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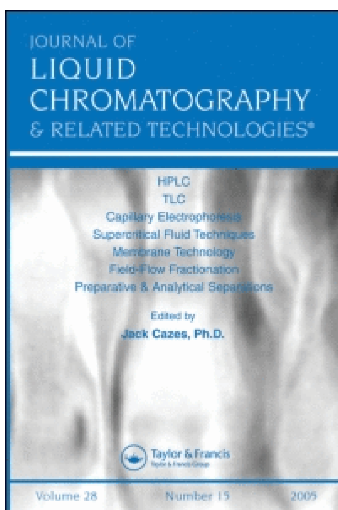

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HIGH-RESOLUTION ANION-EXCHANGE CHROMATOGRAPHY OF ULTRAVIOLET-ABSORBING CONSTITUENTS OF HUMAN ERYTHROCYTES

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ABSTRACT

The analysis of nucleic acid components of human erythrocytes was achieved by high-resolution anion exchange chromatography using a column packed with a macroreticular anion-exchange resin and linear gradient elution with ammonium acetate solution, pH 4.5. On the chromatogram of trichloroacetic acid extracts from human erythrocytes, at least 50 components were detected as ultraviolet-absorbing constituents. On assignment of the chromatographic peaks they were found to be nucleic acid components. The analysis was achieved in 120 min and the elution time of guanosine 5'-triphosphate was 64.3 min.

INTRODUCTION

We previously reported that the separation of ultraviolet (UV)-absorbing constituents of body fluids, such as urine (1,2), blood plasma or serum (3) and hemodialysate (3), can be achieved by chromatography using a macro-

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reticular anion-exchange resin. Blood cells are also an important part of the blood and contain literally hundreds of organic compounds. Particularly, a great number of nucleotides are known in the blood cells (4,5). The tremendous advance in studies of free nucleotides is correlated with the introduction and development of ion exchange chromatography (6). Microreticular (7-10) or pellicular (11-15) ion-exchange resins were employed for the separation of nucleic acid components, but they were not satisfactory in term of resolution and time for separation. Therefore, we tried to use a macroreticular anion-exchange resin column for the separation of nucleic acid components in human erythrocytes.

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EXPERIMENTAL

Reagents. Analytical grade ammonium acetate, acetic acid and trichloroacetic acid (TCA) were purchased from Wako Pure Chemical Industries (Tokyo, Japan). The reference compounds, cytosine, cytidine, uridine, uracil, thymidine, thymine, adenosine, adenine, guanosine, guanine, inosine, hypoxanthine, xanthine, riboflavin, 5'-CMP, 5'-CDP, 5'-UMP, 5'-UDP, 5'-AMP, 2'-AMP, 5'-ADP, 5'-ATP, 5'-IMP, 5'-IDP, 5'-GMP, 5'-GDP, 5'-GTP, FMN, FAD and 3',5'-cyclic AMP were purchased from Wako. β-NAD, β-NADH, β-NADP, UDPG, UDPGA, 5'-TMP, 5'-TDP, 5'-TTP, 5'-XMP and 3',5'-cyclic GMP were purchased from Sigma Chemicals (St.Louis,

Mo., U.S.A.), and 5'-CTP, 5'-UTP and 5'-ITP were purchased from P.L. Chemicals Inc., (Milwaukee, Wis., U.S.A.).

Apparatus. The instrument for the analysis was a Hitachi high-speed liquid chromatograph (model 634, Hitachi Co., Tokyo, Japan). It consisted of a gradient device, a high pressure pump, a sample injection valve, an anion exchange resin column, a double-beam spectrophotometer, a 10-mV data recording device and a circulating water bath. Stopped-flow scanning spectrophotometry was performed with a scan-speed of 60 nm/min and a slit-width of 4.0 nm for scanning from 340 to 220 nm.

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The strongly basic macroreticular anion-exchange resin, Diaion CDR-10 (control #520000), is a polystyrene-divinylbenzene copolymer having quaternary ammonium groups and a particle size distribution of 5 to 7-um. This resin was obtained from Mitsubishi Chemical Industries (Tokyo, Japan). Macroreticular resins have a porous structure that allows the rapid diffusion of ions or molecules into the resin beads (16). Diaion CDR-10 have a higher cross linkage (35 %), a larger surface area (100 m^2/g), a higher pore capacity (0.27 me/mL) and a larger pore diameter (400 A) than those of microreticular resins (17,18).

Procedures.

