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# MEASUREMENT OF THE RELEASE OF ADENINE NUCLEOTIDES DURING PLATELET AGGREGATION BY SMALL-BORE-COLUMN ISOCRATIC HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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## SUMMARY

This paper describes a simple and sensitive method for measuring adenosine, adenosine monophosphate, adenosine diphosphate, and adenosine triphosphate in plasma and tissues. This method involves sample preparation by perchloric acid extraction, followed by high-performance liquid chromatography on a small-bore column with UV detection at 254 nm. The minimum detectable amounts were adenosine, 0.60 pmol; adenosine monophosphate, 0.90 pmol; adenosine diphosphate, 1.5 pmol; and adenosine triphosphate, 2.5 pmol. The interassay coefficient of variation for the measurement of these compounds was less than 4%. The influence of varying the pH and the concentration of phosphate, acetonitrile, and ion-pairing reagent are described. This method can be used to measure adenine nucleotide release during platelet aggregation.

## INTRODUCTION

Recent studies have brought to light the importance of thrombus formation in occlusive coronary artery disease. Qualitative angiography[1], postmortem coronary arterial studies[2–4], coronary angioscopy[5], and biochemical studies[6] indicate the importance of thrombosis, superimposed on ruptured atherosclerotic plaques in myocardial infarction. Platelet aggregation is intimately involved in the process of thrombus formation.

Studies intended to delineate the mechanisms of platelet aggregation during thrombus formation have been hampered by the lack of reliable methods to study *in vivo* platelet aggregation. Detection of circulating platelet aggregates was proposed as evidence of *in vivo* platelet aggregation, but this test is difficult to standardize and reproduce[7]. Other methods to detect platelet activation have focused on radioimmunoassays of platelet secretory products, such as Platelet Factor 4, $\beta$-thromboglobulin, and the arachidonic acid metabolite thromboxane $B_2$ ($TxB_2$). Studies in which Platelet Factor 4[8] or $\beta$-thromboglobulin[9,10] are used as markers for intravascular platelet activation have produced equivocal results. This is probably due to sampling problems, leading to release of these products from platelets. Similar artefactual changes due to sampling, combined with imprecise assay methods, have

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cast doubt on the validity of using $TxB_2$ as a marker for *in vivo* platelet aggregation[11,12]. To assess the involvement of platelets in thrombus formation, more sensitive and specific indices of platelet aggregation are necessary.

During platelet aggregation, adenosine diphosphate (ADP) and serotonin are released from dense granules. Unlike serotonin, ADP released from platelets into plasma is not taken up again by the intact non-aggregated platelets, and may serve as a useful index of platelet aggregation. Several methods have been published for the determination of adenine nucleotides in biological materials by high-performance liquid chromatography (HPLC) (see refs. 13–19 and references cited therein). However, because they lack sensitivity, none of these methods can be used reliably to measure the release of adenine nucleotides as an index of platelet aggregation. A sensitive isocratic method is described for the measurement of adenosine (ADE), adenosine monophosphate (AMP), ADP, and adenosine triphosphate (ATP) in plasma by the use of HPLC on a 2.1-mm I.D. reversed-phase $C_{18}$ column with UV detection at 254 nm. This method is also applicable to studies of the metabolism of adenine nucleotides in tissues and cell cultures.

## EXPERIMENTAL

### *HPLC*

The system consisted of a Waters Assoc. 6000A (Milford, MA, U.S.A.) pump with microflow modifications, a Waters Assoc. U6K injector with a 20-$\mu$l injection loop, and a Kratos Spectroflow 783 UV detector (Kratos Analytical, Ramsey, NJ, U.S.A.), equipped with a 12-$\mu$l flow-cell, operated at 254 nm. A Brownlee (Santa Clara, CA, U.S.A.) RP-18 column (220 × 2.1 mm I.D.), attached to a Brownlee RP-18 (15 × 2.1 mm) guard precolumn was used for chromatographic separations. The connecting tubes between the injector, column, and detector were kept as short as possible. Data were collected and analyzed with IBM PC/XT and Nelson Analytical 2600 Series Chromatography software (Cupertino, CA, U.S.A.).

