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Determination of fifteen nucleotides in cultured human mononuclear blood and umbilical vein endothelial cells by solvent generated ion-pair chromatography

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Abstract

The paper describes the development of a method for the determination of 15 nucleotides in cultured mononuclear blood and umbilical vein endothelial cell lysates by solvent generated ion-pair chromatography. The phase system is generated via a mobile phase of 100 m*M* phosphoric acid adjusted to pH 6.2 with triethylamine. Nucleotides are eluted by applying a linear magnesium ion gradient. The method is robust, highly reproducible and easily adaptable to other cell lysates and allows the separation and quantitation of the nucleotides with detection limits in the range from 17 (ADP) to 126 (CDP) pmol in 20-μl aliquots.

Keywords: Solvent generation; Nucleotides

1. Introduction

Mycophenolic acid (MPA), the active metabolite of mycophenolate mofetil (MMF), is an immunosuppressant which acts by reversibly inhibiting inosine-5′-monophosphate dehydrogenase (IMPDH). IMPDH is the key enzyme in the synthesis of guanosine monophosphate (GMP) from inosine monophosphate (IMP), a rate-limiting step in the biosynthesis of purines. Lymphocytes have to rely on the de novo purine synthesis since they lack a purine salvage pathway. Depletion of the guanine nucleotide pool by MPA thus inhibits the proliferation of T and B lymphocytes preventing the production of antibodies and generation of cytotoxic T cells [1,2].

In previous papers we investigated the in vitro effects of MPA on the nucleotide pool of human peripheral blood mononuclear cells (PBMCs) [3] and human umbilical vein endothelial cells (HUVECs) [4]. In order to detect changes in the nucleotide pool of the cells it is necessary to quantitate the nucleotides in the cell lysates by an analytical method which allows the measurement of low amounts of

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neutral and ionised nucleotides in complex biological matrices.

Ion-exchange [5–8] and ion-pair chromatography [9–16] are the most important methods for the determination of nucleotides. Although ion-exchange chromatography is eminently suitable for the analysis of charged compounds it has several disadvantages. Ion-exchange columns are often poorly reproducible from lot to lot and less stable than other LC columns since they have to be operated with eluents of low pH to elute ionic analytes.

Reversed-phase ion-pair chromatography on RP-columns promised to be the method of choice because RP-columns offer higher efficiency than fixed-site ion exchangers and—by exploiting additional secondary equilibria for optimizing the separation—also greater versatility. In addition the chromatographic phase system can easily be washed off the column packing material and renewed via the mobile phase minimizing the effect of irreversible adsorption of sample or matrix components.

In ion-pair chromatography desorption of the analytes is commonly achieved by gradient elution using pH gradients and gradients of organic modifiers, i.e. acetonitrile or methanol. Alternatively, selective desorption has successfully been carried out by introducing magnesium complexation as additional secondary equilibrium [10]. By adding Mg^{2+} as competing counter ion to the mobile phase, Mg–nucleotide complexes are formed, which are much less retained than the corresponding TEA–nucleotide species. This elution method is very selective since hydrophobic matrix components bound to the C_{18} stationary phase are not eluted when they do not form complexes with Mg^{2+}. It has successfully been applied for the determination of the adenosine and guanosine nucleotides in isolated mitochondria of *Saccharomyces cerevisiae* (without giving experimental data) [10] and the determination of the adenosine nucleotides in *Escherichia coli* [16].

The present paper describes the development of a modification of this ion-pair chromatography method extending its application range for 15 nucleotides and applying it for the detection of changes induced by MPA and other immunosuppressive agents in the intracellular nucleotide pool of PBMCs and HUVECs.

2. Experimental

2.1. Reagents

All nucleotides (see Table 1), triethylamine (TEA), mycophenolic acid (MPA), phytohaemagglutinin, prednisolone, cyclosporin A (CsA), Medium 199 (Hepes-modification) and heparin were purchased from Sigma (St. Louis, MO, USA). *o*-H_3PO_4, $HClO_4$ and K_2HPO_4 were obtained from Merck (Darmstadt, Germany). RPMI-1640 Medium (with glutamax-I and Hepes), penicillin–streptomycin, L-glutamine and PBS (without Ca^{2+} and Mg^{2+}) were purchased from Gibco-Life Technologies (Merelbeke, Belgium). Furthermore endothelial cell growth supplement (ECGS) (Upstate Biotechnologies, Waltham, MA, USA), HFN (Chemicon, Hofheim, Germany), collagenase type 2 (Worthington Biochemical, Freehold, NJ, USA) and foetal calf serum (PromoCell, Heidelberg, Germany) were used.

2.2. Preparation of PBMC and HUVEC samples

PBMCs were isolated according to a previously described procedure [3]. HUVECs were isolated and cultured as described in Ref. [4].

