Journal of Chromatography, 229 (1982) 205—210
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1161

Note

Rapid separation of nucleotides from granulocytes by isocratic, reversed-phase high-performance liquid chromatography

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(First received August 18th, 1981; revised manuscript received November 5th, 1981)

The nucleotide profiles of different cells are characteristic for each tissue [1, 2]. Blood cells contain substantial quantities of adenine nucleotides. The levels of different nucleotides and the energy charge available have been related to the metabolic and physiological requirements of blood cell function [3—6]. In the past, blood cell nucleotides have been assayed in several different ways [7—11], including techniques employing firefly luciferase [12—14], an enzymatic method [15], a fluorimetric procedure [16], column chromatography [17] and high-performance liquid chromatography (HPLC) [18—21]. However, a simple, rapid and efficient method for the analysis of neutrophil nucleotides is still unavailable. We have recently developed a rapid method for the separation and evaluation of adenine and guanine nucleotides (AN and GN) from human blood platelets [21]. In the present paper, we have applied

0378-4347/82/0000—0000/$02.75

this procedure for complete separation of granulocyte nucleotides with a minimum of elution time and maximum efficiency.

MATERIALS AND METHODS

Blood for these studies was obtained from normal subjects. It was immediately mixed with trisodium citrate—citric acid—dextrose (CCD) buffer, (citrate 0.1 *M*, citric acid 7 m*M*, dextrose 0.14 *M*, pH 6.5), in a ratio of 9 parts of blood to 1 part anticoagulant [22]. To obtain leukocyte rich plasma (LRP) free of erythrocytes 1 part of 5% dextran (molecular weight 282,000) in saline was added to 3 parts of whole blood, mixed and allowed to sediment spontaneously by placing tubes at a 45° angle for 20—40 min. LRP separated from red blood cells was layered over a double Ficoll—Hypaque gradient [23, 24], and centrifuged at 1650 *g* for 15 min. The neutrophils were harvested from the lower interface, checked for purity (greater than 95% neutrophils), washed twice and resuspended with saline to the desired concentration. Cell counts were determined using a Royco particle counter. The suspension containing neutrophils was centrifuged for 5 min at 180 *g*. Supernatant was discarded and the neutrophil pellet resuspended in 1.5 ml of Hanks Balanced Salt Solution (HBSS). Samples were transferred in 2-ml volume centrifuge tubes and spun for 1.5 min in a Beckman microfuge. After removing the clear supernatant the pellet of neutrophils was precipitated with 100 μl of cold 2 *N* perchloric acid. The precipitate thus formed was sonicated in perchloric acid at low temperature (4°C) and the samples again centrifuged in a microfuge for 1.5 min. The clear supernatant containing neutrophil nucleotides was separated and neutralized with 5 *N* potassium hydroxide to a pH of 5.5—8.0. The neutralized samples were subjected to a freeze—thaw cycle to achieve complete precipitation of the salt. To sediment the salt generated during neutralization all the samples were centrifuged one more time in a microfuge for 1.5 min. The clear neutral extracts were separated and subjected to HPLC for the separation of nucleotides.

Separation of nucleotides

A Waters Assoc. (Milford, MA, U.S.A.) Model 204 high-performance liquid chromatograph was used for the separation of nucleotides. The chromatographic system consisted of a Model 440 fixed-wavelength (254 nm) UV detector, a 6000A solvent delivery system and a U6K universal injector. A Radial-Pak C18 (10-μm particles) with radial compression module (RCM-100) was used for the separation of nucleotides. The solvent system consisted of HPLC grade acetonitrile (Burdick and Jackson Labs, Muskegon, MI, U.S.A.), 10% (v/v), deionized distilled water of pH 7.5, 90% (v/v), and a vial of Pic A (tetrabutylammonium phosphate) (Waters Assoc.). First, Pic A was mixed with water and the pH was adjusted to 7.5 and then acetonitrile was added to make the final solvent system. The final concentration of the Pic A was 0.005 *M* per l of stock solvent used in the separation of nucleotides. Solvent flow-rate was varied depending on the degree of separation needed. Sample size was 20 μl per injection. A Hewlett-Packard 3385A automation system was used to obtain electronic integration of the peaks. Data thus

generated were further processed to obtain values in μmol/10^9 cells. Results presented are mean values for neutrophils obtained from six different normal donors.

