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SEPARATION OF PURINE BASES, NUCLEOSIDES AND NUCLEOTIDES BY A COLUMN-SWITCHING TECHNIQUE COMBINING REVERSED-PHASE AND ANION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

An on-line two-stage column chromatographic technique is described which combines reversed-phase and anion-exchange chromatography for the separation of purine nucleic acid components. The elution program applied, consisting of two gradient programmes, provides a separation of bases and nucleosides on the octadecyl silica column and a separation of the nucleotides on the anion-exchange column to which they have been switched at the beginning of the elution. This method is easy to modify for special problems and can be used when establishing a complete profile of purines.

INTRODUCTION

The observation and study of cell metabolism, *e.g.*, phosphorylation and deamination, when adding a certain substrate to the cell medium, require techniques which allow the separation and determination of bases, nucleosides and nucleotides. The column-switching method described here was developed to determine the purine compounds belonging to these groups simultaneously, *i.e.*, with only one injection in one analysis. The method is not restricted to a particular application, although it was tested by applying it to the monitoring of adenosine transport and metabolism. This system has been studied for a variety of cell types by several authors[1–11]. It has been found that at low exogenous adenosine concentrations (less than 1 μM) transport is slow enough to allow direct phosphorylation of most of the entering adenosine. The remainder is deaminated and rapidly converted into nucleotides via inosine, hypoxanthine and inosine 5′-phosphate (IMP). On the other hand, at adenosine concentrations of 100 μM or more, influx exceeds the maximum velocity of adenosine kinase by a factor of about 100, leading to deamination of most of the substrate. However, since the maximum velocity of adenosine deaminase exceeds those of nucleoside

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phosphorylase and hypoxanthine/guanosine phosphoribosyltransferase by factors of about 5 and 100, respectively, hypoxanthine and inosine migrate rapidly out of the cells and accumulate in the medium.

For the separation, an on-line combination of a reversed-phase (octadecylsilica) and a subsequent anion-exchange column is used. Alkylsilica reversed-phase materials have been successfully employed for the separation of bases and nucleosides and many elution programs for special separation problems are available[12–17]. Nucleotides are weakly retained on these columns[18–21], so that generally only a limited number of them can be resolved. This low retention can be utilized to separate the majority of nucleotides as a group from the more strongly retained bases and nucleosides, and to switch the eluent fraction containing these nucleotides to a subsequent anion-exchange column, where they are separated according to the usual strategies[22]. The combination of these two columns with different retention mechanisms gives rise to high separation power and a high peak capacity[23,24]. While off-line combinations of columns for the separation of nucleic acid components have been described several times[25–32], few on-line applications have been reported till now[33].

EXPERIMENTAL

Instrumentation

The separations were performed on a Varian 5060 chromatograph combined with a Vista data system CDS 401 (Varian, Walnut Creek, CA, U.S.A.). The chromatographic system was equipped with a AH60 injector (Valco, Houston, TX, U.S.A.) using a 50-μl loop, a Varian UV-5 detector, monitoring at a wavelength of 280 or 254 nm and two pneumatic switching valves (Valco AH60).

The reversed-phase column was a prepacked cartridge (250 × 4.0 mm I.D.) containing chemically bonded octadecylsilica (LiChroCart® RP-18; E. Merck, Darmstadt, F.R.G.). The anion-exchange column was a prepacked stainless-steel column (300 × 4.0 mm I.D.) filled with weak anion-exchange material MicroPak AX-5 (Varian).

Reagents and solutions

Solute standards were obtained from Sigma (Taufkirchen, F.R.G.). Chromatographic grade methanol and acetonitrile were obtained from E. Merck (LiChrosolv®). Analytical reagent grade potassium dihydrogenphosphate and orthophosphoric acid were obtained from E. Merck and high-performance liquid chromatographic (HPLC)-grade water (ChromAR®) from Promochem.

The eluent mixture was prepared by the chromatographic system using three pre-prepared solutions: (A) pure methanol; (B) 0.75 *M* potassium dihydrogenphosphate solution, pH 4.75 (adjusted by orthophosphoric acid) and (C) water containing 2% of acetonitrile.

