Determination of ATP and ADP Secretion from Human and Mouse Platelets by an HPLC Assay

Michael von Papen Stepan Gambaryan Claudia Schütz Jörg Geiger

Institute for Clinical Biochemistry and Pathobiochemistry, University of Würzburg, Germany

Keywords

Platelet exocytosis · Delta granule · Nucleotides · Storage pool deficiency · Secretion defects

Summary

Background: Secretion of ADP and ATP is an essential prerequisite for platelet aggregation. Impaired nucleotide secretion can cause aggregation defects and increased bleeding risk. Quantitative determination of platelet nucleotide content and exocytosis is thus of importance for the characterization and diagnosis of bleeding phenotypes. For transgenic animal models with hemostatic defects analysis of potential secretion defects is as well imperative. **Methods:** Supernatants of washed platelets and platelet-rich plasma were analyzed by HPLC for ADP and ATP concentration. Calibration of the HPLC data was accomplished with an internal standard compensating for loss of analyte, detection sensitivity, and interference of the biomatrix. **Results:** HPLC analysis of nucleotide secretion was carried out with human and mouse platelets. Detection limits were determined for washed platelet and platelet-rich plasma samples. In the physiological concentration range linearity with respect to the peak area is maintained. **Conclusion:** The method combines reasonable sensitivity with robustness. The internal standard ensures reliable quantification of nucleotide concentrations even in presence of otherwise interfering substances. The low sample consumption renders possible the application to analysis of small samples like in mouse experiments.

Introduction

Platelet exocytosis is induced by all strong platelet agonists during platelet activation. By the release of substances inducing aggregation and/or coagulation, the initial activation event is amplified and the activation signal propagated [1]. Secretion occurs from alpha granules, dense granules (delta granules), the dense tubular system, and lysosomes. Mainly alpha and delta granule releasate contributes to platelet aggregation [2]. Alpha granules contain adhesion molecules, proteins necessary for the formation and cross-linking of aggregates, as well as coagulation and growth factors [3]. Delta granules contain nucleotides, serotonin, calcium, and polyphosphates [4]. Nucleotides and serotonin transmit platelet stimulation as paracrine factors by recruiting additional platelets to the aggregate and finally stabilizing the aggregate. Defects in platelet degranulation are the cause of severe hemostatic disorders such as gray platelet syndrome or Hermansky-Pudlak syndrome [5–8]. Routine functional platelet tests like light transmission aggregometry or in vitro bleeding time by PFA[®] can indicate defective platelet secretion but do not allow discrimination with regard to the actual cause [9]. Functional defects may arise from impaired loading of granules, defective signal transduction, dysfunctional exocytosis or lack of responsiveness to the released agents. Though these conditions eventually constitute similar phenotypes of platelet dysfunction, genetics and pathobiochemistry are significantly different and demand for distinct therapeutic approaches. Consequently, methods allowing for differentiation of the exact nature of functional defects are essential for reliable diagnosis. Platelet secretion is not only a predictor for hemostasis or thrombosis [10, 11] but growing evidence indicates that certain neurological disorders are reflected by platelet secretion defects [12–14]. Accordingly functional platelet tests may provide appropriate surrogate markers for these diseases.

Several methods have been developed and established to analyze platelet secretion. The most commonly used method today is based on the detection of surface presentation of P-selectin on the platelet membrane by flow cytometry [15]. As P-selectin is almost exclusively found in alpha granules the method is limited to the diagnosis of alpha granule defects. Though exocytosis of dense granules and alpha granules are associated mechanistically, P-selectin surface presentation does not necessarily reflect dense granule function. Dense granule secretion and, in particular, the release of ATP and ADP from the platelet as secondary event during platelet aggregation have in recent years been recognized as a major step in platelet aggregate formation being crucial for the formation of stable aggregates [16–18]. Purinergic receptor activation and signaling can be efficiently quantified by routine tests [19–21] while monitoring of dense granule secretion is commonly only accomplished in a semi-quantitative manner. Current methods are either based on determination of the release of tracer from pre-loaded platelets [22], on fluorometry of derivatized serotonin [22] or on bioluminescence of an ATP-consuming reaction [23]. These techniques either require extensive pre-analytics and/or sample preparation or are highly susceptible to interferences by sample components.

We have chosen to develop HPLC separation and detection of platelet-derived nucleotides to compensate for the known shortcomings and optimize the method for small sample size, as required for clinical applications or transgenic mouse research.