After stimulating PBMCs with phytohaemagglutinin (2.5 $\mu g/10^6$ cells) and HUVECs with TNF-

Table 1
Nucleotides and their abbreviations

Nucleotide	Abbreviation
Cytidine-5′-monophosphate	CMP
Uridine-5′-monophosphate	UMP
Cytidine-5′-diphosphate	CDP
Cytidine-5′-triphosphate	CTP
Uridine-5′-diphosphate	UDP
Guanosine-5′-monophosphate	GMP
Inosine-5′-monophosphate	IMP
Uridine-5′-triphosphate	UTP
Guanosine-5′-diphosphate	GDP
Inosine-5′-diphosphate	IDP
Guanosine-5′-triphosphate	GTP
Inosine-5′-triphosphate	ITP
Adenosine-5′-monophosphate	AMP
Adenosine-5′-diphosphate	ADP
Adenosine-5′-triphosphate	ATP

α (10 ng/ml) the cells were co-incubated with varying concentrations of MPA (final concentration 0.5–100 μ*M*) or MPA in combination with constant amounts of cyclosporin A (100 ng/ml) and prednisolone (1 μ*M*). The cells were incubated for 68 h in a humidified incubator set at 37 °C and 5% CO_2.

After removing the culture medium and washing cell lysis was carried out by adding 150 μl of cold 0.42 *M* $HClO_4$ solution and incubating for 30 min at −18 °C. The cell extract was then neutralised with 150 μl of an 1 *M* K_2HPO_4 solution (>pH 7.2). After centrifugation 250 μl of the extract were collected, immediately frozen and stored at −18 °C until chromatographic analysis. Before injection into HPLC samples were thawed, centrifuged at 5000 rpm for 2 min and filtered through a 0.45-μm PTFE membrane filter (Sartorius, Göttingen, Germany).

The stability of nucleotides towards hydrolysis by perchloric acid used for cell lysis was investigated by carrying out the sample preparation steps described above using 50 μl of nucleotide standard solutions which had been incubated with 150 μl of cold 0.42 *M* $HClO_4$ for 30, 60 and 120 min.

2.3. Chromatographic separation of nucleotides

2.3.1. Instrumentation

The HPLC system consisted of a Model L-6200 Merck Hitachi gradient pump and a six-port injection valve Model 7161 (Rheodyne, Cotati, CA, USA) equipped with a 20-μl stainless steel injection loop and a Merck Hitachi UV detector model L-4000 set at 254 nm. Peak integration was carried out by using a HP software (Hewlett-Packard, Little Falls, DE, USA).

The column was thermostated with a column oven (Model bfo-04 dt, W.O. Electronics, Langenzersdorf, Austria). In all experiments the flow-rate was 1 ml/min.

Column void time determined by injecting formamide was t_0=2.224 min with a confidence interval of 0.004 min (n=8; P=0.95).

Resolution (R_s) of adjacent peaks was calculated using standard deviation data as evaluated by the software according to

$$R_s = \frac{t_{R_2} - t_{R_1}}{2(\sigma_{t,1} + \sigma_{t,2})} = 2\frac{t_{R_2} - t_{R_1}}{w_1 + w_2}$$

where t_{R_1} and t_{R_2} are the retention times, $\sigma_{t,1}$ and $\sigma_{t,2}$ are the standard deviations and w_1 and w_2 are the peak widths at base of the first and adjacent second peak.

2.3.2. Phase system

2.3.2.1. Stationary phase. The stationary phases were generated via the mobile phase on Merck 250× 4 mm I.D. Superspher® 100 RP-18 (4 μm) end-capped HPLC columns.

2.3.2.2. Mobile phase. A variety of mobile phases and gradients has been used to investigate the influence of experimental parameters on the retention of nucleotides. In order to get reproducible retention data great care has to be taken in the preparation of the mobile phase. The volatility of TEA makes it difficult to weigh with sufficient reproducibility. A rather long series of experiments showed that the most reproducible method to prepare mobile phases containing phosphoric acid of a certain molarity and TEA consists of weighing in phosphoric acid and titrating it with TEA to the pH desired. (Mobile phases of different pH therefore also contain different concentrations of the ion-pair reagent TEA).

Gradients were formed by mixing two mobile phases, A and B. Mobile phase A consisted of 30 to 100 m*M* H_3PO_4, adjusted with TEA to pH values in the range from 6.20 to 6.76. Mobile phase B consisted of mobile phase A to which $MgSO_4$ was added to obtain a solution which is 5 m*M* in $MgSO_4$.

Prior to use all mobile phases were filtered through a 0.45-μm membrane filter (Supelco) via a vacuum filtration apparatus (Schleicher and Schuell, Dassel, Germany).

Chromatographic conditions applied for determination of nucleotides in the lysates of PBMCs and HUVECs are summarised in Table 2.

