biologically important nucleotides. IPLC studies revealed that the capacity factors and resolution of nucleotides were more sensitive to the ionic strength than the methanol content. It was concluded that a well controlled ion concentration (0.08–0.09 *M* sodium chloride) should be maintained in the mobile phase and a linear methanol gradient ranging from 0 to 20% (v/v) was suitable for optimal resolution. Separations of four nucleosides and twelve nucleotides were further improved using a mixed-type reversed-phase column (C_2/C_{18}, MinoRPC). Using these complementary methods, it was possible to reveal the metabolic changes induced by different drug treatments (cyclophosphamide, DL-α-difluoromethylornithine) in the nucleotide pool of P388 leukaemia cells.

INTRODUCTION

In view of the biological significance of purine and pyrimidine nucleotides, their separation and determination are increasingly important biomedical applications of high-performance liquid chromatography (HPLC). Among the different techniques applied [1,2], ion-exchange [3–10] and, more recently, reversed-phase ion-pair liquid chromatography have shown promise for the fractionation of 20–24 nucleosides and nucleotides in complex biological samples [10–21]. However, in the simultaneous determination of various metabolites, both these methods have advantages and disadvantages and further improvements are therefore required

0378-4347/87/$03.50

either by selecting different packing materials and/or by optimization of the chromatographic conditions [2, 6, 10, 11, 13].

In this work the ion-exchange separation of nucleotides on a new packing material, Polyanion SI HR 5/5, and ion-pair liquid chromatography on reversed-phase columns were selected for comparison. The resolution capacity and optimization of these methods were investigated using a wide variety of reference compounds and natural mixtures of tumor cell nucleotides.

EXPERIMENTAL

Materials

All chemicals were of analytical-reagent grade and the water for the solvent systems was glass-distilled twice. High-purity methanol (LiChrosolv, Merck, Darmstadt, F.R.G.) was used throughout. Tetrabutylammonium hydroxide (TBAH, 40%) was obtained from Fluka (Buchs, Switzerland) or Sigma (St. Louis, MO, U.S.A.) and nucleosides and nucleotides from Reanal (Budapest, Hungary). Each reagent and reference compound was checked for purity, especially for degradation products.

A mixture of adenosine 5′-, uridine 5′-, cytidine 5′- and guanosine 5′-mono-, -di- and -triphosphates (AMP, ADP, ATP, UMP, UDP, UTP, CMP, CDP, CTP, GMP, GDP, GTP) containing in specific instances inositol- and orotidine-5′-monophosphates (IMP, OMP) were used. Approximately 5–15 nmol of each dissolved in 50 μl of mobile-phase solvent were applied as an external standard for peak identification and quantitation. DL-α-Difluoromethylornithine (DFMO; RMI 71782) was kindly donated by the Centre de Recherche Merrell International (Strasbourg, France).

Sample preparation

P388 ascites leukaemia cells were maintained in BDF_1 inbred male mice by serial intraperitoneal (i.p.) transplantation and harvested on different days of tumour progression as previously described [9]. The treatment of leukaemia-bearing mice with a 2% solution of DFMO as sole drinking fluid has also been reported [9]. A single dose of 70 mg/kg cyclophosphamide (VEB Jenapharm. Rudolfstadt, G.D.R.) was applied by i.p. injection to the groups of tumourous mice three days following transplantation. Leukaemia cells were harvested and investigated 12–24 h after drug administration. Tumour cells were collected by centrifugation and homogenized in 0.7 *M* ice-cold perchloric acid (100 μl per 10^6 cells) using a vortex mixer. After centrifugation at 3000 *g* for 20 min at 0°C the supernatants were collected and neutralized with potassium hydrogen carbonate (10 mg per 100 μl). The supernatants were recentrifuged at the same speed until clear, then stored at −20°C until taken for analysis by HPLC.