Chromatographic procedure

The chromatographic elution programme for a complete determination of the purine pool consisted of two gradients (Table I). It includes the reconditioning of the columns after each experiment for 10 and 20 min, respectively. When analyzing cell materials, the reversed-phase column was rigorously washed by a methanol–

TABLE I

GRADIENT ELUTION PROGRAMMES USED FOR THE SEPARATION OF BASES AND NUCLEOSIDES ON THE ODS COLUMN AND OF NUCLEOTIDES ON THE ANION-EXCHANGE COLUMN

Volume between mixing of solvents and the top of the column is 2 ml. If not explicitly specified, the flow-rate is 1.0 ml/min. Solvents: A = pure methanol; B = 0.75 *M* aqueous potassium dihydrogen phosphate solution, pH 4.75; C = water containing 2% acetonitrile.

Time (min)			*Solvents (%)*							*Comments*
			A	*B*	*C*		*A*	*B*	*C*	
Programme "RP" applied to the ODS column										
0	to	6.5	1	–	99	to	1	1	98	Flow-rate = 0.7 ml/min
6.5	to	10.6	1	1	98	to	15	3	82	Flow-rate = 1.0 ml/min
10.6	to	14.1	15	3	82	to	20	10	70	
14.1	to	19.7	20	10	70	to	1	–	99	
19.7	to	30.0	1	–	99	to	1	–	99	Reconditioning
Programme "AX" applied to the anion-exchange column										
0.0	to	12.0	2	3	95	to	2	7	91	Flow-rate = 1.0 ml/min
12.0	to	16.0	2	7	91	to	–	33	67	
16.0	to	26.0	–	33	67	to	–	38	62	
26.0	to	28.0	–	38	62	to	–	74	26	
28.0	to	43.0	–	74	26	to	–	75	25	
43.0	to	53.0	–	75	25	to	2	3	95	
53.0	to	83.0	2	3	95	to	2	3	95	Reconditioning

acetonitrile–water gradient program after three experiments. The column-switching programme is specified in Table II: column I, ODS: column II, anion-exchange material. The procedure starts with the elution of the first column (programme RP in Table I). A small fraction of about 3.5 ml eluted at the beginning of the reversed-phase chromatogram is transferred to the second column. After switching of this fraction, the elution on the ODS column is continued by increasing the methanol concentration in the mobile phase as specified in Table I, and the eluted compounds

TABLE II

COLUMN-SWITCHING PROGRAMME

The positions "LOAD" and "INJECT" refer to Fig. 4.

Time (min)			*Valve A*	*Valve B*	*Comments*
0.0	to	1.6	LOAD	INJECT	ODS column only*
1.6	to	5.2	LOAD	LOAD	ODS and anion-exchange columns*
5.2	to	30.0	LOAD	INJECT	ODS column only*
30.0	to	32.5	LOAD	INJECT	Purging of capillaries
32.5	to	115.5	INJECT	INJECT	Anion-exchange column only**
115.5	to	117.5	INJECT	LOAD	Purging of capillaries

* Elution programme "RP" (see Table I) running.
** Elution programme "AX" (see Table I) running.

are passed directly into the detector. When the separation on the reversed-phase column is complete, the second column is eluted using a phosphate-buffer gradient of increasing ionic strength. The temperature was 30°C during all measurements.

Sample preparation

The standard samples contain purines at concentrations of 1–3 μmol/l.

Human red blood cells obtained from healthy donors were washed two times with sodium chloride and resuspended in 150 m*M* sodium chloride, 20 m*M* 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid (HEPES) (E. Merck) pH 7.4 to give a final cell count of 10^8 per ml. To start the reaction, 50 μl of 10 m*M* adenosine were added to 5 ml of cells to give a final concentration of 100 μmol/l. At a chosen time, 450 μl of the incubated mixture were mixed with 50 μl of 5 *M* perchloric acid and extracted for 5 min at 4°C. Then the samples were centrifuged at 10 000 *g* in an Eppendorf laboratory centrifuge for 1 min. The supernatants were neutralized with 3 *M* potassium hydroxide–0.5 *M* dipotassium hydrogenphosphate and kept at 4°C for 20 min, followed by a further centrifugation at 10 000 *g* to remove the potassium perchlorate. The neutralized supernatants were stored at −20°C until required.

