Journal of Chromatography, 493 (1989) 45–52
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4794

APPLICATION OF THE FAST PROTEIN LIQUID CHROMATOGRAPHIC SYSTEM AND MonoQ HR 5/5 ANION EXCHANGER TO THE SEPARATION OF NUCLEOTIDES

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(First received January 11th, 1989; revised manuscript received March 22nd 1989)

SUMMARY

The applicability of a new type of anion exchanger, MonoQ® HR 5/5, and the Pharmacia-LKB fast protein liquid chromatographic (FPLC) system to the separation of nucleotides is described. The elution characteristics of adenosine-5′-, cytidine-5′-, uridine-5′-, guanosine-5′-mono-, -di- and -triphosphates and inositol-5′-monophosphate reference compounds, and of nucleotides originating from various biological samples, are optimized by varying the concentration gradient programme with ammonium phosphate buffer. Some practical examples of biological interest for monitoring the metabolic changes of nucleotides are presented.

INTRODUCTION

Recent developments in high-performance liquid chromatography (HPLC) have led to improved separations of several metabolites of physiological importance [1–3]. The HPLC analysis of nucleotides has acquired growing interest in relation to the metabolism of nucleic acids and to interferences with various drug treatments [4–9]. Apart from the variability and versatility of reversed-phase and ion-pair techniques [6,9,10], ion-exchange liquid chromatographic methods are still in use for the separation of complex mixtures of

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natural nucleotides [11–15]. Well proven MonoBeads®, particularly MonoQ HR 5/5, a strong anion exchanger operated by the fully automated fast protein liquid chromatography (FPLC) system (Pharmacia–LKB Instruments, Uppsala, Sweden), proved to be appropriate for the separation of thirteen nucleotides of natural origin. In this work, optimization of the chromatographic conditions and applicability of the proposed method are demonstrated.

EXPERIMENTAL

Reagents

Ammonium phosphate buffer (pH 7.0) was prepared from high-purity reagents (25% ammonium solution, Suprapur; 85% orthophosphoric acid, Selectipur; Merck, Darmstadt, F.R.G.) and dissolved in water distilled twice in a glass apparatus. Other chemicals were of analytical-reagent grade. Reference nucleotides [adenosine-, cytidine-, uridine-, guanosine-5′-mono-, -di- and -triphosphates (AMP, ADP, ATP, CMP, CDP, CTP, UMP, UDP, UTP, GMP, GDP and GTP) and inositol-5′-monophosphate (IMP)] were purchased from Serva (Heidelberg, F.R.G.) and Reanal (Budapest, Hungary).

Sample preparation

Procedures for handling and collecting biological samples (tissues, tumour cells) have been detailed elsewhere [5,8,16]. The nucleotide content of cell homogenates was generally extracted with ice-cold 0.7 *M* perchloric acid ($25 \cdot 10^6$ cells, or 100 mg/ml wet tissue), centrifuged (3000 *g*, 20 min, 0°C) and neutralized with solid potassium hydrogencarbonate (10 mg per 100 μl). After centrifugation, the clear supernatants were assayed by HPLC or FPLC. A mixture of reference compounds containing 0.5–5 nmol per 10 μl of each was prepared from stock solutions (1 mg/ml). The purity and concentration of the standards were controlled by separate analyses.

Instrumentation and chromatographic conditions

The reference mixture of nucleotides and those of natural origin were separated on the polymer-based MonoQ HR 5/5 strong anion-exchange column (5 cm × 0.5 cm I.D., 10 μm; Pharmacia–LKB). The FPLC system consisted of two P-500 reciprocating pumps, an MV-7 motor valve for introduction of samples (10–100 μl) and a UV-1 monitor for detection at 254 nm. The FPLC system was controlled by an LCC-500 programmer providing the prints (Fig. 1) and the numerical characteristics (programme, retention times, peak areas, etc.) of the separations. Chromatograms and the gradient programme were also recorded with an REC-482 two-channel recorder. Nucleotides were eluted with ammonium phosphate buffer (pH 7.0) using an improved gradient pro-

gramme in the concentration range 0.02–0.5 *M*. The method was quantified on the basis of peak areas measured with the LCC-500 controller. In order to control and compare the results obtained, ion-pair HPLC of nucleotides was accomplished simultaneously as described recently [9].