Ion-exchange liquid chromatography

Ion-exchange liquid chromatographic separation of tumour cell nucleotides was performed on a novel weak anion-exchange column (5×0.5 cm I.D.) of Polyanion SI HR 5/5 (5 μm) (Pharmacia, Uppsala, Sweden) operated on the Phar-

TABLE I

TECHNICAL PARAMETERS FOR THE ION-EXCHANGE AND ION-PAIR LIQUID CHROMATOGRAPHIC SEPARATION OF NUCLEOTIDES

Parameter	Ion-exchange LC	Ion-pair LC	
Column type	Polyanion SI HR 5/5	ODS-Hypersil	Pharmacia MinoRPC
Size (cm)	5×0.5	25×0.46	20×0.46
Packing material	Silica-based weak anion exchanger	C_{18}	C_2/C_{18}
Particle size (μm)	5	5	5
Elution	Gradient	Isocratic and gradient	
Mobile phase	Ammonium phosphate buffer (pH 7)-0.01 *M* solution A-1.0 *M* solution B	0.025 *M* TBAH phosphate buffered (pH 6) Isocratic (variable): NaCl, 0.08–0.11 *M*; methanol, 1–10% (v/v) Gradient: solution A, 0.08 *M* NaCl; solution B, 0.08 *M* NaCl and 20% (v/v) methanol	
Flow-rate (ml/min)	0.75	0.75	0.70
Sample (μl)	10–100	10–50	
Detection (nm)	254	254	
Apparatus	Pharmacia FPLC	Hewlett-Packard 1084B	
Temperature	Ambient	30°C	

macia fast performance liquid chromatography (FPLC) system [7–9]. The chromatographic conditions are detailed in Table I. The column was equilibrated with 0.01 *M* ammonium phosphate buffer (pH 7, solution A) and 10–100 μl of samples were applied. Nucleotides were eluted using linear gradient programmes (I or II) with an increasing buffer concentration as indicated in Table II. Ammonium phosphate buffer (1 *M*, solution B) was prepared using 40% (w/v) ammonia solution and 85% (w/v) orthophosphoric acid and adjusted to pH 7. Nucleotides were measured by reference to calibration graphs of peak area plotted

TABLE II

ELUTION PROGRAMMES FOR NUCLEOTIDE PHOSPHATES SEPARATED ON A POLYANION SI HR 5/5 COLUMN WITH THE PHARMACIA FPLC SYSTEM

Programme I		Programme II	
Time (min)	Percentage B	Time (min)	Percentage B
0	0	0	0
25	100	25	30
27	100	30	100
28	0	32	100
30	End	32	0
		35	End

against the amount of reference compounds. Retention times were given by the LCC-500 liquid chromatography controller of the FPLC system.

Ion-pair liquid chromatography

Two types of reversed-phase columns, Hypersil ODS (Shandon, London, U.K.) and MinoRPC (Pharmacia) with phosphate-buffered (pH 6) 0.025 *M* tetrabutylammonium hydroxide (TBAH) as pairing agent were applied for the separation of nucleosides and nucleotides. Methanol and ion concentrations ranging from 0 to 20% (v/v) for methanol and from 0.08 to 0.11 *M* for sodium chloride were systematically varied in the mobile phase. Ion concentrations (in the chloride form) were determined by the titrimetric method of Schales and Schales [22]. Separations were performed on a Hewlett-Packard 1084B chromatograph equipped with a Model 79875A variable-wavelength detector, fitted with an automated sample injector and an 79850 LC terminal. The chromatographic characteristics, capacity factor (k'), selectivity, plate number and height of columns (HETP) and peak resolution were calculated in the conventional way [23].