2.4. Analytical data

The HPLC system was calibrated by injecting aqueous standard solutions obtained by diluting a solution which was 200 μ*M* for each nucleotide. This standard solution was diluted to give five

Table 2
HPLC protocol for determination of nucleotides in the lysates of PBMCs and HUVECs

Support material	Superspher® 100 RP-18 (4 μm) endcapped
Mobile phase A	100 m*M* H_3PO_4, pH 6.20 (TEA)
Mobile phase B	100 m*M* H_3PO_4, 5 m*M* $MgSO_4$, pH 6.20 (TEA)
Gradient elution	0–33 min: 3.03% B/min. After reaching 100% B elution was continued with 100% B
Flow-rate (ml/min)	1.0
Column temperature (°C)	22
UV detection (nm)	254
Injection volume (μl)	20
Analysis time (min)	60
Reconditioning time (min)	30

additional standard concentrations: 100, 50, 25, 10 and 5 μ*M*. The analysis function was obtained by linear regression of peak areas on standard concentrations. Aqueous stock solutions of the nucleotides were stored at −18 °C.

Reproducibility of nucleotide determination by HPLC was determined by repeated injection of standard solutions in a concentration range from 5 to 100 μ*M* in a time period of 2 months. Reproducibility of the analysis method including cell lysis was determined by producing and analysing the same cell sample on 6 different days.

3. Results and discussion

In ion-pair chromatography secondary chemical equilibria play a dominant role controlling retention and resolution of analytes. Retention is influenced by a large number of experimental variables, including type and concentration of the ion-pair reagent, pH and ionic strength of the mobile phase, concentration of Mg^{2+} (or organic modifier), the type of the gradient elution program and column temperature. In order to achieve optimum separation of the 15 nucleotides listed in Table 1 the influences of various experimental parameters were investigated in about 130 experiments.

Scouting experiments demonstrated that poor resolution due to clustering of peaks poses problems in two regions of the chromatograms: region 1 containing the peaks of CTP, UDP, GMP and IMP and region 2 containing the peaks of IDP and GTP.

3.1. Influence of column temperature

In ion-pair chromatography temperature is an important experimental parameter since the temperature dependence of secondary equilibria makes these phase systems highly sensitive to temperature effects. In order to investigate the influence of column temperature on retention and resolution of the nucleotides various column temperatures in the range 22–32 °C were applied. Table 3 gives the experimental details and illustrates the effects for selected nucleotide pairs. The results indicate that an increase in column temperature generally reduces the capacity factors of all nucleotides. This reduction, however, causes changes in the resolution of critical pairs which can improve (see UTP–IMP) or deteriorate

Table 3
Influence of the column temperature on capacity factors and resolution. Constant parameters: 30 m*M* H_3PO_4, pH 6.64, mobile phase gradient: 2% B/min. After reaching 100% B elution was continued with 100% B

	Temperature (°C)				
	22	25	27	29	32
k'					
CTP	4.81	4.37	4.40	4.16	3.99
GMP	6.22	5.30	5.17	4.77	4.26
IMP	6.90	5.75	5.60	5.18	4.64
UTP	6.77	6.21	6.17	5.81	5.44
GTP	10.65	9.39	9.15	8.50	7.69
ATP	24.96	21.68	20.76	19.17	17.02
R_s					
GMP–CTP	4.20	2.27	2.07	1.64	0.88
IMP–GMP	1.90	0.96	0.94	0.89	0.85
UTP–IMP	0.39	1.22	1.33	1.64	1.95

(see GMP–CTP) with increasing column operating temperature. In some cases the retention changes induced will even lead to a change in elution order for critical pairs as IMP–UTP. At 22 °C IMP ($k' = 6.90$) was eluted after UTP ($k' = 6.77$) while at temperatures ≥25 °C the elution order is changed and IMP was eluted before UTP. Since higher column temperatures in more cases resulted in a decrease and not an increase of resolution of adjacent nucleotides, a column temperature of 22 °C was selected and kept constant in all the following experiments.

3.2. Influence of mobile phase phosphoric acid concentration

Since it is desirable to keep the number of mobile phase components to a minimum it was decided to proceed without separately investigating the influence of the interdependent concentrations of TEA, pH and phosphoric acid, which might have been possible by keeping the amount of phosphoric acid and TEA constant and introducing an additional base (such as NaOH) to adjust the pH. In order to investigate the combined influence of mobile phase phosphoric acid and TEA concentration mobile phases with 30, 60, 90 and 100 m*M* phosphoric acid were prepared and adjusted to the pH desired by titrating with TEA. Mobile phases with higher concentrations of H_3PO_4 made it necessary to add higher amounts of TEA to obtain a certain pH value.

Table 4 illustrates the influence of phosphoric acid concentration by showing the results from a series of experiments with a mobile phase adjusted to pH of 6.64. The results indicate that retention of the nucleotides is differently affected. These differences cause significant changes in the resolution of adjacent peaks which, again, lead to either increased or decreased resolution. Resolution values of several critical nucleotide pairs are included in Table 4. IMP and UTP were co-eluted with a H_3PO_4 concentration of 30 m*M*. By increasing the H_3PO_4 concentration k' of IMP was decreased whereas k' of UTP remained nearly unaffected. The resolution of the two nucleotides thus increased up to $R_{UTP-IMP} = 4.1$ at 100 m*M* H_3PO_4. Resolution between IMP and GMP was only slightly changed over the H_3PO_4 concentration range investigated, because both monophosphates were influenced in the same manner. However, since retention of GMP was decreased but retention of CTP remained nearly constant the resolution of these two nucleotides was reduced as a result of increasing phosphoric acid concentration.