RESULTS AND DISCUSSION

Fig. 1 shows the retention behaviour of purines on the ODS column with the applied gradient programme. A good separation is obtained for purine bases and

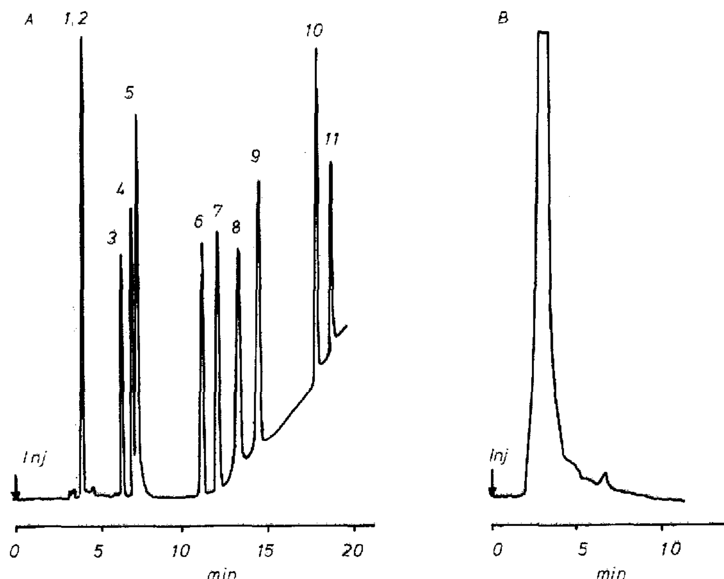

Fig. 1. Retention behaviour of purines on the ODS column. (a) Chromatogram of nine purine bases and nucleosides as well as AMP and adenosine 3′,5′-monophosphate (cAMP). Particle diameter: 5 μm. For the applied gradient programme see Table I. Temperature: 30°C. Peaks: 1 = AMP; 2 = uric acid; 3 = cAMP; 4 = hypoxanthine; 5 = xanthine; 6 = inosine; 7 = guanosine; 8 = 2′-deoxyinosine; 9 = 2′-deoxyguanosine; 10 = adenosine; 11 = 2′-deoxyadenosine. (b) Chromatogram of the main purine mono-, di- and triphosphates. Depending on the packing materials used, a significantly higher retention of AMP and cyclic AMP was sometimes observed.