RESULTS AND DISCUSSION

As a result of progress in the preparation of polymer-based packing materials, uniform resin spheres with a nearly monodisperse particle size distribution (MonoBeads, Pharmacia–LKB) were designed to overcome the problems encountered by the sensitivity and pH lability of silica-based ion exchangers. From a collection of references [17] it can be concluded that MonoQ has been applied successfully in many biochemical separations [4,7,11–17]. Apart from the wide variety of practical examples, MonoQ has not yet been adapted for

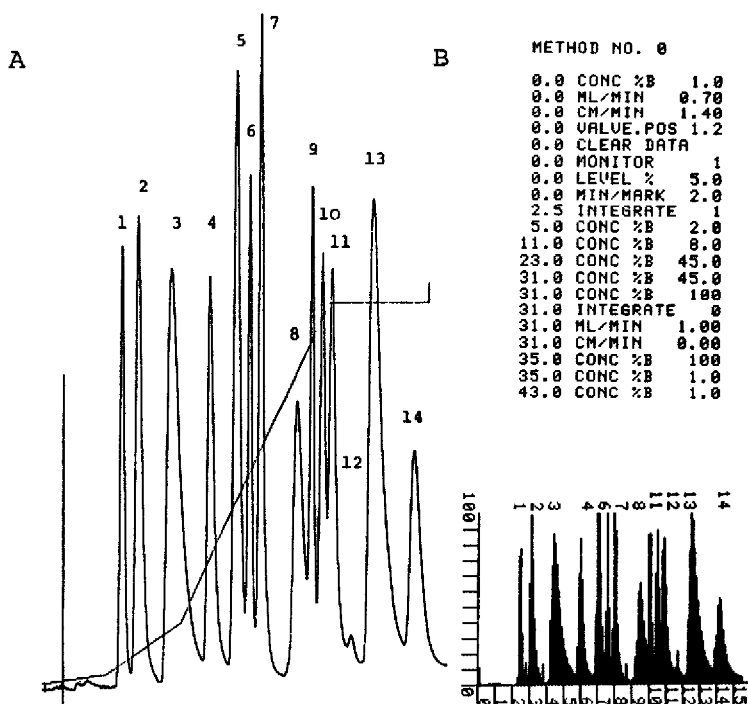

Fig. 1. (A) Ion-exchange chromatographic separation of nucleotides (reference compounds injected in amounts varying from 0.5 to 10 nmol per 50 μl) on a MonoQ HR 5/5 column (5 cm×0.5 cm I.D., 10 μm) using a concentration gradient programme of ammonium phosphate buffer (pH 7.0). Flow-rate, 0.7 ml/min; detection, 254 nm (0.01 a.u.f.s.) at ambient temperature. Peaks: 1=CMP; 2=UMP; 3=AMP; 4=IMP; 5=GMP; 6=CDP; 7=UDP; 8=ADP; 9=CTP, 10=UTP; 11=GDP; 12=unknown; 13=ATP; 14=GTP. For abbreviations, see Experimental. (B) Elution programme (method) and chromatogram printed by the LCC-500 controller.

the fractionation of nucleotides. In this work, the applicability and selectivity of MonoQ for the resolution of thirteen nucleotides of biological interest using the FPLC system and a carefully selected elution programme are demonstrated.

Improvements in the shape of concentration gradient of ammonium phosphate buffer in the range 0.02–0.5 *M* are shown in Fig. 1. In preliminary experiments it was found that a low concentration (0.01 *M*) and a shallow gradient of buffer not exceeding a rate of 0.002 *M*/min for 5 min, then 0.01 *M*/min for 6 min, was necessary first for the optimum separation of mono- and diphosphates. Further alterations in the slope (0.03 *M*/min for 12 min) did not improve further the fractionation of the ADP–CTP–UTP–GDP complex. However, in comparison with the reference mixture, a higher resolution of critical pairs (ADP–CTP, CTP–UTP, UTP–GDP) could be obtained in the biological samples owing to the uneven distribution of nucleotides (see Figs. 2–4).