Table 4
Influence of mobile phase phosphoric acid concentration on retention and resolution[a]

	H_3PO_4 concentration (m*M*)			
	30	60	90	100
k'				
UMP	–	–	2.36	2.21
CTP	4.77	4.91	4.66	4.43
UDP	–	–	5.57	5.14
GMP	6.46	5.83	5.30	4.91
IMP	6.96	6.44	5.93	5.51
UTP	6.96	7.50	7.41	7.12
GDP	–	–	9.58	8.91
IDP	–	–	10.94	10.28
GTP	10.91	11.19	10.82	10.28
ITP	12.12	12.71	12.47	11.92
AMP	17.92	16.50	15.11	13.81
ADP	20.84	23.33	23.26	22.11
ATP	25.57	26.60	26.00	24.38
R_s				
UTP–IMP	0	2.52	3.31	4.07
GMP–CTP	4.27	2.27	1.66	1.68
IMP–GMP	1.03	1.36	1.35	1.50

[a] Constant parameters: pH 6.64, column temperature: 22 °C, mobile phase gradient: 2% B/min. After reaching 100% B elution was continued with 100% B. –, not determined.

Since the resolution of the critical pair UTP–IMP dramatically increases with increasing phosphoric acid concentration and ATP—the most strongly retained nucleotide which marks the end of the chromatogram—shows the smallest k' value in 100 m*M* H_3PO_4 it was decided to keep this concentration constant and proceed by exploring the possibility to improve the resolution of other critical pairs by varying the mobile phase pH value.

3.3. Influence of mobile phase pH

The influence of mobile phase pH value on retention and resolution of the nucleotides was

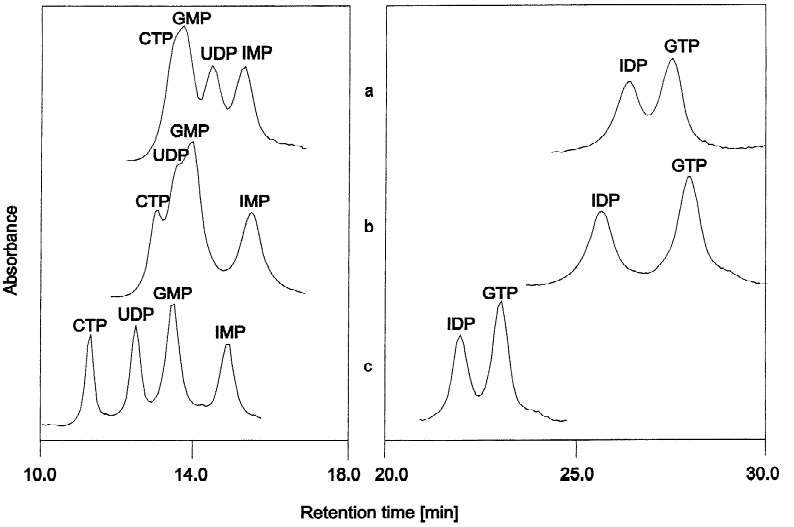

Fig. 1. Influence of pH value and steepness of the Mg^{2+} gradient on two critical regions of the chromatogram. Mobile phase A: 100 m*M* H_3PO_4, pH adjusted with TEA. Mobile phase B: 5 m*M* solution of $MgSO_4$ in mobile phase A. (a) pH 6.6; gradient: 0 min: 100% A, 0–17 min: 1.18% B/min, 17–49 min: 2.50% B/min. (b) pH 6.4; gradient: 0 min: 100% A, 0–17 min: 1.18% B/min, 17–49 min: 2.50% B/min. (c) pH 6.4; gradient: 0 min: 100% A, 0–33 min: 3.03% B/min. After reaching 100% B elution was continued with 100% B. Column temperature: (a)–(c) 22 °C.