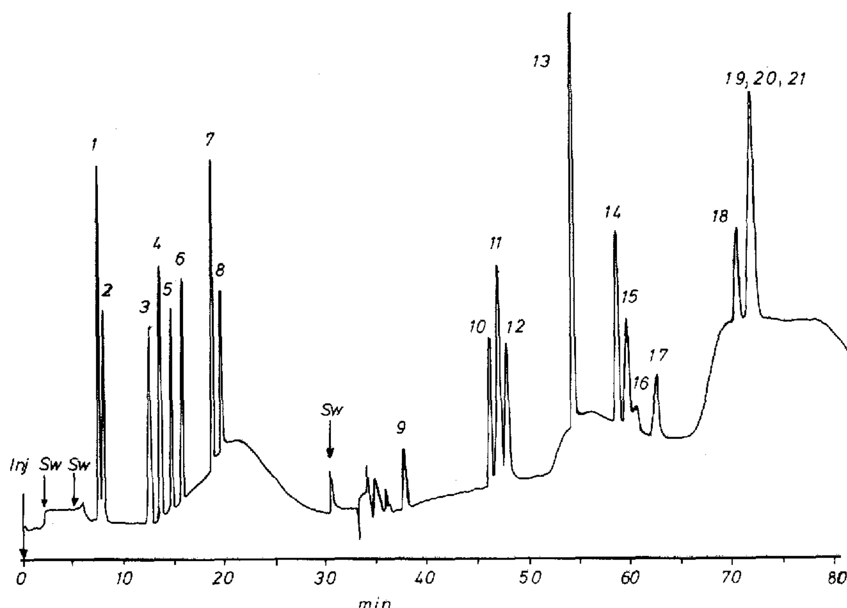

Fig. 2. Two-column HPLC with ODS column followed by an anion-exchange column. Chromatogram of a synthetic mixture containing purine bases, nucleosides and nucleotides. Peaks: 1 = hypoxanthine; 2 = xanthine; 3 = inosine; 4 = guanosine; 5 = 2'-deoxyinosine; 6 = 2'-deoxyguanosine; 7 = adenosine; 8 = 2'-deoxyadenosine; 9 = uric acid; 10 = IMP; 11 = AMP; 12 = guanosine 5'-phosphate; 13 = xanthosine 5'-phosphate; 14 = inosine 5'-diphosphate; 15 = adenosine 5'-diphosphate; 16 = guanosine 5'-diphosphate; 17 = xanthosine 5'-diphosphate; 18 = inosine 5'-triphosphate; 19 = ATP; 20 = GTP; 21 = XTP. A small eluent fraction of about 2.5 ml was switched from the first to the second column. The most important switching events are indicated by "SW" arrows. The elution program and all switching events are described in the Experimental section and are listed in the Tables I and II, respectively. Temperature: 30°C.

nucleosides. The nucleotides are eluted very early in the chromatogram, due to the high solubility of phosphates in water at pH >3. A complete profile of all purine nucleotides is therefore difficult to obtain on this material.

Fig. 2 shows the chromatograms obtained by two-stage HPLC, where the ODS column is followed by an anion-exchange column. The first 2.5 ml eluted from the ODS column after t_{R0} (zone between the first and the second "Sw" arrows in Fig. 2) are transferred to the anion-exchange column. This fraction contains all tri- and diphosphates, most of the monophosphates, some of the cyclic nucleotides, orotic acid and uric acid. The nucleotides not contained in this fraction are some cyclic phosphates and, depending on the reversed-phase material used, sometimes adenosine 5'-phosphate (AMP) and ribosylthymine 5'-phosphate (TMP). The bases, nucleosides and deoxynucleosides (and the remaining cyclic phosphates and eventually AMP and TMP) are separated on the ODS column (left part of the chromatogram in Fig. 2), and thereafter (right part of the chromatogram after the third "Sw" arrow) the nucleotides and the acids on the anion-exchange column. A separation of adenosine 5'-triphosphate (ATP), guanosine 5'-triphosphate (GTP) and xanthosine 5'-triphosphate (XTP) is not obtained on this particular column used. The drifting of the baseline is due to the strong gradients. If the sample contains the pyrimidine bases

cytosine and uracil as well as cytidine, these compounds are transferred with the nucleotides to the anion-exchange column by the procedure described.

Fig. 3 illustrates an analysis of biological materials: human erythrocytes which had an enhanced adenosine take up and therefore showed elevated levels of its metabolites. This chromatogram is analogous to that of the standard mixture in Fig. 2.

The column-switching technique described allows one to establish a profile of the most important bases, nucleosides and nucleotides in one injection and in one experiment. Important features of this approach are:

(i) In this arrangement of columns there is no deconditioning of the surface layers of the second column by the carry-over.

(ii) The gradient programme applied on the second column enables a peak-sharpening effect at the top of the second column which is needed for a good efficiency of the subsequent separation.

(iii) The overall separation problem is subdivided into one separation problem for the bases and nucleosides and another for the nucleotides. For these sub-problems many different and well established elution programmes have already been developed for single column chromatography[12–17]. These programmes are readily available also for this two-column approach, since the mobile phases for the individual columns can be optimized almost independently of each other for special problems. If one is interested only in a limited number of compounds, the elution programme can also be simplified or can be optimized for specific applications. The rôle of the first column can even be reduced to that of a sample clean-up and protection of the anion-exchange column.