Material and Methods

Material

AMP, ADP, ATP, 2-MeS-ADP (2-methylthio-5'-ADP) were obtained from Sigma-Aldrich (Taufkirchen, Germany) at the highest purity available. 6-Cl-PuDP (6-chloro purine 5' diphosphate) was obtained from Biolog (Bremen, Germany). All other chemicals were obtained from Merck (Darmstadt, Germany) at HPLC grade.

Methods

Platelet-rich plasma (PRP) and washed platelets (WP) were prepared from whole human blood as described previously [24]. The blood was obtained from healthy volunteers who had not taken any medication affecting platelet function within 2 weeks prior to the experiment. After informed consent the blood was drawn by venipuncture and collected in citrate tubes. The whole blood (WB) was centrifuged at $300 \times g$ for 10 min to obtain PRP. WP were prepared by centrifugation of PRP at $380 \times g$ for 10 min and resuspension of the resulting pellet in phosphate-buffered saline (PBS; 137 mmol/l NaCl, 2.7 mmol/l KCl, 10 mmol/l Na_2HPO_4 , 2 mmol/l KH_2PO_4 , pH = 7.4). WP and PRP were adjusted with PBS to a cell density of 2×10^6 platelet/ml.

Mouse platelets were prepared from mouse WB collected from the orbital sinus of anesthetized mice. PRP and WP were prepared according to published protocols [25]. WP were resuspended in Tyrode buffer (137 mmol/l NaCl, 2 mmol/l KCl, 2 mmol/l MgCl_2 , 12 mmol/l NaHCO_3 , 0.3 mmol/l $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 5.5 mmol/l D-glucose, 5 mmol/l Hepes, pH 7.4), and the number of used WP was adjusted similar to that in human platelets.

As internal standard 6-Cl-PuDP was used, a compound which has been shown to be without effect on platelets [26]. The internal standard was added to the sample to final concentration of 5 $\mu\text{mol/l}$. For HPLC analysis 200 μl of PRP or WP were stopped with 20 μl (WP samples) or 300 μl (PRP samples) ice-cold EGTA (ethylene glycol tetraacetic acid) solution (5 mmol/l) and 100 μl silicon oil. The samples were thoroughly mixed and centrifuged for 90 s at $6,500 \times g$. The aqueous phase above the silicon oil layer was removed and mixed with 200 μl (1.5 ml for PRP samples) 100% ethanol. The precipitate was removed by centrifugation at $20,000 \times g$ for 10 min at 4 °C. The supernatant was evaporated in the vacuum at room temperature. PRP samples were solved in 200 μl HPLC grade water and extracted with 300 μl ether to remove lipophilic components. Dry samples were stored at –20 °C until analysis.

Nucleotide recovery was determined for PRP and WP samples by spiking with the appropriate nucleotide. ATP, ADP, and AMP were added to EGTA-stopped samples at concentrations between 0.2 and 15 $\mu\text{mol/l}$ and treated just as normal human platelet samples.

For functional tests with human platelets, 200 μl samples of PRP or WP were challenged with established platelet receptor stimuli such as thrombin, TRAP-6, and AY-NH2 for thrombin receptors, collagen and convulxin for GPIb (glycoprotein Ib), U46619 for the thromboxane receptor, and 2-MeS-ADP for purinergic receptors. The stimulation was carried out by addition of the stimulant to the platelet sample in the water bath at 37 °C. The samples were stopped with a EGTA/silicon oil mixture and treated as described above.

The dried samples were dissolved in 200 μl of mobile phase buffer (buffer A: 90 mmol/l KH_2PO_4 , 10 mmol/l K_2HPO_4 , 4 mmol/l tetrabutylammonium sulfate (TBAS), pH 6.0) and vortexed. To remove insoluble material the dissolved samples were centrifuged 10 min at $20,000 \times g$. HPLC separation was carried out on a Merck® LC18 column (150 $\times$ 4.6 mm, 5 μm) using a Hitachi® HPLC system equipped with a L7250 autosampler, L7100 pump, L7300 column oven, D7000 controller and L7400 UV-detector. 20 μl of each sample were analyzed using a linear gradient of 100% buffer A to 60% buffer B (buffer B: 80 mmol/l KH_2PO_4 , 20 mmol/l K_2HPO_4 , 4mmol/l TBAS, 30% methanol, pH 7.2) over 30 min at 25 °C. After each run the column was eluted with 100% buffer B for 5 min and equilibrated again with buffer A for 5 min. The nucleotides were detected by their UV absorption at 260 nm. A standard curve for all adenine nucleotides was measured for each set of samples with concentrations ranging from 0.2 to 15 $\mu\text{mol/l}$ (4–300 pmol) and 5 $\mu\text{mol/l}$ 6-Cl-PuDP as internal standard added.