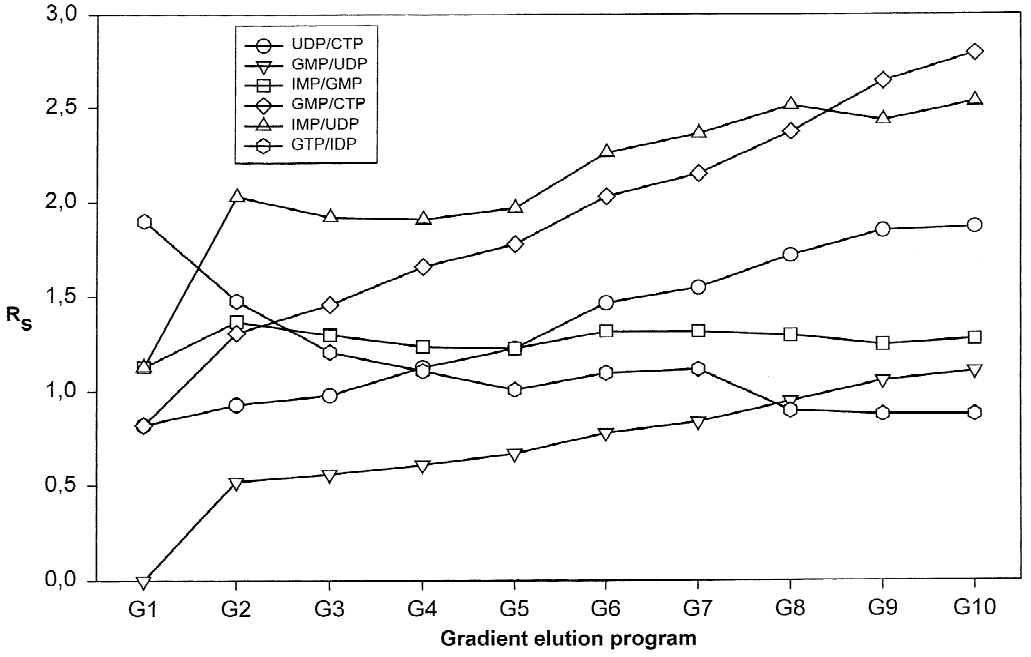

Fig. 2. Influence of Mg^{2+} gradient steepness on the resolution (R_s) of adjacent peaks in critical sections of the chromatogram. Gradient elution programs: G1: 0–17 min: 1.18% B/min, 17–49 min: 2.50% B/min. G2: 0–17 min: 1.41% B/min, 17–47 min: 2.53% B/min. G3: 0–17 min: 1.59% B/min, 17–46 min: 2.52% B/min. G4: 0–17 min: 1.82% B/min, 17–44 min: 2.56% B/min. G5: 0–17 min: 2.0% B/min, 17–43 min: 2.54% B/min. G6: 0–17 min: 2.24% B/min, 17–41 min: 2.58% B/min. G7: 0–40 min: 2.50% B/min. G8: 0–33 min: 3.03% B/min. G9: 0–28 min: 3.57% B/min. G10: 0–25 min: 4.0% B/min. After reaching 100% B elution was continued with 100% B.

Table 5
Influence of mobile phase pH value on retention and resolution[a]

	pH					
	6.40	6.50	6.55	6.60	6.66	6.76
k'						
CMP	1.37	1.29	1.24	1.23	1.19	1.12
UMP	2.57	2.40	2.30	2.27	2.19	2.07
CDP	3.16	3.09	3.05	3.04	2.96	2.88
CTP	5.53	5.23	5.02	5.00	4.83	4.60
UDP	5.53	5.23	5.32	5.30	5.19	5.09
GMP	5.53	5.23	5.02	5.00	4.83	4.60
IMP	6.29	5.91	5.69	5.63	5.44	5.09
UTP	8.77	8.30	8.09	7.93	7.75	7.53
GDP	9.88	9.63	9.48	9.42	9.26	9.02
IDP	11.37	11.12	10.95	10.89	10.72	10.45
GTP	12.61	11.96	11.64	11.39	11.17	10.81
ITP	14.38	13.68	13.33	13.08	12.83	12.43
AMP	15.70	14.83	14.32	14.14	13.74	13.00
ADP	23.73	23.24	23.00	22.79	22.55	22.15
ATP	27.86	26.64	25.99	25.45	24.98	24.20
R_s						
GMP–CTP	0	0	0	0	0	0
UDP–GMP	0	0	0.79	0.74	0.95	1.04
IMP–UDP	1.53	1.17	0.84	0.90	0.70	0
GTP–IDP	2.35	1.77	1.33	0.94	1.00	0.79
AMP–ITP	2.15	2.13	1.45	1.74	1.75	1.00

[a] Constant parameters: 100 m*M* H_3PO_4, column temperature: 22 °C, mobile phase gradient: 0–17 min: 1.18% B/min, 17–49 min: 2.5% B/min. After reaching 100% B elution was continued with 100% B.

investigated in the range from pH 6.40 to 6.76. (Since adjustment of the pH value was carried out by adding the appropriate amount of TEA an increase in mobile phase pH coincided with a higher concentration of TEA).

Fig. 1 illustrates the effect of different optimisation strategies on the resolution of critical pairs. Fig. 1a and b demonstrates how a small change of pH (ΔpH 0.2) not only changes the resolution but also the elution order.

Table 5 shows how the capacity factors of the nucleotides depend on mobile phase pH values in the range from pH 6.40–6.76. For all the nucleotides increasing pH leads to a decrease in retention values but the slope of this decrease is different for different nucleotides resulting in changes in resolution of some critical pairs. These changes result in a deterioration of the resolution for IMP–UDP, GTP–IDP and AMP–ITP with only a slight improvement for UDP–GMP and no change at all for GMP–CTP.

Since higher mobile phase pH values led to lower resolutions of adjacent peaks in several regions of the chromatogram further attempts to improve the resolution of critical pairs started from a mobile phase of pH 6.40 and investigated the possibilities offered by magnesium gradient elution programs.