RESULTS AND DISCUSSION

Despite the high resolution and speed of HPLC, the choice of ion exchangers in the determination of nucleic acid constituents is limited [3–6, 10]. The Pharmacia FPLC system with the Polyanion SI HR 5/5 column has given promising results in the separation of nucleotides [7–9]. The application of the Pharmacia FPLC system for measuring natural polyamines has already been described [24]. The Polyanion SI HR 5/5 column contains silica-based polyethylenimine-coated microparticulate beads with an extremely narrow particle size distribution (5 μm $\pm$ 3%), and provides high resolution at medium pressures (1–2 MPa) in a column of relatively short length (5 cm). Fig. 1 shows that a reasonable resolution of ten characteristic nucleotides can be achieved using a very simple linear gradient of buffer concentration (Table II, programme I). To improve the resolution of mono- and diphosphates (OMP–ADP), a shallow gradient of buffer concentration was introduced first (from 0.01 to 0.3 *M* in 25 min), then elution was continued with a steeper gradient up to 1 *M* applied over 5–10 min (Table II, programme II). The retention times of the different nucleotides obtained by these methods are summarized in Table III. Fig. 1B shows that the gradient of programme I separated the nucleotides of P388 leukaemia cells and allowed the determination of changes induced by different drug treatments (Fig. 1C and D).

Administration of cyclophosphamide to tumour-bearing animals resulted in a marked increase in the nucleotide pool of leukaemia cells. In contrast, the DFMO treatment elevated preferentially specific precursors of DNA biosynthesis (uridine and cytidine phosphates), as reported elsewhere [9].

Modifications to the experimental conditions and reagents of the ion-exchange technique were explored. Although linear correlations were obtained between the amount of nucleotides and their peak areas at 254 nm, the individual calibration graphs gave different slopes, indicating the necessity for carefully selecting mixtures of reference compounds in order to measure accurately particular nucleo-

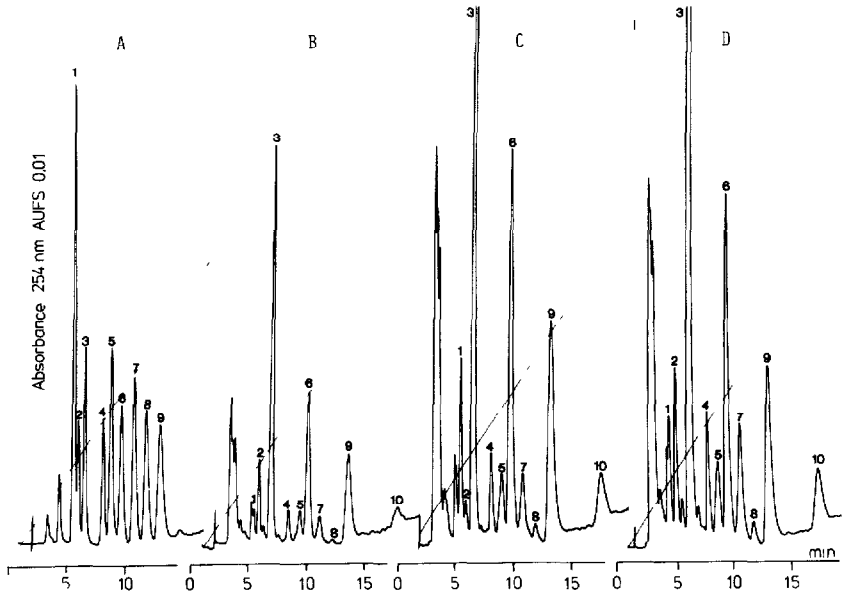

Fig. 1. Ion-exchange chromatographic separation of nucleotides on a 5-μm Polyanion SI HR 5/5 column (5×0.5 cm I.D.) using a linear concentration gradient of ammonium phosphate buffer (pH 7). For details of the chromatographic conditions, see Table I. (A) Separation of reference compounds. (B) Nucleotide pattern of 3-day-old P388 leukaemia cells. (C) Effects of cyclophosphamide on the nucleotide composition of 3-day-old P388 leukaemia cells following 24 h of in vivo drug administration. (D) Effects of DFMO on the nucleotide composition of 3-day-old P388 leukaemia cells. Peaks: 1=UMP; 2=CMP; 3=AMP; 4=UDP; 5=CDP; 6=ADP; 7=UTP; 8=CTP; 9=ATP; 10=GTP.