Sample preparation. Fresh heparinized blood from a normal healthy adult was centrifuged for 5-min at 3,500

r.p.m. at 5°C. The plasma and white blood cells were pipetted out, and the settled layer was used as the red blood cell (erythrocyte) sample. The preparation of TCA extracts of erythrocytes was performed by the method of Miech (19). One milliliter of whole blood or 0.5-mL of erythrocytes was pipetted up, and added dropwise to 2-mL of cold 12% TCA aqueous solution and then stirred rapidly with a Vortex mixer. After centrifugation for 5-min at 3,500 r.p.m., the upper layer was separated through a filter (0.22-um pore size, Millipore type GS), then 100 or 50-uL was injected onto the column.

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Preparation of anion-exchange resin column. A stainless steel column (50-cm in length and 4-mm I.D.) was packed with the Diaion CDR-10 as described by Scott and Lee (20). The column was packed using 6.0 M ammonium acetate buffer (pH 4.5) and run for 1-hr at 15 MPa. The column was connected to a circulating water bath maintaining the column temperature within ±1°C of the selected setting.

Anion-exchange chromatography. A blood sample was introduced onto the column. The constituents of the sample were eluted with a linear ammonium acetate gradient at an average flow-rate of 0.70 mL/min. The linear acetate gradient was formed with a two-chamber gradient generator placing 30-mL of distilled water in the first gradient

chamber, and 30-mL of 6.0 M ammonium acetate buffer (pH 4.5) in the second chamber. The column temperature was raised from 22 to 70°C over the first 30 min, and then maintained at 70°C to the end of the run. Due to the increase in the column temperature, the inlet pressure changed from 10.5 to 7.0 MPa over the first 25 min, and then on introduction of the acetate gradient, the inlet pressure changed from 7.0 to 13.0 MPa to the end of the run. The column effluent was monitored with a double beam spectrophotometer operated at 254 nm.

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RESULTS

Analyses of whole blood and blood plasma by the direct injection method.

50-uL of whole blood or 100-uL of blood plasma was directly chromatographed on the macroreticular anion exchange resin column. UV-absorbance chromatograms are shown in Figure 1 (whole blood) and Figure 2 (plasma). The UV-absorbing constituents of plasma have three protein peaks (eluted at 3.0, 4.1 and 22.2 min) and one uric acid peak (eluted at 32.0 min)(3). While the chromatogram of whole blood showed many peaks, the majority of them came from blood cells since the plasma have only four main peaks.

We have usually analyzed body fluids by the direct injection method, especially when rapid treatment of

sample was required. However, the direct injection of whole blood samples caused the clogging of the column and increased pressure. After ordinaly analyses of five to ten samples, regeneration of the column was required. For the regeneration, 2.0 M sodium hydroxide aqueous solution was run through the column for 1-hr at room temperature and at a flow-rate of 0.5 mL/min.

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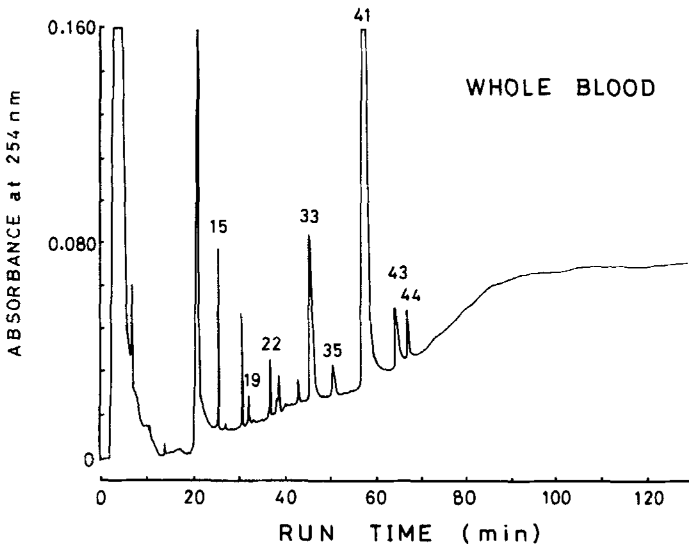

FIGURE 1. Chromatogram of human whole blood with the direct injection method.
Run conditions: column, 50 x 0.4 cm I.D., packed with Diaion CDR-10; eluent, ammonium acetate buffer, pH 4.5, varying concentration from 0 to 6.0 M by linear gradient; temperature, increasing from 22 to 70°C over the first 30-min, then 70°C to the end of the run; average flow rate, 0.70 mL/min; average pressure, 10.5-MPa; sample volume, 50-uL.