RESULTS AND DISCUSSION

Extracts of granulocytes and an extract containing standards were analyzed using Radial-Pak™ column. Solvent elution was isocratic and flow-rate was 6 ml/min. Complete separations of all the major nucleotides from the mixtures were achieved at ambient temperatures in less than 8 min (Fig. 1). Retention times for GMP/AMP, GDP, ADP, GTP and ATP were 1.1, 2.3, 3.0, 4.9 and 7.5 min, respectively. To test the efficacy of this method, recoveries of the standards added to the neutrophil suspension were measured. The range of recovery was 94—100% for all nucleotides except for GDP (65—75%).

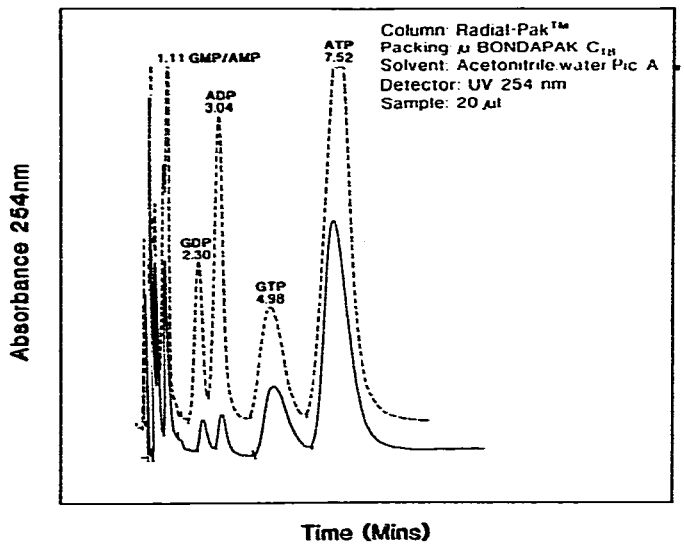

Fig. 1. Separation of adenine and guanine nucleotides by isocratic elution at ambient temperature. Chromatogram in solid lines was obtained by analyzing a granulocyte extract, whereas the one shown with dotted lines was obtained by analyzing a granulocyte extract with added adenine and guanine nucleotide standards.

Identification of individual peaks of adenine and guanine nucleotides was done by comparison of retention times of standards added to the neutrophil extract and assayed under identical conditions. In addition, a standard mixture containing both adenine and guanine nucleotides was added to a neutrophil extract and run under identical conditions as a supplementary confirmation of peaks. Enzymatic peak shift method was used to follow the loss of ATP and GTP and increases in ADP and GDP, respectively. In addition, cells were incubated with adenine and guanine and the increases in the level of phosphorylated compounds were followed. No attempts were made to identify several fast eluting compounds which may be precursors, metabolites or other UV-absorbing components.

Since in this mode of elution both GMP and AMP eluted as a single peak, the standards were run at a slower flow-rate (1 ml/min). A profile of nucleotide standards separated under such conditions is presented in Fig. 2. Under this condition, all the adenine and guanine nucleotides were completely separated. Using similar conditions, neutrophil extracts were analyzed to obtain separation of AMP and GMP (Fig. 3). There are slight differences in the retention times of individual nucleotides. However, if the runs are repeated with stan-

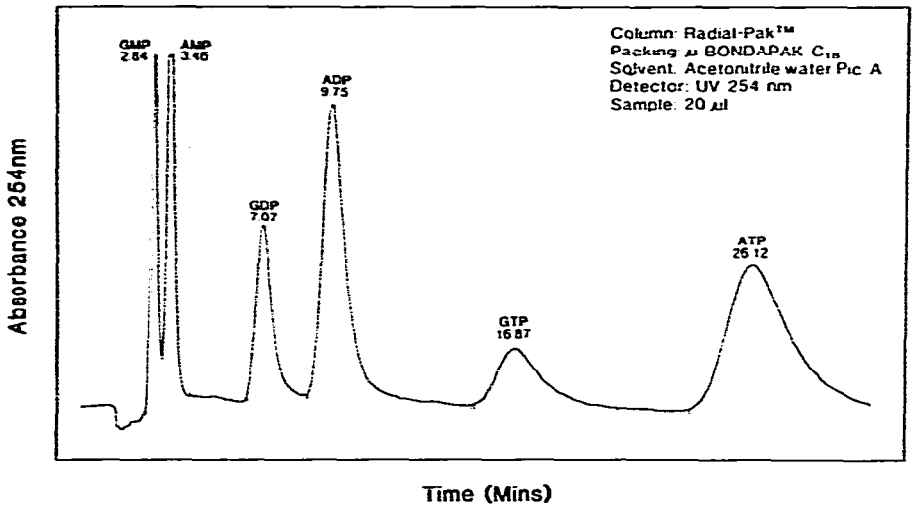

Fig. 2. Separation of various nucleotide standards from a mixture by isocratic elution. Elution time for separating all the nucleotides was less than 26 min. The range of recovery for all the nucleotides was excellent.