3.4. Influence of magnesium ion gradient elution programs

The influence of ten different magnesium ion gradient elution programs on retention of the nucleotides was investigated. Table 6 summarises the capacity factors obtained. The results demonstrate that varying the steepness of the gradient had a minor effect on the elution of the nucleotide monophosphates, whereas elution of diphosphates was more affected and elution of triphosphates was influenced most. Figs. 1 and 2 show the effect of Mg^{2+} gradient steepness on resolution of some adjacent peaks. Increasing the steepness of the magnesium ion gradient led to higher resolution

Table 6
Influence of steepness of Mg^{2+} gradient on retention of nucleotides

	k'									
	Gradient elution program[a]									
	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10
CMP	–	–	–	–	–	–	1.20	1.21	–	–
UMP	–	–	–	–	–	–	2.24	2.26	–	–
CDP	–	–	–	–	–	–	2.72	2.73	–	–
CTP	4.80	4.68	4.61	4.50	4.44	4.36	4.17	4.12	4.05	3.99
UDP	5.18	4.98	4.95	4.90	4.87	4.81	4.64	4.63	4.60	4.56
GMP	5.18	5.18	5.18	5.17	5.16	5.11	4.99	5.02	5.03	5.01
IMP	5.85	5.84	5.83	5.82	5.81	5.76	5.62	5.65	5.65	5.64
UTP	–	–	–	–	–	–	6.60	6.46	–	–
GDP	–	–	–	–	–	–	8.14	8.02	–	–
IDP	10.55	10.36	10.19	10.0	9.87	9.67	9.27	9.11	8.93	8.75
GTP	11.59	11.23	10.98	10.69	10.52	10.25	9.80	9.59	9.39	9.21
ITP	13.21	12.84	12.56	12.23	12.04	11.74	11.23	11.00	10.74	10.52
AMP	14.67	14.67	14.60	14.55	14.48	14.36	13.99	14.05	14.03	13.94
ADP	–	–	–	–	–	–	19.83	19.36	–	–
ATP	–	–	–	–	–	–	22.97	22.67	–	–

[a] Gradient elution programs: G1: 0–17 min: 1.18% B/min, 17–49 min: 2.50% B/min. G2: 0–17 min: 1.41% B/min, 17–47 min: 2.53% B/min. G3: 0–17 min: 1.59% B/min, 17–46 min: 2.52% B/min. G4: 0–17 min: 1.82% B/min, 17–44 min: 2.56% B/min. G5: 0–17 min: 2.0% B/min, 17–43 min: 2.54% B/min. G6: 0–17 min: 2.24% B/min, 17–41 min: 2.58% B/min. G7: 0–40 min: 2.50% B/min. G8: 0–33 min: 3.03% B/min. G9: 0–28 min: 3.57% B/min. G10: 0–25 min: 4.0% B/min. After reaching 100% B elution was continued with 100% B. −, not determined.

values for the adjacent nucleotide pairs UDP–CTP, GMP–UDP, GMP–CTP and IMP–UDP. Resolution of IMP–GMP remained nearly constant but resolution of the pair GTP–IDP decreased. It is thus necessary to choose an adequate compromise in magnesium ion gradient steepness to achieve the separation of all fifteen nucleotides in a single run.

3.5. Parameters selected for the determination of nucleotide profiles in cell lysates

The results discussed in Sections 3.1–3.4 indicate that an adequate separation of the 15 nucleotides listed in Table 1 can be achieved applying the following conditions: mobile phase phosphoric acid concentration 100 m*M*, adjusted with TEA to pH 6.40, a gradient elution program starting with 0% B and changing to 100% B in 40 min and a column temperature of 22 °C.

An additional small modification was, however, applied to optimise the resolution in the region of the chromatogram where most of the changes in the intracellular nucleotide pool induced by mycophenolic acid were expected. Since MPA acts by reversibly inhibiting inosine-5′-monophosphate dehydrogenase the largest effects are expected in the guanosine nucleotide pool. Thus, by carrying out several further experiments we tried to improve resolution of the nucleotide pairs GMP–UDP and GTP–IDP without dramatically decreasing the resolution of other adjacent peaks. By slightly decreasing mobile phase pH value to 6.2 and applying a slightly steeper gradient program (starting with 0% B and changing to 100% B in 33 min) it was possible to increase $R_{GMP-UDP}$ from 0.84 to 1.44 and $R_{GTP-IDP}$ from 1.12 to 1.60.

