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Note

Rapid separation of platelet nucleotides by reversed-phase, isocratic, high-performance liquid chromatography with a radially compressed column

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In the past, platelet adenine nucleotides have been assayed in several different ways [1—5], including a firefly luciferase [6], an enzymatic method [7], a fluorimetric procedure [8], column chromatography and high-performance liquid chromatography (HPLC) [9]. A simple, rapid and efficient method is still unavailable for routine analysis of clinical and research samples. Several years ago we described a method using ion-exchange HPLC for separating and quantitating adenine nucleotides of human blood platelets [10]. Other laboratories subsequently improved the technique for separation by employing linear gradients [11, 12]. However, no information was provided regarding reversed-phase separation of platelet nucleotides and the linear gradient methods described took considerable lengths of time for complete separation of all the nucleotides. In the present paper we describe a method for complete separation of platelet nucleotides which is carried out in an isocratic mode with minimum of elution time and maximum efficiency.

MATERIALS AND METHODS

Blood for these studies was obtained from normal voluntary donors. The procedures used to obtain blood, mix the samples 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 blood to 1 part anticoagulant, and isolate platelet-rich plasma (PRP) by centrifugation at room temperature, have been described in several recent publications [13—15]. Nucleotides were determined using platelets obtained from 1-ml samples of fresh PRP. Cell count was determined by phase optics or a coulter system. Each 1-ml PRP sample was mixed with 0.5 ml of CCD and centrifuged for 1.5 min in a Beckman microfuge to obtain

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platelet pellets. Supernatant plasma was discarded and the platelet pellet precipitated with 100 μl of cold, 2 *N* perchloric acid. After sonication of the precipitate in perchloric acid at low temperature, the samples were again sedimented in a microfuge for 1.5 min. The clear supernatant thus obtained containing platelet 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. Model 204 high-performance liquid chromatograph was used for the separation of nucleotides. The chromatographic system consisted of a Model 440 fixed-wavelength (245 nm) UV detector, a 6000A solvent-delivery system and a UK6 universal injector. A 30-cm stainless-steel column packed with μBondapak C_{18} (10-μm particles), or a Radial-Pak C_{18} (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.) 20% (v/v), deionized distilled water of pH 7.5, 80% (v/v), and a vial of tetrabutylammonium phosphate, (Pic A) (Waters Assoc., Milford MA, U.S.A.). Final concentration of the Pic A was 0.005 *M* per liter of stock solvent used in the separation of nucleotides. Solvent flow-rate was 1 ml/min when the μBondapak C_{18} column was used and 6 ml/min when the Radial-Pak column was used. Sample size was 10 μl per injection. A Hewlett-Packard 3385A automation system was used to obtain electronic integration of the peaks. Data thus generated were further processed through a programmable Cannon calculator (Canola 1614P) to obtain values in μmol for 10^{11} platelets. Results presented are mean values for platelets of six different normal donors.

RESULTS AND DISCUSSION

Human platelets contain substantial quantities of adenine nucleotides and small quantities of guanine nucleotides. A relative absence of nucleotide precursors in platelets has also been demonstrated. The uptake of mono-, di- or triphosphate nucleotides is negligible. Metabolites such as inosine monophosphate and hypoxanthine are released to the supernatant and, as such, do not form a major component of the platelet extracts.

In the present study a mixture of various nucleotides was analyzed using the conventional μBondapak C_{18} column. Solvent elution was isocratic and the flow-rate was 1 ml/min. Complete separation of all the nucleotides at ambient temperature was achieved in approximately 24 min (Fig. 1). Retention times for hypoxanthine and adenine were close to one another, but were well separated. To achieve a better separation of nucleotides, samples were analyzed using radially compressed columns. The separation obtained at ambient temperature of various nucleotide standards on a Radial-Pak column is presented in Fig. 2. Elution time for the separation of the eight standards from the mixture

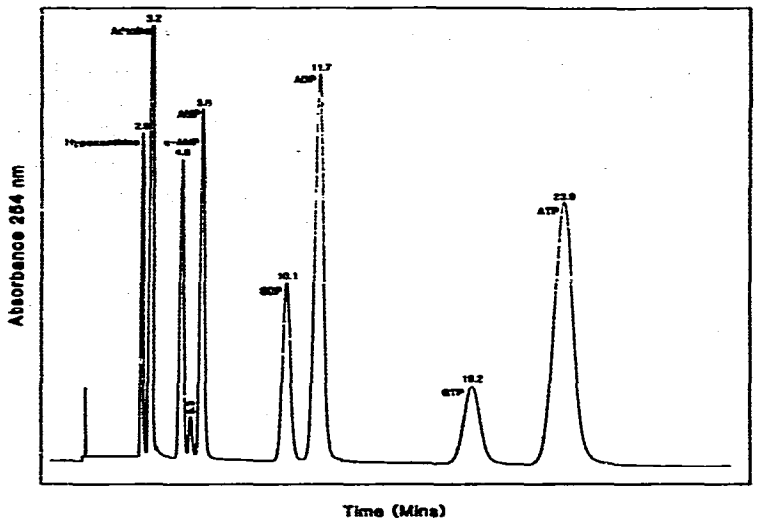

Fig. 1. Separation of various nucleotide standards from a mixture by isocratic elution using a μBondapak C_{18} column. Complete separation of all the nucleotides (including c-AMP from AMP) at ambient temperatures was achieved in approximately 24 min. Chromatographic conditions: column, 30 cm × 4 mm; packing, μBondapak C_{18}; solvent, acetonitrile—water—Pic A; detector, UV 254 nm; sample, 10 μl.