From the data in Table I it can be concluded that the retention times were reproducible with an average coefficient of variation (C.V.) of 2.76%, whereas 7.71% was found for the peak-area values. The sensitivity of the method was 0.01–0.02 nmol per 50 μl for all nucleotides tested (0.05 a.u.f.s.), and the accuracy was better than 97% for the determination of individual fractions in the biological samples, verified by the recovery of internal standards. It should

TABLE I

REPRODUCIBILITY OF RETENTION TIMES AND PEAK AREAS FOR NUCLEOTIDES SEPARATED ON A MonoQ HR 5/5 COLUMN

Mean values ± S.D. were calculated from eleven separate assays in a concentration range of nucleotides of 1–10 nmol.

Nucleotide[a]	Retention time		Peak-area
	t_R (min)	C.V. (%)	C.V. (%)
CMP	4.72 ± 0.32	6.78	8.28
UMP	5.94 ± 0.38	6.46	7.01
AMP	8.33 ± 0.28	3 36	6.38
IMP	11.54 ± 0.45	3.86	9.02
GMP	13.91 ± 0.30	2.17	7.99
CDP	15.17 ± 0.15	0.98	7.54
UDP	15.95 ± 0.23	1 42	8.74
ADP	18.67 ± 0.33	1.78	4.09
CTP	20.15 ± 0.27	1.32	11.26
UTP	20.87 ± 0.34	1.64	6.17
GDP	21.66 ± 0.38	1.73	8.03
ATP	24.82 ± 0 51	2.06	9.19
GTP	28.29 ± 0.67	2.38	6.75
Average		2.76	7.71

[a]For abbreviations, see Experimental.

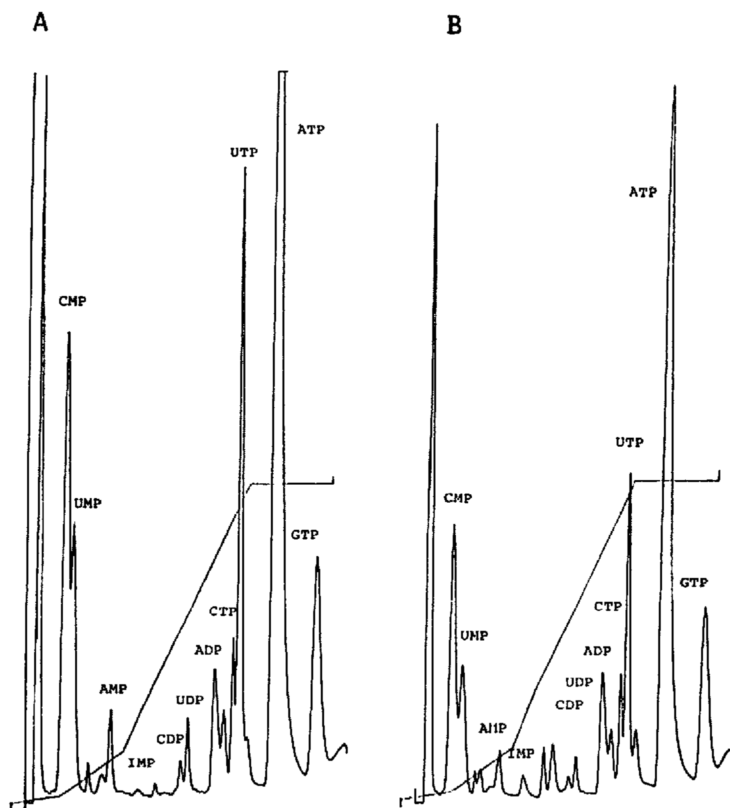

Fig. 2. Nucleotide patterns of (A) untreated (control) K562 human leukaemia cells and (B) after 96 h of in vitro haemin treatment. Chromatographic conditions and abbreviations as in Fig. 1.

be noted that the order of elution of some nucleotide pairs (guanosine–adenosine, cytidine–uridine) is reversed on MonoQ compared with that on other ion exchangers [9], indicating that molecular interactions different from ionic forces may also influence the selectivity of the stationary phases. In this respect, MonoQ has favourable selectivity.