tides in complex biological extracts. With regard to the buffer components, no commercial sodium or potassium phosphates of analytical-reagent grade were found to be suitable for the gradients, especially below 0.01 a.u.f.s., because of their content of UV-absorbing impurities. Purification of phosphate buffers for HPLC has been described [3]. In our experience, the best results were obtained when ammonium phosphate buffer was prepared from analytical-reagent grade constituents. Even under these conditions the baseline of UV absorbance increased markedly and a "ghost" peak of cumulated impurities appeared at 0.8 *M* buffer concentration that sometimes impeded the determination of GTP. This observation indicated that 1 *M* ammonium phosphate buffer should be applied for systematic cleaning of columns before they are re-equilibrated and used. The separation of nucleotides on Polyanion SI HR 5/5 was sensitive to the quality of counter ion applied. Poor resolution was achieved by increasing the ion strength with acetate, chloride, sulphate or carbonate ions, suggesting that a counter ion with three negative charges was necessary for optimal separation.

Various reversed-phase techniques have opened up new perspectives for the analysis of nucleosides and nucleotides [10–19]. To improve the efficiency of the reversed-phase technique we used phosphate-buffered TBAH as a pairing agent and the appropriate sodium chloride and methanol concentrations for the separation of nucleotides. These were investigated in detail.

TABLE III

RETENTION TIMES FOR NUCLEOTIDE PHOSPHATES SEPARATED ON A POLYANION SI HR 5/5 COLUMN WITH THE PHARMACIA FPLC SYSTEM

Nucleotide*	Retention time (min)	
	Programme I	Programme II
UMP	3.94	5.17
CMP	4.37	5.98
AMP	5.26	7.27
IMP	5.63	7.94
UDP	7.12	12.07
CDP	7.90	13.53
GMP	8.43	14.01
OMP	9.22	14.57
ADP	9.29	16.51
UTP	10.46	20.47
CTP	11.91	23.63
ATP	13.71	27.62
GDP	15.01	28.45
GTP	20.75	(31)

*For abbreviations, see Experimental.

It has been established that the surface concentration of pairing agents is dependent on the length of apolar hydrocarbon chains comprising the reversed phase, and also the extent of carbon loading of the particles [19, 20]. Similar correlations were found between the hydrophobic character (chain length) of pairing agents and their surface concentrations [11, 13, 15, 25, 26]. With regard to stationary phase–solute interactions, it has been suggested that in certain instances the mixed-type (C_2/C_{18}) reversed phases are better for the separation of biologically active complex molecules. In agreement with others [19, 21, 25], we observed in a preliminary study that a saturating concentration of 0.025–0.03 *M* TBAH in the mobile phase was necessary for optimization of the pairing mechanism. Increasing the concentration of TBAH in the mobile phase did not lead to higher retention times or better resolution. Lowering of the pairing agent concentration resulted in poorly resolved fractions and a parallel decrease in capacity factors. Therefore, an optimal TBAH concentration of 0.025 *M* was used throughout the experiments.

To demonstrate the effects of sodium chloride and methanol on the resolution, some critical pairs of nucleotides (AMP–UDP, ADP–UTP and UTP–GTP) were selected. Fig. 2 shows that the separation of mono- and some diphosphates (UMP, CMP, IMP, CDP, AMP) was improved by lowering the salt concentration, resulting in a simultaneous increase in the retention times of ATP and other di- and triphosphates. Resolutions, summarized in Table IV, indicate that the optimal molarity of sodium chloride was between 0.09 to 0.1. In a separate experiment, decreasing the methanol content in the mobile phase increased similarly the resolution of monophosphates (Fig. 3). Table V shows, however, that at higher