The chemicals used were of the highest grade available. Acetonitrile and perchloric acid were obtained from Aldrich (Milwaukee, WI, U.S.A.). Potassium dihydrogenphosphate, potassium hydroxide, tetrabutylammonium hydrogensulfate (TBAHS), and triethylamine (TEA) were obtained from Fluka (Ronkonkoma, NY, U.S.A.). Nucleotide standards and adenosine were obtained from Sigma (St. Louis, MO, U.S.A.). Milli-Q Water (Millipore, Bedford, MA, U.S.A.) was used for preparing the mobile phase. Standards were prepared by dissolving an accurately weighed amount of standard (10 mg) in 10 ml of 0.1 *M* phosphate buffer (pH 6.5), which was stored at −70°C.

Mobile phases were prepared fresh daily by adding 10 ml of acetonitrile to 450 ml of 0.125 *M* phosphate buffer (pH 6.5), containing 4 m*M* TBAHS, followed by 1 ml of TEA. The pH of the mobile phase was adjusted to 6.5 with 8 *M* potassium hydroxide, and finally the volume was made up to 500 ml with 0.125 *M* phosphate buffer (pH 6.5). The mobile phase was filtered through a 0.45-$\mu$m Nylon filter (Rainin Instruments, Woburn, MA, U.S.A.) and degassed for 5 min before use. This mobile phase contained 2% (v/v) acetonitrile 12.5 m*M* TEA, and 4 m*M* TBAHS, and produced excellent resoluion at pH 6.5. Chromatography was carried out at room temperature with a mobile phase flow-rate of 0.5 ml/min. Various flow-rates were tested. Slower

flow-rates led to a loss in resolution and higher flow-rates produced undesirable back pressure (> 3000 p.s.i.). Generally, equilibration of the column was assumed when a steady baseline was observed at a detector range of 0.002 a.u.f.s. with a 0.1-s rise time. When the column was not in use, the mobile phase was recycled through the column to keep the chromatographic conditions stable.

*Platelet aggregation and sample preparation*

For platelet aggregation studies, blood was collected in plastic tubes containing 3.8% sodium citrate (1 ml for 9 ml of blood), and platelet-rich plasma was separated immediately by centrifugation at 102 *g* for 15 min at room temperature. After removing the upper two-third of platelet-rich plasma, the remainder was again centrifuged at 4000 *g* for 10 min to yield platelet-poor plasma. Platelet-rich plasma samples were kept tightly capped at room temperature until analysed. For platelet aggregation, a platelet-rich plasma sample of 360 $\mu$l was prewarmed for 1 min by stirring in siliconized glass cuvettes placed in a Sienco dual-channel aggregometer (Morrison, CO, U.S.A.). Aggregation was induced by adding 10 $\mu$l of 120 m*M* solution of calcium chloride and 3 $\mu$g of collagen (Helena Labs., Beaumont, TX, U.S.A.) dissolved in 0.01 *M* Tris-buffered saline (pH 7.4). During aggregation, the percentage change in light transmission of platelet-rich plasma (set at 10%) relative to platelet-poor plasma (set at 90%) was monitored. When platelet aggregation was 60% completed (usually 2–3 min after adding collagen), the cuvette was plunged into ice–water and then centrifuged immediately at 4000 *g* for 10 min at 4°C to prepare platelet-poor plasma by removing the platelets. The nucleosides and nucleotides were extracted from platelet poor plasma by a modified perchloric acid method. To 500 $\mu$l of platelet-poor plasma, 500 $\mu$l of 10% (v/v) perchloric acid was added, followed by 50 $\mu$l of 1 *M* potassium dihydrogenphosphate, and mixed for 10 s on a vortex mixer. The sample was then centrifuged at 4000 *g* for 5 min at 4°C. A 500-$\mu$l aliquot of the clear supernatant was transferred to a 75 × 12 mm polypropylene tube, and 30 $\mu$l of 8 *M* potassium hydroxide was added. After mixing, the tube was placed in an ice-bath for 30 min. The precipitate formed was removed by centrifugation at 30 000 *g* for 10 min at 4°C. A 300-$\mu$l aliquot of the supernatant was transferred to another 75 × 12 mm polypropylene tube, and 30 $\mu$l of 1.5 *M* phosphate buffer (pH 6.5) was added to adjust the pH to 6–7.