By comparing the advantages and disadvantages of the methods applied, ion-pair liquid chromatography was undoubtedly time-consuming but provided more selective simultaneous separations of both nucleosides and nucleotides [9,10]. Conversely, the ion-exchange technique using the fast and precise automation of the FPLC system with MonoQ seemed to be more convenient for routine work, although uncertainty in the elution parameters of monophosphates (CMP and UMP, C.V. 6–7%; see Table I) occurred owing to the inevitable low buffer concentration.

The applicability of MonoQ to the separation of nucleotides is illustrated

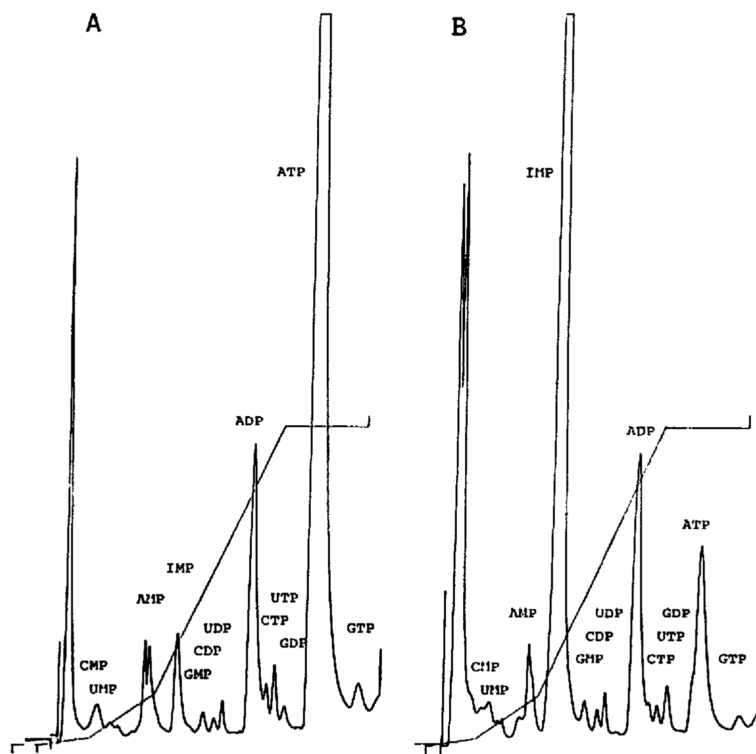

Fig. 3. Nucleotide composition of a pork meat sample (A) freshly cut and (B) after storage at room temperature for 1 h. Chromatographic conditions and abbreviations as in Fig. 1.

with some practical examples of biochemical analyses. Fig. 2 shows the in vitro changes in the nucleotide pool of K562 human leukemia cells induced by haemin treatment for differentiation [16]. It seems clear that after 96 h of haemin treatment the intracellular contents of nucleotides were reduced; in particular, ATP, CTP, UTP and GTP levels were reduced by 53, 40, 52 and 47%, respectively, in comparison with the control cells.

In another application, in the quality control of food chemistry, the catabolic rearrangement of nucleotides due to the storage of pork meat was monitored on MonoQ. Fig. 3A demonstrates that a composition of a sample taken immediately from the freshly cut tissue is characterized by high ATP (7.15 nmol/mg) and lower ADP (1.16 nmol/mg), AMP (0.237 nmol/mg) and IMP (0.074 nmol/mg) levels, respectively. As a result of fast post-mortem enzymatic reactions (Fig. 3B), a pronounced increase in IMP (0.671 nmol/mg) and ADP (1.53 nmol/mg) occurred within 1 h, with a simultaneous decrease in ATP (6.06 nmol/mg) (the data given represent the averages of five separate assays) [18].