Table 2 summarises the experimental parameters of the analysis method developed. Fig. 3A shows a chromatogram of an aqueous standard solution containing the 15 nucleotides. It was obtained by using a column which had already been used for all the experiments needed to develop the separation method—including the generation of about 130 phase systems. The same column was then also used for analysing about 460 samples of PBMC and HUVEC

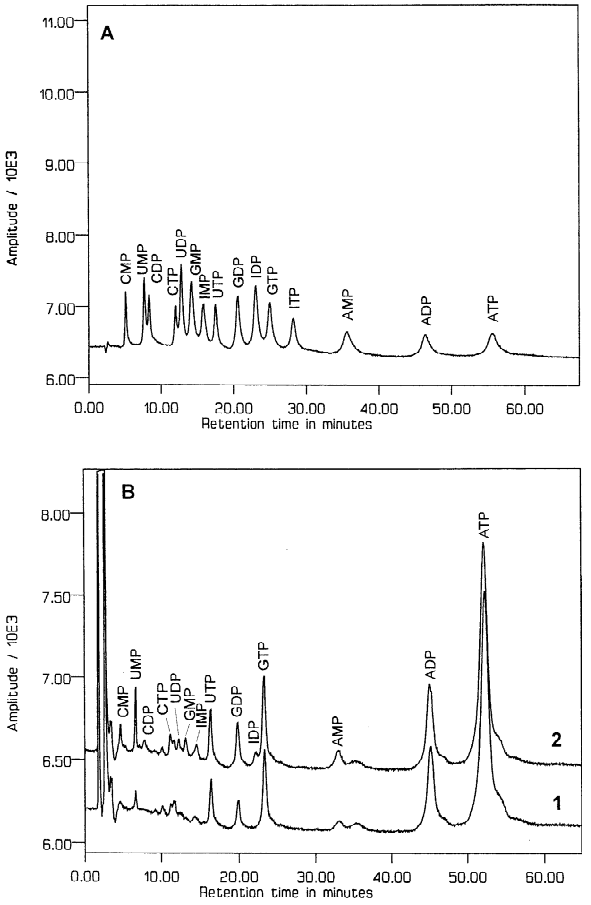

Fig. 3. (A) Chromatogram of an aqueous standard solution with a 12.5 μ*M* concentration of each of the 15 nucleotides. (B) Trace 1: Chromatogram of an unspiked lysate of unstimulated PBMCs containing 6.06×10^5 cells per injection (20 μl). Trace 2: Chromatogram of the cell lysate spiked with 0.032 nmol of each of 14 nucleotides. For chromatographic conditions see Table 2.

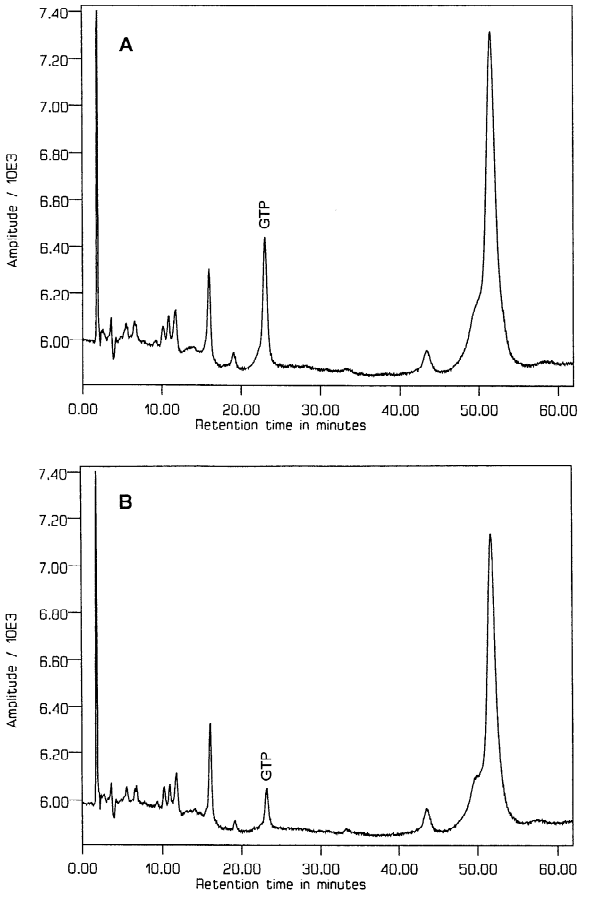

Fig. 4. (A) Chromatogram of the lysate of PHA stimulated PBMCs. (B) Chromatogram of the lysate of PHA stimulated PBMCs co-incubated with 100 μ*M* MPA. For chromatographic conditions see Table 2.

lysates. The following chromatograms were selected from this series to demonstrate the potential of the method developed. Fig. 3B shows the chromatogram obtained from an unspiked and spiked PBMC lysate. Fig. 4 demonstrates the applicability of the method for quantifying changes in the intracellular nucleotide pool by enabling a comparison of the chromatograms of stimulated PBMCs (Fig. 4A) and PBMCs co-incubated with MPA (Fig. 4B). (Incubation with MPA significantly decreased the intracellular concentration of GTP whereas the concentration of other nucleotides was not affected).

Fig. 5 shows the first chromatogram obtained by analysing a standard mixture of the 15 nucleotides with a new column of the same type. A comparison of Fig. 3A and Fig. 5 shows that generating the phase system via the mobile phase on an new column packed with the same packing material results in a separation column with excellent resolution without the necessity of additional adjustment of the mobile phase composition or operational parameters. The differences observed possibly include slight differences in the retention characteristics of the new packing material but are mainly due to the changes in retention and column packing characteristics caused by the intensive use described above.