<u>Analysis of the TCA extracts from whole blood and from erythrocytes.</u>

A chromatogram of the TCA extracts from whole blood is shown in Figure 3. 50-uL of the TCA extracts is equivalent to about 17-uL of original whole blood. The chromatogram shows about 50 peaks.

A chromatogram of the TCA extracts from erythrocytes is shown in Figure 4. 100-uL of the TCA ectracts from erythrocytes is equivalent to about 20-uL of original erythrocytes and to about 40-uL of original whole blood. This chromatogram also shows about 50 peaks, but there is

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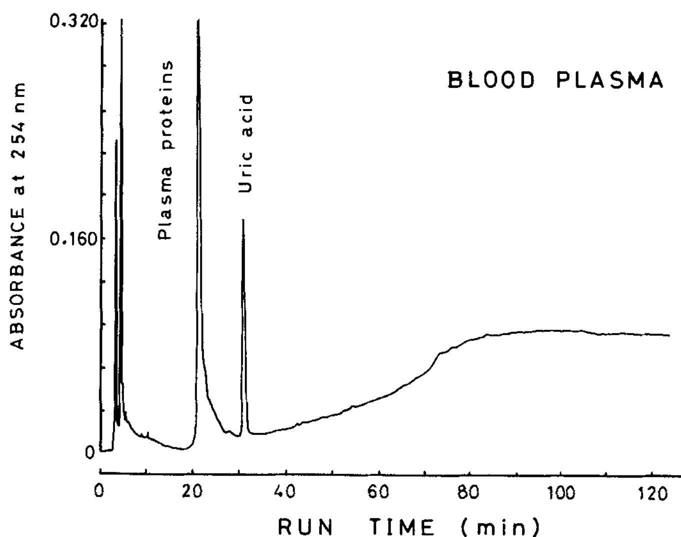

FIGURE 2. Chromatogram of human blood plasma with the direct injection method.
Run conditions were the same as in FIGURE 1.
Sample volume: 100-uL.

no peaks of uric acid. The difference, between Figure 3 and Figure 4, correspond to the chromatogram of TCA extracts from plasma. Another noticeable difference between the TCA extracts from whole blood and from erythrocytes is in the quantities of UV-absorbing constituents when calibrated to the whole blood. The content of triphosphate nucleotides (No.41 ATP and No.43 GTP) are higher and the content of nucleosides (No.8), monophosphate (No.19 AMP and No.22 GMP) and diphosphate (No.33 ADP and No.35 GDP) nucleotides are lower in whole blood than in erythrocytes. These difference could

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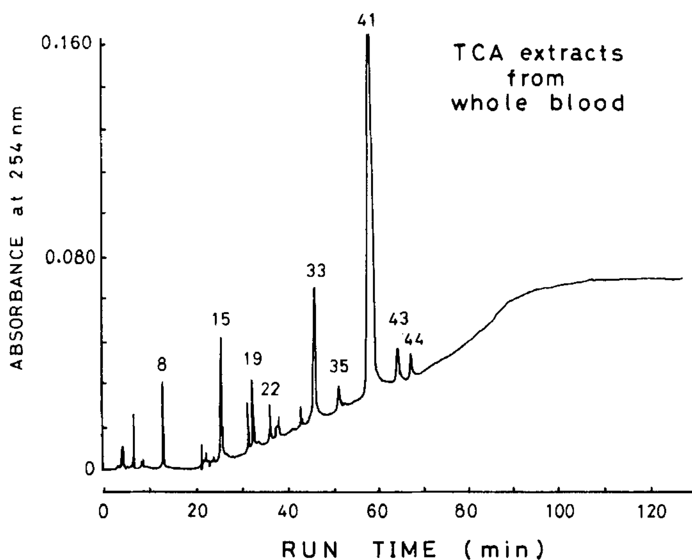

FIGURE 3. Chromatogram of TCA extracts from whole blood. Run conditions were the same as in FIGURE 1. Sample volume: 50-uL.

be explained by the rapid degradation of labile triphosphates and the production of di- and monophosphates even in the process of separation of erythrocytes and plasma. Because of this, direct extraction from whole blood is recommended for the determination of the true profile of nucleotides in blood cells.