Platelet aggregation was carried out with an APACT (Haemochrom, Essen, Germany) aggregometer as described [19]. Briefly, 0.2 ml of PRP or WP were placed in a cuvette containing a stir bar and incubated for 5 min at 37 °C. 100% aggregation was calibrated with platelet-poor plasma (for PRP samples) or 1% BSA (for WP samples). Agonists and antagonists were added and aggregation was observed for 5 min under stirring (1,000 rpm).

Data Analysis

The HPLC traces were background corrected with a blank sample (20 μl buffer A). The signals were integrated and assigned to the respective nucleotide according to the retention time of the nucleotide standards. With the internal standard, peak area ratios were calculated for each nucleotide standard and a calibration curve determined. From the sample data the signal ratio was calculated for each nucleotide signal in relation to the internal standard sample and the nucleotide concentration calculated from the standard curve. Finally the calculated concentrations were converted to pmol/ 10^9 platelet using the platelet count determined on a Sysmex® KX21N blood cell counter.

Fig. 1. Efficient separation of natural and synthetic purine nucleotides by HPLC. Original traces from HPLC experiments with standards solution containing ATP, ADP, AMP, 6-Cl-PuDP (continuous line), 2-MeS-ADP solved in PBS (dotted line) and WP spiked with ATP, ADP, AMP, and 6-Cl-PuDP (dashed line) are shown. Retention time is given in minutes, signal intensity in arbitrary units of absorption at 260 nm.

Results

Separation and Quantification

Mixtures of natural (AMP, ADP, ATP) and synthetic nucleotides (2-Cl-ADP, 2-Cl-ATP, 2-MeS-ADP and 6-Cl-PuDP) at concentrations ranging from 1 to 15 $\mu\text{mol/l}$ were used for the optimization of the separation protocol to enable future experiments on nucleotide effects on nucleotide secretion and nucleotide degradation. Any interaction of the nucleotides and effects on the separation could be excluded (fig. 1; supplemental table 1, available at www.karger.com/?DOI=350294). Peak integrals for ATP, ADP, and 6-Cl-PuDP correlate perfectly ($R^2 = 0.99986$, $R^2 = 0.99990$, $R^2 = 0.99974$) with the respective nucleotide concentration; for AMP the correlation is less optimal ($R^2 = 0.99925$) mainly because of significant deviation at small concentrations ($<1 \mu\text{mol/l}$) (supplemental table 2, available at www.karger.com/?DOI=350294). ATP, ADP, and AMP peak areas correlate similarly with 6-Cl-PuDP concentration (fig. 2; supplemental table 2). Sample matrices can severely affect separation and quantification in HPLC; thus we investigated the quality of the separation, linearity of the quantification, and recovery of nucleotides by spiking experiments where nucleotides were added at concentrations ranging from 0.1 to 15 $\mu\text{mol/l}$ to WP suspensions or PRP, which were stopped and separated as described in 'Methods'. The samples were processed identically to platelet samples. The non-calibrated data show a high correlation with the nucleotide concentration (supplemental table 1) and no discrepancy in retention times.

The limit of detection (LOD) and the limit of quantification (LOQ) were determined with nucleotide standards and platelet spiking samples as well, based on a limit of $3 \times \text{SEM}$ (standard error of the mean) for LOD and $5 \times \text{SEM}$ for LOQ (supplemental table 3, available at www.karger.com/?DOI=350294).

Assay Properties

Reproducibility of the method was determined with a dilution series of standards and 3 individual sets of WP samples obtained from different donors. The sample sets contained untreated platelets, thrombin-treated platelets, and total platelet lysates. For each set the measurement was repeated 3 times on 3 different days. For the nucleotide standards the inter-assay variance amounts to CV (coefficient of variation) = 2% for low nucleotide concentration ($\leq 2 \mu\text{mol/l}$) down to 0.4% for high nucleotide concentration ($\geq 10 \mu\text{mol/l}$). The intra-assay CV was determined to be $\leq 15\%$ for unstimulated platelets (low nucleotide content) and $\leq 12\%$ for thrombin-stimulated platelets (high nucleotide content). The inter-assay CV was $\leq 25\%$ for low and $\leq 14\%$ for high nucleotide content (supplemental table 4, available at www.karger.com/?DOI=350294).

Nucleotide Content of Human and Mouse Platelets

Human platelet ATP, ADP, and AMP as well as medium or plasma contents were determined using WP and PRP. Mouse platelet ATP, ADP, and AMP contents were determined using WP. The extracellular ADP content is slightly higher in WP than in PRP, indicating release of nucleotides induced by the conditions during WP preparation (table 1).