Ion-exchange chromatography of nucleotides using MonoQ was also used to

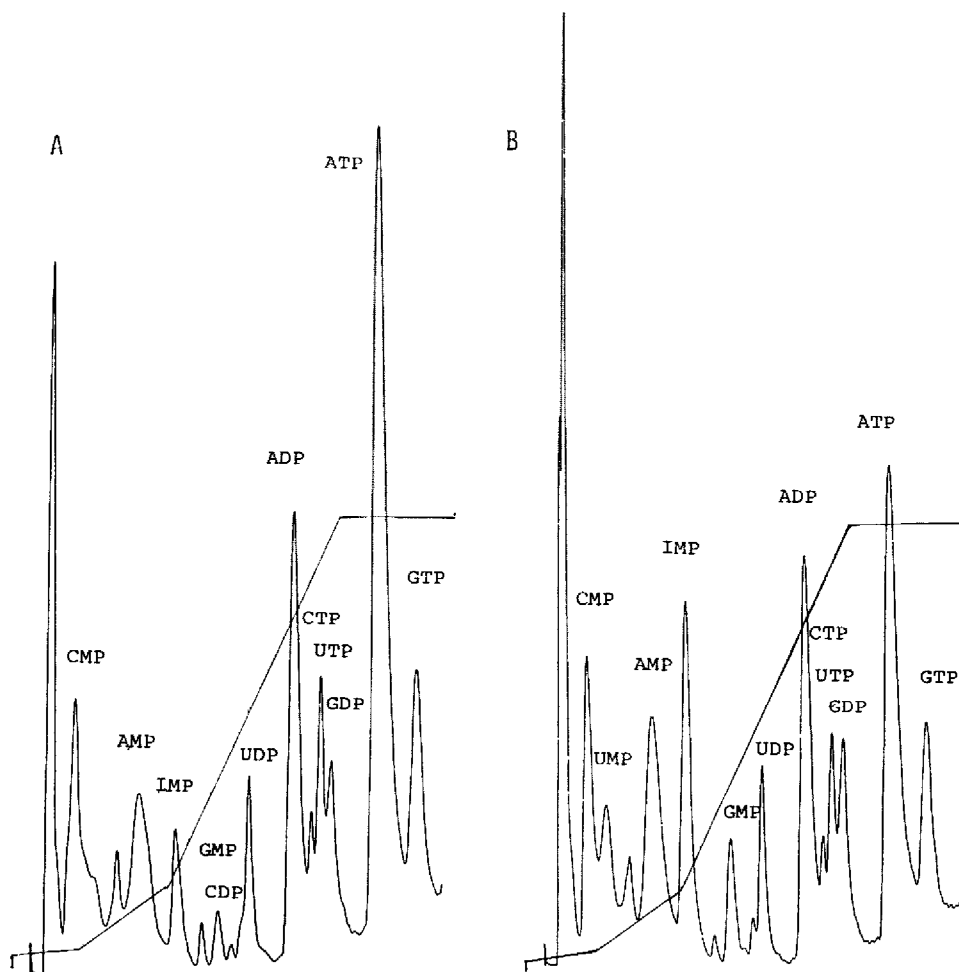

Fig. 4. In vivo changes in the nucleotide content of L1210 leukaemia cells during the tumour growth: (A) one-day-old and (B) two-day-old tumour cells. Chromatographic conditions and abbreviations as in Fig. 1.

follow the metabolic changes in L1210 leukaemia cells during tumour growth in vivo (Fig. 4). Sharp differences, mainly in the distribution of AMP, ADP and ATP, were observed in the nucleotide composition of leukaemia cells of different ages. Within 24–72 h after transplantation, a 24–106% increase in AMP and 9–33 and 40–65% decreases in ADP and ATP levels were measured. Similar observations have also been made with P388 leukaemia, indicating an aerobic–anaerobic shift in the energy metabolism of tumour cells [19].

In conclusion, an efficient separation of thirteen nucleotides, reference com-

pounds representing the main constituents of natural mixtures, can be achieved by using the Pharmacia–LKB FPLC system with a MonoQ HR 5/5 anion-exchange column. This technique allowed the development of a precise and reproducible method for the determination of nucleotides originating from different biological sources. The applicability of MonoQ to bioanalytical problems concerning the metabolic changes of nucleotides in various tissues and cells was demonstrated.

ACKNOWLEDGEMENTS

The authors thank Pharmacia–LKB Instruments for their cooperation. The valuable technical assistance of Mrs. Vera Scheuring, Mrs. Beata Akacs and Mrs. Gyöngyi Salamon is gratefully acknowledged.

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