<u>Assignment of the chromatographic peaks.</u>

The assignment of peaks in a chromatogram was performed in the following way: (1) by comparing the retention time of a peak to those of the standard compounds;

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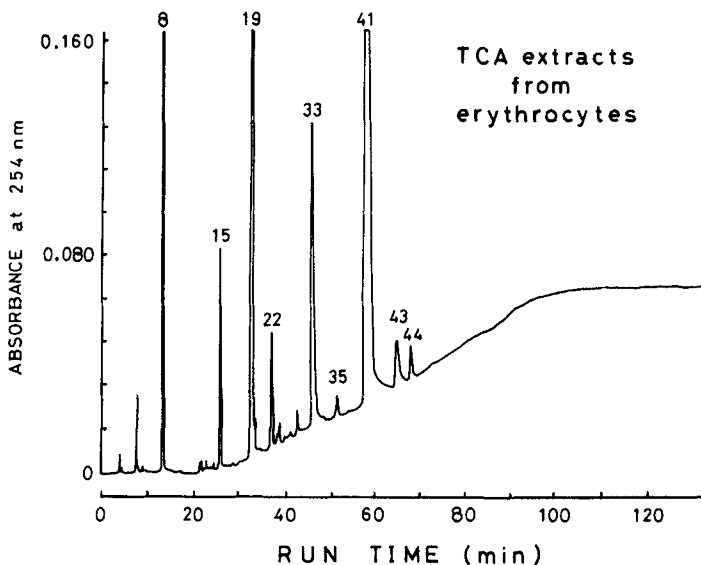

FIGURE 4. Chromatogram of TCA extracts from erythrocytes. Run conditions were the same as in FIGURE 1. Sample volume: 100-uL.

(2) by injecting the standard compounds along with the sample; (3) by measuring the UV-spectrum of a peak at the peak maximum by stopped-flow scanning spectrophotometry. The estimation of retention times of nucleic acid components and related compounds was repeated and mean values are given in Table 1. A representative chromatograms of a mixture of standard compounds is shown in Figure 5. The elution order of the nucleic acid bases was cytosine, uracil, thymine, adenine and guanine. This elution order was observed also in the case of nucleosides, cytidine, uridine, thymidine, adenosine and guanosine, and in the case of mono-, di- and triphosphate nucleotides. Cyclic 3',5'-AMP or cyclic 3',5'-GMP has a larger retention time than 5'-AMP or 5'-GMP, respectively. This elution order corresponds with that suggested in the case of N-bases and nucleosides by Singhal and Cohn (21) and in the case of nucleotides by Brown (12). The retention time of β-NADH, FAD and β-NADP in the chromatography using the macroreticular anion-exchange resin are remarkably different from those in the pellicular anion-exchange resin (12).

For the determination of the UV-spectrum, the flow was stopped at a peak maximum and the spectrum was measured. Then, when the absorbance came down to the base-line, again, the flow was stopped and the spectrum was measured. The latter was used as a control for the preceding peak, and the difference spectrum was made. The UV-spectra of

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TABLE I

Retention times of standard nucleic acid components and related compounds.

N-bases and Nucleosides			Nucleotides								
			mono-phosphates			di-phosphates			tri-phosphates		
	peak number	*retention time(min)*		*peak number*	*retention time(min)*		*peak number*	*retention time(min)*		*peak number*	*retention time(min)*
Cytosine	1	5.5	NAD	15	25.5	UDPG	27	39.5	NADP	37	52.0*
Cytidine	2	8.1	(DPN)			NADH	28	41.0*	(TPN)		53.6*
Uridine	3	9.0	5'-CMP	16	28.4	(DPNH)		41.6*	5'-CTP	38	54.9
Uracil	4	11.0	5'-UMP	17	31.5	5'-CDP	29	42.0	5'-UTP	39	57.0
Thymidine	5	13.1	5'-TMP	18	32.0	FAD	30	45.1	5'-TTP	40	58.0
Thymine	6	14.0	5'-AMP	19	33.0	5'-UDP	31	45.5	5'-ATP	41	58.4
Adenosine	7	15.5	5'-IMP	20	34.1	5'-TDP	32	45.8	5'-ITP	42	60.2
Inosine	8	17.1	2'-AMP	21	34.9	5'-ADP	33	46.2	5'-GTP	43	64.3
Hypo-	9	17.5	5'-GMP	22	38.0	5'-IDP	34	48.0	unknown	44	67.0
xanthine			3',5'-	23	40.0	5'-GDP	35	51.8			
Adenine	10	19.0	cyclic-			UDPGA	36	54.8			
Xanthine	11	21.5	AMP								
Ribo-	12	23.8	FMN	24	42.8						
flavin			3',5'-	25	43.1						
Guanosine	13	34.0	cyclic								
Guanine	14	35.0	GMP								
			5'-XMP	26	44.9						

* The two retention data for NADH and NADP were considered to originate from the mixture of 2'- and 5'- of adenosine of these standards.