A standard curve was constructed for each assay from four different amounts of adenosine, AMP, ADP, and ATP, added to 1 ml of platelet poor plasma (PPP) and carried through the entire procedure. The amount of standard added, covered the range from 1.25 ng to 10 ng.

## RESULTS

When a mixture of standards, containing ADE, AMP, ADP, and ATP in 0.125 *M* phosphate buffer (pH 6.5) was injected, ADE was eluted at 5.90 min, AMP at 4.66 min, ADP at 8.97 min, and ATP at 17.53 min. The $k'$ values were: ADE = 4.87, AMP = 3.63, ADP = 7.94, and ATP = 16.5, respectively (Fig. 1). When standards in phosphate buffer were extracted with perchloric acid and injected, identical results were obtained. The recovery was determined by injecting a known amount of the nucleotides in mobile phase and comparing the results with values obtained when the

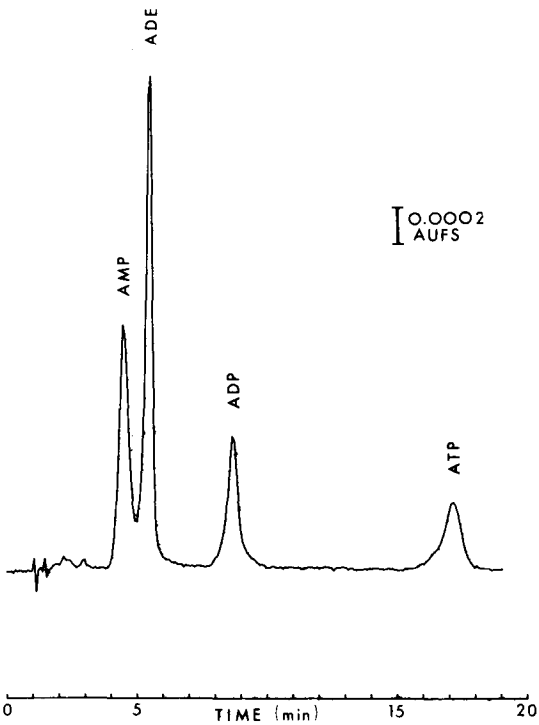


Fig. 1. Chromatogram showing the retention time for adenosine (ADE), adenosine monophosphate (AMP), adenosine diphosphate (ADP), and adenosine triphosphate (ATP). A 2-ng amount of each was dissolved in the mobile phase and injected. For conditions, see text.

same concentrations were added to platelet-poor plasma and carried through. The recovery for all four compounds was 92.6 ± 0.5% (mean ± S.D.) ($n$ = 12). The standard curve was linear for all four compounds throughout the range tested ($r$ = 0.99; $p < 0.001$). The sensitivity of the method (at a signal-to-noise ratio of 3) for ATP was 2.5 pmol, for ADP 1.5 pmol, for AMP 0.90 pmol, and for ADE 0.60 pmol (amount injected on the column). The inter-assay coefficient of variation was determined by adding 5 ng of ADE, AMP, ADP, and ATP to four different plasma samples and assaying on four different days. The value was less then 4% for all the compounds.

Platelet-poor plasma had no detectable levels of ADP or ATP, whereas low concentrations of AMP and ADE were detected (Fig. 2, Table I). With platelet aggregation, there was a significant increase in the concentation of AMP, ADP, and ATP in platelet poor-plasma (Fig. 3, Table I).