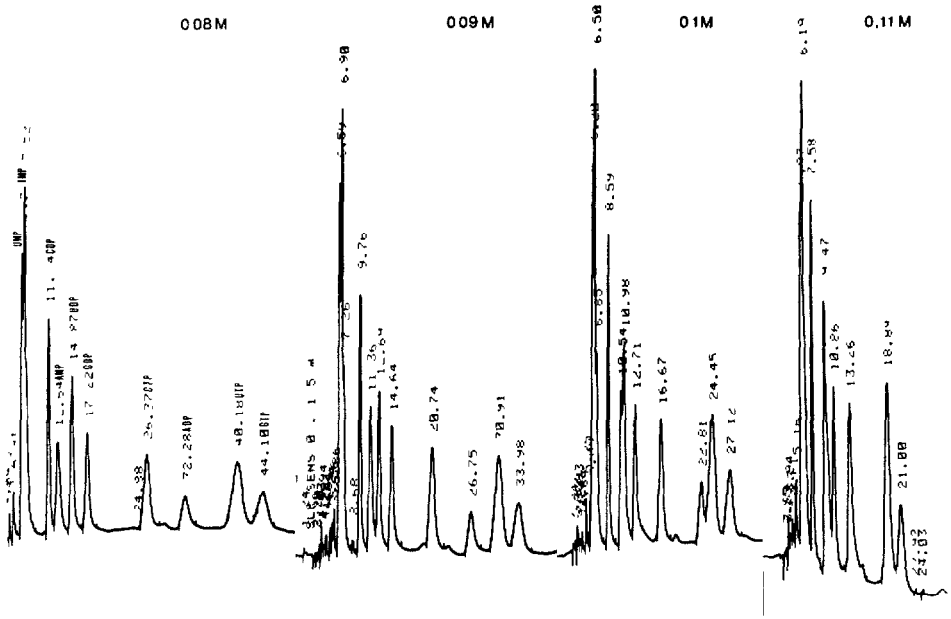

Fig. 2. Effect of sodium chloride concentration (0.08, 0.09, 0.1 and 0.11 *M*) in the mobile phase on the ion-pair liquid chromatographic separation of nucleotides. Hypersil ODS column, 5 μm (25 $\times$ 0.46 cm I.D.). Eluent: 0.025 *M* TBAH, phosphate-buffered (pH 6), containing 5% (v/v) methanol.

methanol concentrations the resolutions of the pairs ADP–UTP and UTP–GTP were improved and ATP was eluted earlier. To demonstrate the effects of sodium chloride and methanol on the capacity factor, ADP was studied in greater detail. Fig. 4 shows that, in general, the ion-pair liquid chromatographic separation of nucleotides is more sensitive to the ionic strength than to the concentration of the organic modifier (methanol) used. Therefore, from a practical viewpoint it is advisable to control the chloride concentration systematically using a simple and sensitive method as described by Schales and Schales [22]. Apart from this observation, there were no significant differences in the separation efficiency when sodium chloride was replaced with the chlorides of K^+, Li^+, Cs^+ or ammonium ions at the same concentrations. The application of iodide or bromide was not suitable owing to their high UV absorbances. Phosphate, perchlorate or acetate

TABLE IV

EFFECT OF SODIUM CHLORIDE CONCENTRATION ON THE RESOLUTION OF SOME CRITICAL PAIRS OF NUCLEOTIDES

Methanol concentration: 5%, v/v.

Sodium chloride (*M*)	Resolution		
	AMP-UDP	ADP-UTP	UTP-GTP
0.08	1.89	2.73	1.12
0.09	1.35	1.84	1.18
0.10	0.53	0.86	1.24