A two-column technique in the reversed order, *i.e.*, anion-exchange column

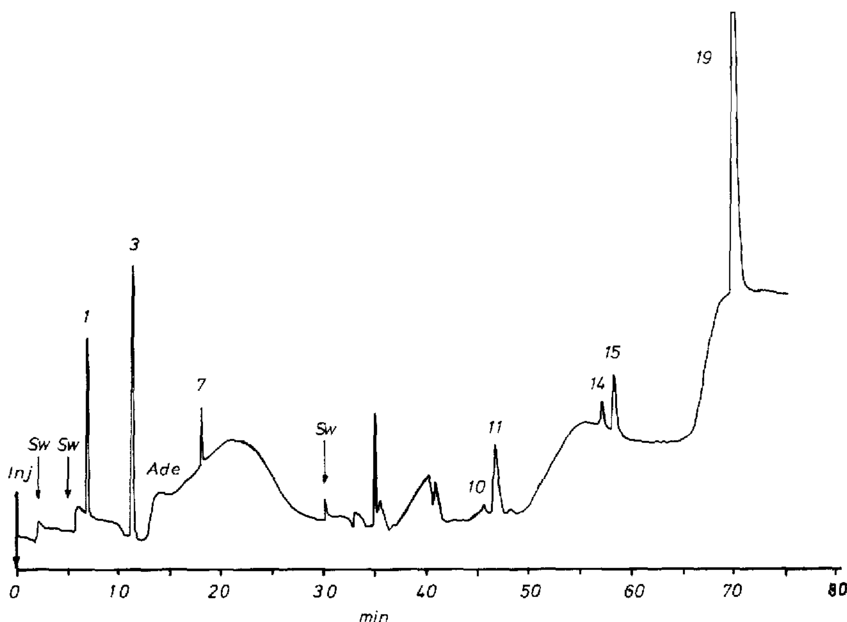

Fig. 3. Two-column HPLC of ultra-centrifuged lyzed human erythrocytes, grown in a medium spiked with adenosine (final concentration 100 μM) and incubated there for 5 min. Subsequent preparation for analysis as described in the Experimental section. Chromatographic conditions and symbols as in Fig. 2.

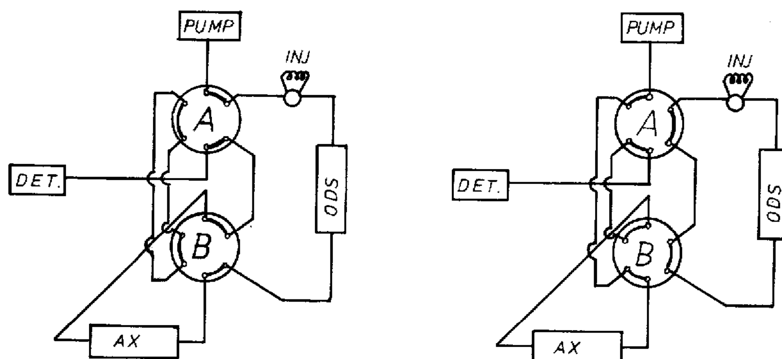

Fig. 4. The column-switching arrangement. AX = Anion-exchange column.

followed by a reversed-phase column, can also be used with independent elution programmes for each column if the bases and nucleosides are only weakly retained on the anion-exchange column. In this case the mono-, di- and triphosphates are separated in this order on the first column by increasing the ionic strength, whereas the early eluted and switched bases and nucleosides can be analyzed on the second column by increasing the modifier concentration. This method has some advantages when pyrimidine bases and nucleosides have to be determined together with the nucleotides, or for removing ionic components prior to reversed-phase chromatography of bases and nucleosides. This order of columns, however, is not recommended if the anion-exchange material requires the addition of an organic solvent component for the elution of bases and nucleosides, as is necessary for instance with certain anion-exchange resins[34]. In this case the range of application may be significantly reduced because of deconditioning of the second column by the switched eluent fraction.

CONCLUSIONS

A two-stage HPLC column-switching method is presented which allows the establishment of a profile of purine bases, nucleosides and nucleotides with one injection and in one run.

In the method described the first of the two columns is filled with ODS and serves mainly for the separation of bases and nucleosides. The nucleotides, switched in a small fraction to the second column, are separated by anion exchange. The gradient programme applied to the second column produces a peak-sharpening effect. The subdivision of the overall separation problem into two sub-problems, and the possibility to optimize the elution programmes for the two columns almost independently of each other, allow one to take advantage of the large number of elution programs already developed for single column chromatography. This combination of two columns with different retention mechanisms offers a high separation power and a high peak capacity.

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