The purity of the detected ADE, AMP, ADP, and ATP peaks was determined by several methods: (1) the peak areas were increased in direct proportion to added ADE, AMP, ADP, and ATP; (2) no ADE, AMP, ADP, or ATP peaks were detected when samples were incubated with a mixture of hexokinase, myokinase, and adenylic acid deaminase, which metabolized the adenine nucleotides; (3) the absorption at 254 and 280 nm were compared for all four compounds. The mean $areas_{254}/areas_{280}$ ratio for all four compounds were similar for the sample peaks and the injected standards with a value of 3.42 for ADE, 3.49 for AMP, 3.83 for ADP, and 2.75 for ATP.

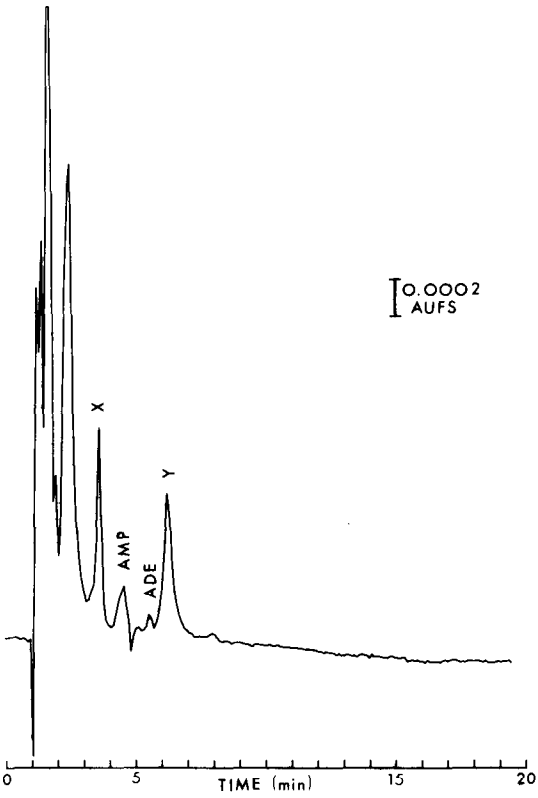


Fig. 2. Chromatogram of platelet-poor plasma. X and Y are unknown peaks.

Several chromatographic parameters were examined to determine their role in enhancing peak separation without significantly increasing the analysis time for each sample. The percentage of acetonitrile used (Fig. 4), the concentration of phosphate buffer used (Fig. 5), the concentration of TBAHS used (Fig. 6), and the pH (Fig. 7) had a major effect on retention characteristics of the adenine nucleotides. The pH selected for the mobile phase did not significantly alter the peak retention characteristics of the column, even after four months of continuous use ($>$ 1000 sample injections).

TABLE I

THE CHANGE IN PLASMA CONCENTRATION OF ADENOSINE (ADE), ADENOSINE MONOPHOSPHATE (AMP), ADENOSINE DIPHOSPHATE (ADP), AND ADENOSINE TRIPHOSPHATE (ATP) DURING PLATELET AGGREGATION

Concentrations are expressed as pmol/ml of plasma ($n$ = 8) (mean ± S.D.).

| | *ADE* | *AMP* | *ADP* | *ATP* |
|---|---|---|---|---|
| Pre-aggregation | 2.4 ± 0.7 | 152 ± 21 | <1.5 | <2.5 |
| Post-aggregation | 3.1 ± 0.6 | 1396 ± 139* | 2372 ± 212* | 2601 ± 237* |

* $p < 0.001$ (unpaired $t$ test).