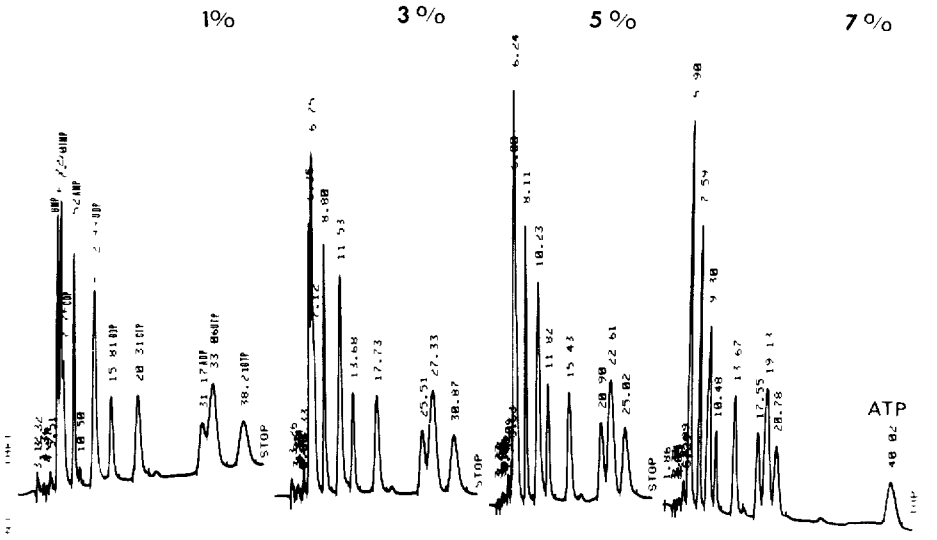

Fig. 3. Effect of methanol content (1, 3, 5 and 7%) in the mobile phase on the ion-pair liquid chromatographic separation of nucleotides. Sodium chloride concentration: 0.1 *M*. Chromatographic conditions as in Fig. 2.

gave similar separations to those obtained with sodium chloride. For practical reasons, phosphate was selected to maintain the pH of the buffer system.

In conclusion, the range of chromatographic parameters suitable in the ion-pair technique for the optimal separation of metabolically important nucleotides in biological samples is relatively narrow, and the solvent system should contain 0.08–0.1 *M* sodium chloride and 5–7% (v/v) methanol in a phosphate-buffered (pH 6) 0.025 *M* TBAH solution. With regard to the selectivity of the ion-pair liquid chromatographic method, it should be noted that using isocratic conditions neither one specific mobile phase composition nor any combination of the mobile and stationary phases are capable of separating all of the nucleotides of biological interest in the same system and/or with a reasonable retention time. In general, the optimal composition of the mobile phase is governed mainly by the specific aims of the analysis.

TABLE V

EFFECT OF METHANOL CONTENT ON THE RESOLUTION OF SOME CRITICAL PAIRS OF NUCLEOTIDES

Sodium chloride concentration: 0.10 *M*.

Methanol (%, v/v)	Resolution	
	ADP-UTP	UTP-GTP
1	0.57	1.62
3	0.75	1.30
5	0.87	1.08
7	0.92	0.84

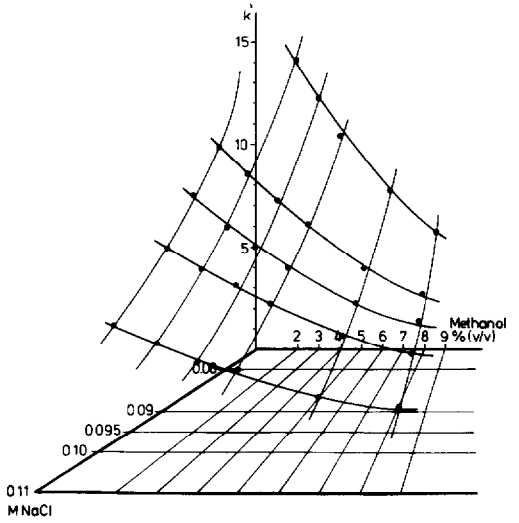

Fig. 4. ADP isotherms for capacity factors (k') at different methanol and sodium chloride concentrations in the mobile phase. Hypersil ODS column, 5 μm (25×0.46 cm I.D.). Eluent: 0.025 M TBAH, phosphate-buffered (pH 6). Detection: 254 nm. Temperature: 30°C.