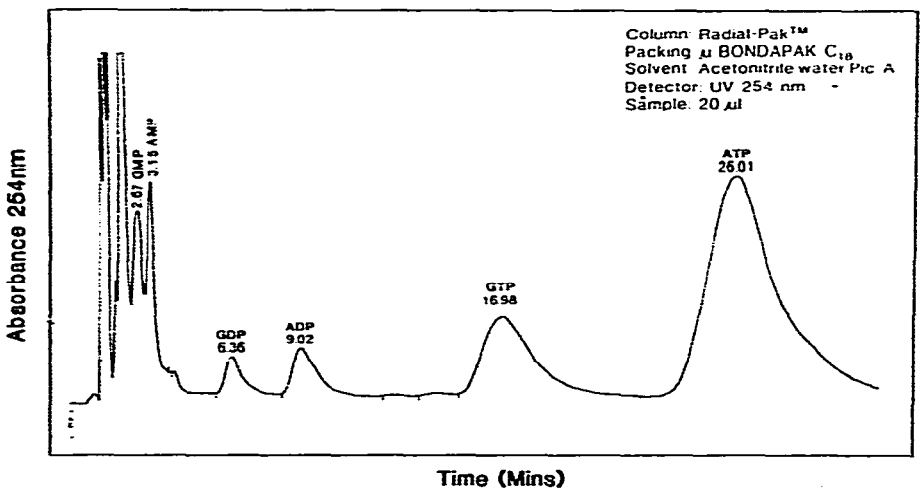

Fig. 3. Separation of granulocyte nucleotides by isocratic elution. Retention times for adenine and guanine nucleotides matched closely with those of standards obtained under identical conditions (Fig. 2). The values obtained by this method for various nucleotides were higher than those reported by others [18] using linear gradient elution to achieve complete separation of various nucleotides.

dards added to a biological extract one could get relatively matching retention times (Fig. 1). Mean values obtained for each nucleotide are presented in Table I. Values obtained for all the nucleotides were higher by this method than those reported by Scholar et al. [18] using a gradient elution technique employing ion-exchange chromatography.

The two major nucleotides, ATP and GTP, together, constituted over 84% of the total nucleotides. Since UTP, UDP and UMP are not separated from the adenine and guanine nucleotides they probably coelute with these products. However, the data from Scholar et al. [18] using an ion-exchange technique which separates all three nucleotides shows that the contribution of UTP is less than 3% towards the total nucleotides. Values obtained for ATP and GTP by reversed-phase chromatography were 40% higher than those reported by Scholar et al. [18]. Therefore, the presence of uridine nucleotides may not account for the higher values observed using our method. The reason for higher values may be better separation techniques employed for obtaining cells, method of handling cells, more complete extraction and the high resolution obtained by improved column technology. Ratios for ATP/ADP and AN/GN were 10.9 and 2.9, respectively.

In summary, this is a preliminary report on the nucleotide profiles of normal granulocyte populations. The effect of storage on the nucleotides and their function will be published elsewhere. Results of these studies show that adenine and guanine nucleotides of granulocytes could be separated rapidly and efficiently by this method.

TABLE I

NUCLEOTIDES OF HUMAN BLOOD GRANULOCYTES*

Relative values obtained for granulocyte nucleotides by isocratic reversed-phase high-performance liquid chromatography. The values obtained using this method are higher than those reported by Scholar et al. [18].

	Nucleotides found (μmol/10^9 cells)**	
	Present paper	Scholar et al. [18]
GMP	0.19 ± 0.02	—
AMP	0.10 ± 0.02	—
GDP	0.03 ± 0.01	0.012
ADP	0.11 ± 0.03	0.09
GTP	0.26 ± 0.02	0.16
ATP	1.2 ± 0.05	0.75

*UTP, UDP, and UMP are not separated from the adenine and guanine nucleotides.
**Mean ± standard error (n = 6).

ACKNOWLEDGEMENTS

Supported by USPHS grants HL-11880, AM-06317, HL-06314, CA-12607, CA-08832, CA-11996, GM-AM-22167, HL-20695, HL-16833, AM-15317, AM-17697 and supported in part by a research grant from the American National Red Cross.

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