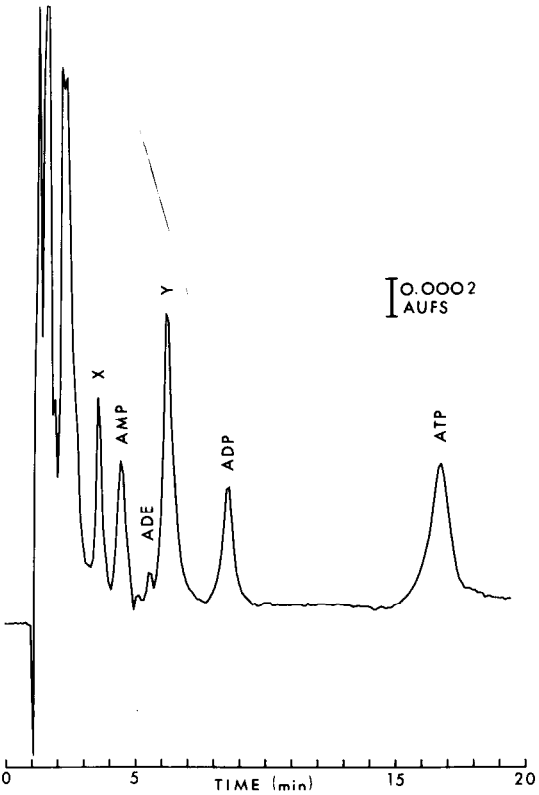


Fig. 3. Chromatogram of platelet-poor plasma, prepared from platelet-rich plasma following platelet aggregation. X and Y are unknown peaks.

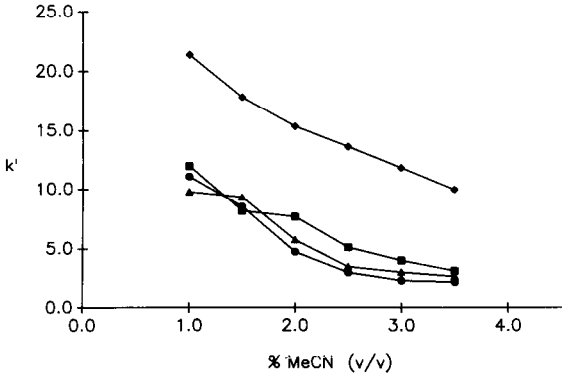


Fig. 4. Change in capacity factors ($k'$) for ADE (▲), AMP (●), ADP (■), and ATP (◆) with variation in acetonitrile (MeCN) concentration. Other parameters were the same as in text.

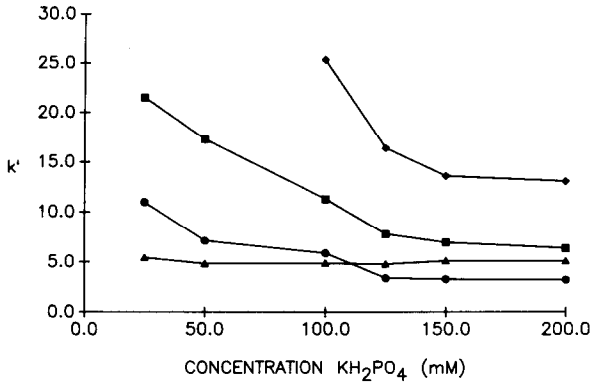


Fig. 5. Effect of potassium phosphate buffer concentration on capacity factors for ADE (▲), AMP (●), ADP (■), and ATP (◆). Other parameters were the same as in text.

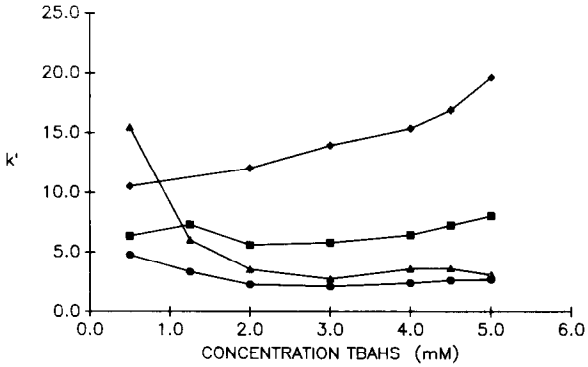


Fig. 6. Change in capacity factors for ADE (▲), AMP (●), ADP (■), and ATP (◆) with variation in TBAHS concentration. Other parameters were the same as in text.