To shorten the time of analysis and improve the resolution a gradient elution by increasing the methanol concentration in the mobile phase from 0 to 20% (v/v) in 45 min was introduced. Fig. 5 shows that using a simple gradient programme for methanol (Table I) and the mixed type (C_2/C_{18}) Pharmacia MinoRPC column, an exceptionally good and simultaneous fractionation of four nucleosides and twelve nucleotide phosphates was achieved. In addition, Fig. 5B shows that six prominent monophosphates including inositol and orotidine 5′-derivatives can also be separated satisfactorily.

On comparing the ion-exchange and ion-pair liquid chromatographic methods it is apparent that they represent two different but complementary means of identifying and quantifying nucleotides [6, 10]. The Pharmacia FPLC system with the Polyanion SI HR 5/5 column has the desirable qualities (speed, low pressure, well controlled gradient formation) for the routine analysis of complex mixtures of nucleotide phosphates. However, this work has shown that the packing material in this system is highly sensitive to the impurities of the reagents used, and nucleosides are not separated (eluted at about the void volume) under these conditions. Impurities can be eliminated by submitting the column systematically to the purification procedures recommended by the manufacturer. The ion-pair technique, on the other hand, appeared to be more flexible and offered more possibilities for changing the stationary and/or mobile phase parameters. One of the most valuable features of this method is the relative insensitivity of the reversed phase to the impurities. In practice, washing of the column with pure methanol solved these problems. The methods developed in this work have allowed an investigation of metabolic changes, especially in nucleotide levels induced by tumour progression or by the different drug treatments in P388 leukaemia cells (Fig. 1B–D), to be carried out.

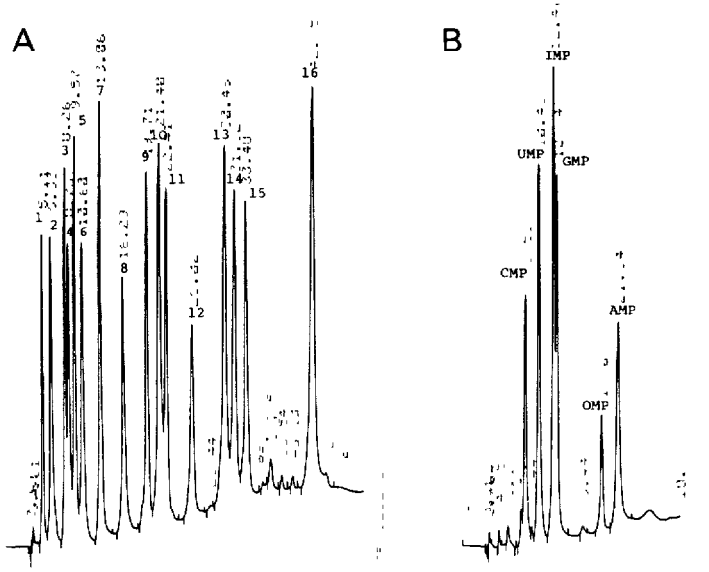

Fig. 5. Ion-pair liquid chromatographic separation of nucleosides and nucleotide phosphates on a Pharmacia MinoRPC 5-μm column (20×0.46 cm I.D.) with gradient elution of methanol ranging from 0 to 20% (v/v) in 45 min. Eluent: 0.025 *M* TBAH, phosphate-buffered (pH 6), containing 0.08 *M* sodium chloride. Flow-rate: 0.70 ml/min. Detection: 254 nm. (A) Peaks: 1 = cytidine; 2 = uridine; 3 = adenosine; 4 = CMP; 5 = guanosine; 6 = UMP; 7 = GMP; 8 = CDP; 9 = UDP; 10 = AMP; 11 = GDP; 12 = CTP; 13 = UTP; 14 = GTP; 15 = ADP; 16 = ATP. (B) Fractionation of six nucleotide monophosphates in the same system.