Nucleotide Secretion of Stimulated Human and Mouse Platelets

Human platelet secretion was investigated with human WP resuspended in PBS buffer and PRP. The platelets were stimulated with collagen (10 $\mu\text{g/ml}$), the collagen receptor agonist convulxin (10 nmol/l), 10 mU/ml thrombin, the thrombin receptor-activating peptides TRAP-6 (10 $\mu\text{mol/l}$) or AYNH2 (200 $\mu\text{mol/l}$), or the thromboxane A2 analogue U46619 (1 $\mu\text{mol/l}$). The ATP concentration increases within the first 30 s after stimulation to the maximum, regardless of the stimulant,

Table 1. Nucleotide content of human and mouse plasma, buffer, and platelets and platelets^a

	Human PRP		Human WP		Mouse WP	
	basal	total	basal	total	basal	total
ADP	0.83 ± 0.67	4.85 ± 1.68	1.85 ± 1.15	5.20 ± 3.15	0.10 ± 0.00	0.46 ± 0.04
ATP	1.54 ± 1.04	8.39 ± 0.99	1.57 ± 1.48	9.43 ± 7.08	0.36 ± 0.05	1.76 ± 0.38

^aBasal extracellular (basal) of human platelet PRP (n = 5) and WP (n = 7), washed mouse platelets (n = 4) and total (total) nucleotide content of human and mouse platelets is shown. Concentrations are means given in nmol/10⁸ platelets ± standard deviation.

Fig. 2. Peak areas of ATP, ADP and AMP signals correlate with the respective concentrations and with the concentration of the internal standard.

Linear regression for ATP (triangles), ADP (diamonds), AMP (squares), and 6-Cl-PuDP (triangles down) peak area with the respective nucleotide concentration (A) and the internal standard 6-Cl-PuDP (B) are shown. Data are means of 5 experiments, given in micromolar nucleotide concentration and arbitrary units for the peak area.

Table 2. Relative amount of released nucleotides from human platelets depending on stimulating agent in WP^a

Agonist	% of total content	
	ADP	ATP
Collagen 10 μg/ml	75.5 ± 13.53	59.3 ± 32.19
Convulxin 10 nmol/l	44.9 ± 8.04	26.7 ± 14.49
Thrombin 10 mU/ml	44.0 ± 24.10	27.0 ± 7.96
TRAP6 10 μmol/l	86.2 ± 47.22	41.2 ± 12.15
AYNH2 200 μmol/l	99.3 ± 54.37	44.9 ± 15.89
U46619 1 μmol/l	32.6 ± 3.42	29.7 ± 10.53
Releasable [27]	84.0	33.0

^aThe data are means of 3 (collagen, convulxin, AYNH2) or 5 (thrombin, TRAP6, U46619) independent experiments ± standard deviation. The observed differences were not found to be significantly different (p>0.05). Literature data for the maximal releasable amount of nucleotides were taken from [27].

Table 3. ATP and ADP release from human and mouse platelet suspensions^a

	Human		Mouse	
	thrombin	% of total	thrombin	% of total
ADP	2.14 ± 1.17	44	0.16 ± 0.01	35
ATP	2.27 ± 0.67	27	0.52 ± 0.05	29

^aPlatelets were stimulated with 10 mU/ml thrombin. Concentrations are given as means of 5 (human) or 4 (mouse) experiments in nmoles/10⁸ platelets ± standard deviation. The total refers to the amount released in relation to the total nucleotide content of platelets in the sample.

while ADP concentration increase is delayed taking 60–90 s until the maximum is reached (fig. 3). Convulxin and collagen released more than 60% of the total ADP and ATP content, thrombin and thrombin receptor peptides 40–50%, and U46619 30% (table 2). This is in line with established data [27] on non-secretable ATP which makes up about 65% of total platelet ATP. The role of ADP as paracrine stimulator of nucleotide secretion was investigated with the established strong purinergic receptor agonist 2-Me-SADP which is clearly separated from other nucleotides on the HPLC column. 2-Me-SADP induces a small but significant secretion of

Fig. 3. Time course of thrombin, convulxin and thromboxane evoked ADP (A) and ATP (B) secretion and aggregation (C) of human platelets. ATP and ADP were determined from washed human platelet suspensions stimulated by 1 $\mu\text{mol/l}$ U46619 (squares, continuous line) 10mU/ml thrombin (diamonds, dashed line) or 1 nmol/l convulxin (triangles, dotted line) in the time intervals indicated. Insets show early changes in ADP and ATP secretion within the first 60 s. Platelet aggregation was measured with an Apat aggregometer. Data are means of 3 (convulxin) or 5 (thrombin, U46619) individual experiments, given in nmol/ 10^8 platelets. The error bars indicate the SD of the mean.