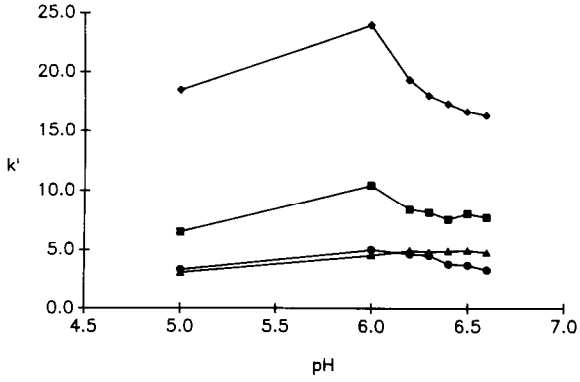


Fig. 7. Effect of pH changes on capacity factors for ADE (▲), AMP (●), ADP (■), and ATP (◆). Other parameters were the same as in text.

## DISCUSSION

It is well known that application of gradient elution to routine quantitative analysis can impair reproducibility[20]. In fact, the column equilibrium between analyses easily results in peak shifting[21]. Further, these methods have limited sensitivity. Therefore, for the simultaneous measurement of ADE, AMP, ADP, and ATP, a highly sensitive isocratic reversed-phase method was developed by optimization of the condition of separation.

Tetrabutylammonium ions (TBA) bind to the stationary phase of the $C_{18}$ column due to hydrophobic interactions of the aliphatic groups of the packing material with the butyl groups of TBA. Due to this interaction, positively charged groups arise on the stationary phase. This modulates the retention of nucleotides as a function of the number of negatively charged phosphate groups. Thus, $k'$ tends to increase as the number of nucleotidyl phosphate groups increases (triphosphate > diphosphate > monophosphate) (Fig. 1).

An increase in TBA cation concentration increases the equilibrium concentration of TBA cations retained on the $C_{18}$ column surface. Thus, as the concentration of TBAHS increases, the $k'$ of ATP increases dramatically due to the association of the nucleotidyl phosphate groups with the increased number of TBA cations (Fig. 6). Below 2 m*M* TBAHS concentration, ADE showed large increases in $k'$ due to greater hydrophobicity of the column as a result of the reduced equilibrium concentration of TBA cations retained. The $k'$ values of AMP, ADP, and ATP do not increase substantially below 2 m*M* TBAHS, since the hydrophobic interactions with the column are reduced by the phosphate groups.

An increase in acetonitrile decreases the equilibrium concentration of TBA cations retained. This suggests a greater hydrophobic interaction of the butyl groups of the TBA cations with acetonitrile, thus decreasing the concentration of TBA on the stationary phase[22,23]. An increase in acetonitrile concentrations therefore leads to a reduction of the $k'$ for all four compounds (Fig. 4).

Increasing the mobile-phase counter-anion concentration by addition of salt or buffer decreases the amount of nucleotide retained due to mass-action effects[22]. The degree of variation in retention is dependent on both the selectivity and concentration of the counter-anion. Therefore, increasing the concentration of potassium dihydrogenphosphate reduces the $k'$ of all the nucleotides due to anion exchange of the dihydrogenphosphate anion with the TBA cations; effectively reducing the amount of TBA cations available for binding the nucleotidyl phosphate groups (Fig. 5).

In addition, lowering the pH reduces the $k'$ of the nucleotides (Fig. 7). In this case, the nucleotidyl phosphate groups have a reduced interaction with the the TBA cations on the stationary phase because of partial protonation of the phosphate groups themselves.

When TEA was not added to the mobile phase, tailing of the peaks was observed, suggesting that the column was not fully endcapped. Use of 12.5 m*M* TEA decreased this effect. It is known that even after exhaustive endcapping, steric hindrance prevents derivatization of all the silanol groups. The effect of these functional groups was modified with TEA, which was routinely used in all experiments. To obtain high sensitivity, a 2.1 mm I.D. column was used. To minimize the peak broadening, the connecting tubes were kept as short as possible and the micro-bore manifold was

installed on the detector to connect the column directly to the detector cell.