** Standard compounds were dissolved in distilled water and separated by HPLC.

peak No. 41 and 43 in Figure 4 are shown respectively in Figure 6. The wavelength of the absorbing maximum of the difference spectrum of other peaks are listed in Table 2 and they were good agreement with those of the standard compounds (22).

DISCUSSION

The nuclectide profiles of the whole blood and erythrocytes of humans and other animals were previously reported by Brown (5). She used pellicular anion-exchange

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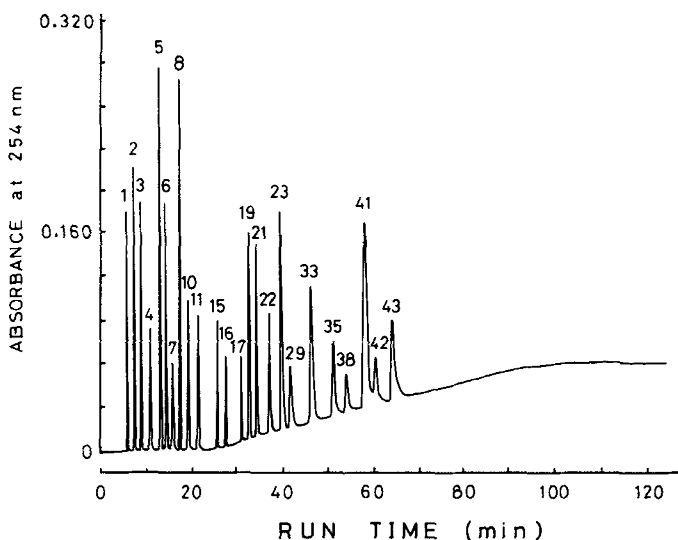

FIGURE 5. Representative chromatogram of nucleic acid components and related compounds. Run conditions were the same as in FIGURE 1. Sample: 100-uL of a mixture of standard compounds containing 2.5 ug/100-uL (except for 5 ug/100-uL of 5'-ATP and 5'-GTP).

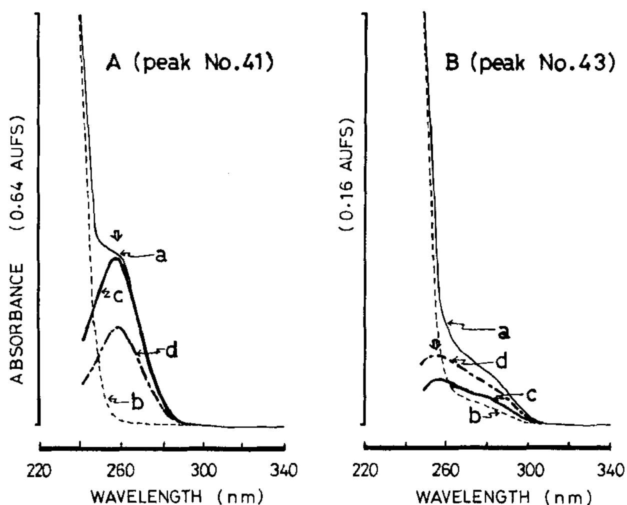

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FIGURE 6. UV-spectra of peaks by stopped-flow scanning spectrophotometry.
A (peak No.41) and B (peak No.43).
Curve a (←——): UV-spectrum of the peak at peak maximum, curve b (←---): UV-spectrum of control, curve c (←·—): the difference spectrum (curve a minus curve b), and curve d (←——): the difference spectrum of a standard compound under the same conditions as for curve a.
⇩ indicates absorbance maximum.

TABLE 2

The Wavelength of The Absorbance Maximum of The Difference Spectrum of Peaks

Peak number	*Wavelength (λ_{max} nm)*	*Assignment of the peak*
8	250	Inosine
15	258	NAD(DPN)
19	257	5'-AMP
22	255	5'-GMP
33	257	5'-ADP
35	255	5'-GDP
41	257	5'-ATP
43	255	5'-GTP

resins for the separation of nucleotides and microreticular resins for the separation of purine and pyrimidine bases and nucleosides. However, chromatography using a pellicular anion-exchange resin is lower in ion-exchange capacity and in resolution (13-15), and chromatography using microreticular resins requires a relatively longer separation time resulting lower sample throughput rates (8-10) compared with the present system. Furthermore, the overall analysis of nucleic acid components, including N-bases, nucleosides and nucelotides, could not be achieved with conventional anion-exchange resin column. Thus, the chromatography using the macroreticular anion-exchange resin described above showed better separation of nucleic acid components and related compounds.

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