ACKNOWLEDGEMENTS

The authors thank Dr. Stephen Oldfield and Imre Holenyi of Pharmacia Biotechnology International (Uppsala, Sweden) for generous support and cooperation, and the Centre de Recherche Merrell International (Strasbourg, France) for providing DL-α-difluoromethylornithine. The valuable technical assistance of Miss Edit Paulik, Mrs. Vera Scheuring, Mrs. Andrea Szobi, Miss Beata Sohonyai and Mrs. Susan Németh is gratefully acknowledged.

REFERENCES

1 D. Hunting and J.F. Henderson, Methods Cancer Res., 20 (1982) 245.
2 R.E. Majors, H.G. Barth snd C.H. Lochmüller, Anal. Chem., 56 (1984) 300R.
3 J.D. Karkas, J. Germershausen and R. Liou, J. Chromatogr., 214 (1981) 267.
4 R.A. De Abreu, J.M. Van Baal, J.A.J.M. Bakkeren, C.H.M.M. De Bruyn and E.D.A.M. Schretlen, J. Chromatogr., 227 (1982) 45.
5 E.P. Harmsen, Ph. de Tombe and J.W. de Jong, J. Chromatogr , 230 (1982) 131.
6 E. Freese, Z. Olempska-Beer and M. Eisenberg, J. Chromatogr., 284 (1984) 125.
7 J. Richey, Am. Lab., 14 (1982) 104.
8 O. Heby, S.M. Oredsson and M. Kanje, Adv. Enzyme Regul., 22 (1984) 243.
9 T. Kremmer, M. Boldizsár and L. Holczinger, Exp. Cell Biol., 54 (1986) 8.
10 S.M. Payne and B.N. Ames, Anal. Biochem., 123 (1982) 151.
11 E. Tomlinson, T.M. Jefferies and C.M. Riley, J. Chromatogr., 159 (1978) 315.

12 R.A. Hartwick, S.P. Assenza and P.R. Brown, in A. Zlatkis (Editor), Advances in Chromatography, University of Houston, Houston, TX, 1979, p. 725
13 T.F. Walseth, G. Graff, M.C. Moos, Jr. and N.D. Goldberg, Anal. Biochem., 107 (1980) 240.
14 J.B. Crowther, R. Jones and R.A. Hartwick, in A. Zlatkis (Editor), Advances in Chromatography, University of Houston, Houston, TX, 1981, p. 517.
15 J.H. Knox and J. Jurand, J. Chromatogr., 203 (1981) 85.
16 R.P. Agarwal, P.P. Major and D.W. Kufe, J. Chromatogr., 231 (1982) 418.
17 H. Martinez-Valdez, R.M. Kothari, H.V. Hershey and M.W. Taylor, J. Chromatogr., 247 (1982) 307.
18 S.P. Assenza, P.R. Brown and A.P. Goldberg, J. Chromatogr., 277 (1983) 305.
19 A. Sauter, J. Chromatogr., 325 (1985) 314.
20 W.R. Melander and Cs. Horváth, in M.T.W. Hearn (Editor), Ion-Pair Chromatography, Theory and Biological and Pharmaceutical Applications, Marcel Dekker, New York, Basle, 1985, p. 27.
21 P.A. Perrone and P.R. Brown, in M.T.W. Hearn (Editor), Ion-Pair Chromatography, Theory and Biological and Pharmaceutical Applications, Marcel Dekker, New York, Basle, 1985, p. 259.
22 O. Schales and S.S. Schales, J. Biol. Chem., 140 (1941) 879.
23 J.H. Knox, High-Performance Liquid Chromatography, Edinburgh University Press, Edinburgh, 1977.
24 T. Kremmer, M. Boldizsár and L. Holczinger, Chromatographia, 20 (1985) 79.
25 Á. Bartha and Gy. Vigh, J. Chromatogr., 260 (1983) 337.
26 Á. Bartha, Gy. Vigh, H. Billiet and L. De Galan, J. Chromatogr., 303 (1984) 29.