The method is sensitive enough to measure nucleoside and nucleotide concentrations in 500 $\mu$l of plasma or less. Platelet-poor plasma showed non-detectable levels of ADP and ATP (Table I). With platelet aggregation, there was a marked increase in concentration of ATP, ADP, and AMP. This suggests that during platelet aggregation, all three nucleotides are released. This contrasts with previously published results[23]. However, the possibility cannot be excluded that the ADP and/or ATP released form platelets may be rapidly metabolized to produce AMP and may contribute to the observed results.

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REFERENCES

1 J. A. Ambrose, S. L. Winters, R. R. Arora, A. Eng, A. Riccio, R. Gorlin and V. Fuster, *J. Am. Coll. Cardiol.*, 7 (1986) 472.
2 D. C. Levin and J. T. Fallon, *Circulation*, 66 (1982) 316.
3 E. Falk, *Circulation*, 71 (1985) 699.
4 M. J. Davies and A. C. Thomas, *Br. Heart J.*, 53 (1985) 363.
5 C. T. Sherman, F. Litvack, W. Grundfest, M. Lee, A. Hickey, A. Chaux, R. Kass, C. Blanche, J. Mateloff, L. Morgenstern, W. Ganz, H. J. C. Swan and J. Forrester, *N. Engl. J. Med.*, 315 (1986) 913.
6 D. J. Fitzgerald, L. Roy, F. Catella and G. A. Fitzgerald, *N. Engl. J. Med.*, 315 (1986) 983.
7 M. A. Packham, *Thromb. Haemost.*, 40 (1978) 174.
8 G. C. White and A. A. Marouf, *J. Lab. Clin. Med.*, 97 (1981) 369.
9 M. D. Rubenstein, R. T. Wall, D. S. Bain and D. C. Harrison, *Am. Heart J.*, 102 (1981) 363.
10 C. W. Pumphrey and J. Dawes, *Am. J. Cardiol.*, 50 (1982) 1258.
11 G. A. Fitzgerald, A. K. Pedersen and C. Patrono, *Circulation*, 67 (1983) 1174.
12 A. K. Pedersen, M. L. Watson and G. A. Fitzgerald, *Thromb. Res.*, 33 (1983) 99.
13 E. Juengling and H. Kammermeier, *Anal. Biochem.*, 102 (1980) 358.
14 D. W. Nierenberg, A. L. Pogolotti, Jr. and D. V. Santi, *J. Chromatogr.*, 190 (1980) 212.
15 B. Levitt, R. J. Head and D. P. Westfall, *Anal. Biochem.*, 137 (1984) 93.
16 G. Crescentini and V. Stocchi, *J. Chromatogr.*, 290 (1984) 393.
17 G. K. Bedford and M. A. Chiong, *J. Chromatogr.*, 305 (1984) 183.
18 Z. Olempska-Beer and E. B. Freese, *Anal. Biochem.*, 140 (1984) 236.
19 J. L.-S. Au, M.-H. Su and M. G. Wientjes, *J. Chromatogr.*, 423 (1987) 308.
20 G. Lencini, E. Buzzi, M. Marcesca, M. Mazzei and A. Balbi, *Anal. Biochem.*, 165 (1987) 379.
21 N. A. Parris, *Instrumental Liquid Chromatography (Journal of Chromatography Library*, Vol. 27), Elsevier, Amsterdam, 2nd ed., 1984, p. 305.
22 P. G. Rigas and D. J. Pietrzyk, *Anal. Chem.*, 60 (1988)454.
23 A. M. Pimenov, Yu. V. Tikhonov and P. T. Toguzov, *J. Liq. Chromatogr.*, 9 (1986) 1003.
24 H. Holmsen, *Sem. Hematol.*, 22 (1985) 219.