TABLE I

RELATIVE VALUES FOR PLATELET NUCLEOTIDES OBTAINED BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

The values obtained by other investigators using linear gradient elution are presented for comparison. Values are given in μmol per 10^{11} cells.

Method	ATP	ADP	AMP	GTP	GDP	ATP : ADP ratio
Scholar et al. 1973 [9]	5.7	3.5	—	0.9	0.9	1.6
Rao et al. 1974 [10]	7.4	4.0	3.0	—	—	1.8
Parks et al. 1975 [11]	5.0	3.5	0.8	—	—	1.4
D'Souza and Glueck 1977 [12]	3.8	3.5	0.3	0.45	0.4	1.1
Rao et al.* 1981 - present paper	4.3 ± 0.2	3.0 ± 0.2	0.6 ± 0.08	0.7 ± 0.05	0.4 ± 0.05	1.4

*Mean ± standard error (n = 6).

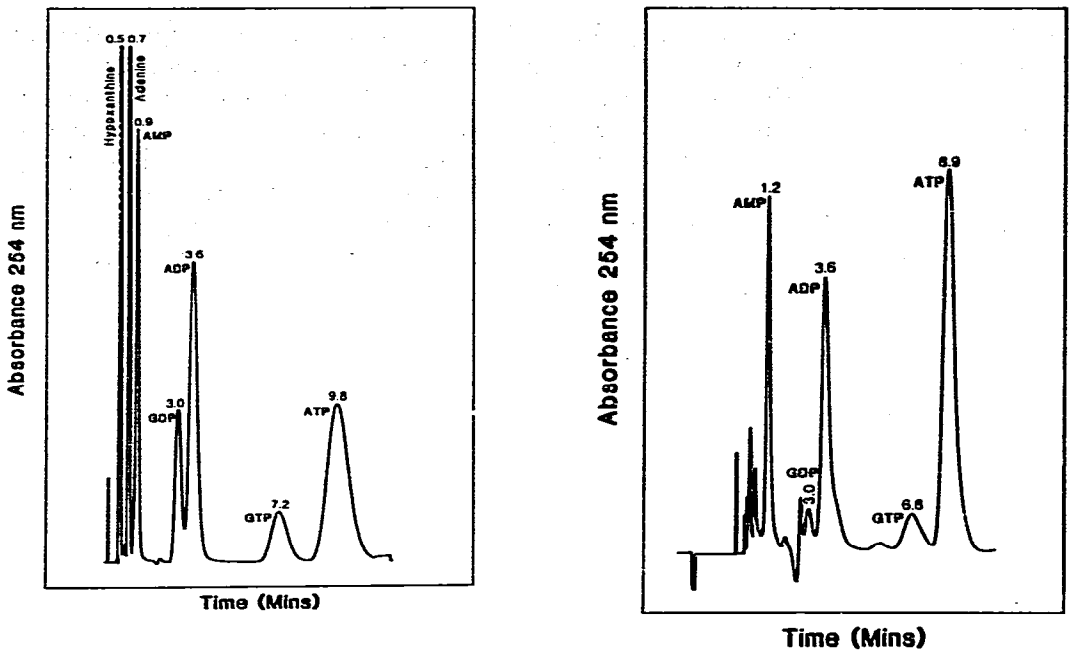

Fig. 2. Separation of various nucleotide standards from a mixture by isocratic elution with a radially compressed column. Elution time for separating all the nucleotides was less than 10 min. The range of recovery for all the nucleotides was excellent. Chromatographic conditions as in Fig. 1 except that column used was Radial-Pak.

Fig. 3. Separation of platelet nucleotides by isocratic elution using a radially compressed column. Identification of individual peaks was done by comparison of retention times of standards added to the platelet extract and assayed under identical conditions. Also, standard additions of known nucleotides to platelet extracts and their analysis served as a supplementary confirmation for peak identification. Retention times for AMP, ADP and ATP were 1.2, 3.6 and 8.9 min. The values obtained using this method for AMP, ADP, ATP and ATP : ADP ratios, agree with results obtained by others using linear gradient elution to achieve complete separation of nucleotides. Chromatographic conditions as in Fig. 2.

was less than 10 min. To test the efficacy of this analytical method, recoveries of standards added to the platelet rich plasma were measured. The range of recovery was 94—100% for all the nucleotides examined.

Nucleotide profile obtained by using a platelet extract on the Radial-Pak column is presented in Fig. 3. Complete separation was achieved in less than 9 min. In addition to adenine nucleotides, guanine nucleotides (GDP, GTP) were also separated by this method. Mean values obtained for each nucleotide are presented in Table I. Values obtained by other investigators using HPLC are presented for comparison [9—12]. Retention times for AMP, ADP and ATP were 1.2, 3.6 and 8.9 min. According to published reports elution time for complete separation of nucleotides by linear gradient techniques is over 70 min [9, 11, 12]. Values obtained for ATP in the present paper are lower than those reported by Parks et al. [11], but closer to the results of D'Souza and Glueck [12]. ATP:ADP ratios obtained by this method matched with the

values obtained by Parks et al. [11]. The value of 0.6 μM for AMP is in between the values published by Parks et al. [11] and by D'Souza and Glueck [12].

Results of these studies demonstrate that nucleotides can be separated rapidly and quantitated accurately by reversed-phase chromatography using isocratic elution. The time taken for each analysis could be further reduced by using radially compressed columns. The method is fast, efficient and provides excellent separation of platelet nucleotides. The values obtained using this method agree with results published by others using linear gradient elution to achieve complete separation of nucleotides [9, 11, 12].

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