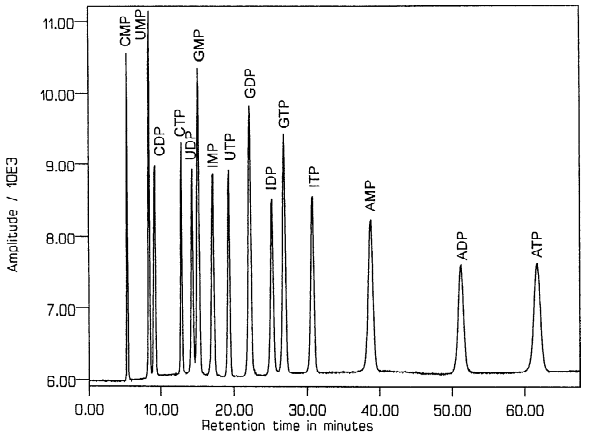

Fig. 5. First chromatogram of an aqueous standard solution with a 25 μ*M* concentration of each of the 15 nucleotides obtained after generating the phase system on a new column. For chromatographic conditions see Table 2.

3.6. Calibration and detection limits

The method was calibrated by measuring six aqueous solutions containing all 15 nucleotides in the concentration range from 5 to 200 μ*M*. Correlating peak areas to concentrations yielded linear calibration curves with regression coefficients in the range of $0.99801 < r < 0.99996$. The detection limits are calculated as the upper 95% confidence limit of the intercept of the linear calibration curve. Table 7 lists the detection limits together with typical results obtained by the analysis of cell lysates.

An eventual loss of nucleotides by hydrolysis during the cell lysis step was investigated by incubating standard solutions of nucleotides with 0.42 *M* $HClO_4$ for 30, 60 and 120 min and comparing the results with the results of direct injection of the same standard solutions. In accordance with similar results from the literature [16] we found >95% recovery for all nucleotides tested.

3.7. Reproducibility and stability

Reproducibility of HPLC analysis was determined by repeated injection of standard solutions containing all 15 nucleotides in various concentrations in the range from 5 to 100 μ*M*. The results obtained in a time period of 2 months show a relative standard deviation (RSD) in the range from 3 to 5%.

RSD values of the whole analysis method including cell lysis determined by analysing the same cell sample on 6 different days were in the range from 6 to 11%.

In order to maintain the quality of the columns it is important to operate according to procedures which guarantee the regeneration of the phase system and remove matrix components which can be strongly adsorbed mainly in the first segment of the column. After each gradient elution the phase system

Table 7
Detection limits and typical cellular nucleotide concentrations in PBMC and HUVEC lysates

Nucleotide	Detection limit (pmol/injection)	PBMCs[a] (pmol/10^6cells)	HUVECs[a] (pmol/10^6 cells)
CMP	34	N.d.	N.d.
UMP	49	N.d.	N.d.
CDP	126	N.d.	N.d.
CTP	102	216±60	2166±230
UDP	73	288±59	2151±211
GMP	63	N.d.	N.d.
IMP	85	N.d.	N.d.
UTP	124	452±105	3255±221
GDP	95	274±76	N.d.
IDP	44	N.d.	N.d.
GTP	76	459±95	2040±150
ITP	119	N.d.	N.d.
AMP	63	256±83	N.d.
ADP	17	1004±342	851±108
ATP	20	2660±558	6319±648

[a] Mean values±95% confidence interval; HUVECs, $n=10$; PBMCs, $n=9$; N.d., below detection limit.

was regenerated by flushing the column with mobile phase A for 30 min with a flow-rate of 1.0 ml/min.

In order to remove strongly adsorbed matrix components the column was washed with two methanol–water gradients after each analysis run of 15 biological samples.

If the analysis had to be interrupted for more than 8 h the chromatographic system was washed with bidistilled water at a low flow-rate of 0.1 ml/min in order to remove the deposition of inorganic components.

4. Conclusion

This paper describes the development of a method which can be applied to characterise the composition of the nucleotide pool of cultured human mononuclear blood and umbilical vein endothelial cells and the determination of changes in the nucleotide pool induced by the immunosuppressant mycophenolic acid. The method allows the separation of 15 nucleotides (CMP, UMP, CDP, CTP, UDP, GMP, IMP, UTP, GDP, IDP, GTP, ITP, AMP, ADP, ATP) in the respective cell lysates using an ion-pair system generated on an RP-18 silica column via a mobile phase consisting of 100 m*M* phosphoric acid adjusted to pH 6.2 with triethylamine. Nucleotides are eluted by applying a linear Mg^{2+} gradient. The method is robust, highly reproducible and—based on the information on the influence of experimental variables given in the paper—easily adaptable to the lysates of other cell types or different analytical tasks. Using UV detection at 254 nm the method allows the determination of the nucleotides with detection limits in the range from 17 (ADP) to 126 (CDP) pmol in 20-μl aliquots.

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