Fig. 4. Stimulation of ADP release by 2-MeS-ADP in absence and presence of calcium ions.

WP were stimulated with 5 or 10 $\mu\text{mol/l}$ 2-MeSADP in absence or presence of 1 mmol/l CaCl_2 and nucleotide accumulation in the supernatant was determined after 5 min incubation at 37 °C. Data are means of 3 independent experiments given in nmol/ 10^8 platelets. The error bars indicate the SD of the mean (* $p < 0.05$).

ADP and ATP which can be further enhanced by addition of calcium (fig. 4).

While washed platelet supernatant can be analyzed directly, PRP requires additional treatment to remove lipophilic components which significantly affect separation and can clog the HPLC column. The secretion responses are not significantly different in PRP and WP. Both kinetics and maximal secretion are almost identical (table 2).

The secretion of mouse platelets was investigated with WP stimulated with 10 mU/ml thrombin (table 3). The peak area readings were found to be in the range of the calibration standards (fig. 5). The total and released amount of nucleotides in mouse platelets is about 30–50% of the amount observed with human platelets, which is in line with data on size and storage capacity of mouse platelets [28]. The ATP/ADP ratio however appears to be clearly higher in mouse platelets than in human platelets.

Discussion

Platelet dense granule secretion not only enhances initially stimulated platelet aggregation but is indispensable for the formation of a stable aggregate [16, 17]. Notably the nucleotides ADP and ATP contribute significantly by activating several purinergic receptors on the platelet membrane [18]. Defective nucleotide signaling can account for platelet function disorders, resulting in severe bleeding [8]. An adjusted

Fig. 5. Basal, releasable and total ADP (**A**) and ATP (**B**) of washed mouse platelets in relation to the standard curves for ADP or ATP. Washed mouse platelet supernatants were analyzed for ADP and ATP content without stimulation (basal) and after stimulation with 10 mU/ml thrombin (releasable). Total ADP and ATP content was determined by lysis of washed mouse platelets (total). The standard curve was determined with ADP and ATP solutions in PBS at concentrations of 1, 2, 3, 5, 10, and 12 $\mu\text{mol/l}$ and is given as means $\pm$ SD. The standard curve was calculated by linear regression. The data are given as peak area in arbitrary units of the respective HPLC traces.

therapy of a hemostatic dysfunction of unknown origin requires detailed analysis of possible causes and the underlying mechanisms [9, 30]. Receptor defects or impaired signal transduction but also deficiency or absence of nucleotide secretion can be the cause for nucleotide signaling defects. Dysfunctional secretion may in turn result from storage deficiency or from receptor or signal transduction disorders in the exocytosis pathway. Identification of the immediate cause of a functional defect in secretion requires therefore data on the stored nucleotides as well as on the time course and absolute amount of secreted nucleotides.

Dense granule secretion is mostly quantified by monitoring the main components of these granules, either nucleotides or serotonin. Serotonin can be detected by covalent modification generating a fluorescent derivative. This can then be quantified by fluorometry using a standard curve [22]. While the method is quite robust, it requires cumbersome sample preparation and is rather insensitive. Direct quantification of serotonin is also possible by electrochemical detection after HPLC separation [31] or by ELISA [32]. Alternatively radiolabeling of platelets with ^{14}C serotonin is used which offers improved sensitivity, but requires pre-loading with the radiotracer [22]. The fluorescent compound mepacrine has frequently been used for secretion monitoring. Platelets incubated with the dye accumulated it in dense granules and release it upon stimulation [33]. However, serotonin and nucleotide content and release from platelets are not necessarily quantitatively linked. Measurement of the individual nucleotides can thus not be substituted for by quantification of serotonin alone. Analysis of nucleotide secretion currently is mostly carried out by a bioluminescence assay [34]. The method is based on the consumption of free ATP by the enzyme luciferase which oxidizes its substrate luciferin to oxy-luciferin. The oxy-luciferin is initially present in an excited electronic state which decays under emission of visible light. At saturating concentrations of substrate the enzyme activity is only dependent on the supply of ATP. The area under curve of the luminescence signal is thus proportional to the amount of ATP available. The high sensitivity of luminescence detection and the use of PRP rendering pre-analytical procedures unnecessary are major advantages of this technique. However, only ATP can be detected directly; ADP can only be quantified after prior phosphorylation to ATP by creatine kinase and creatine phosphate. Likewise the robustness of the method is rather limited. Data quality is strongly affected by enzyme activity and substrate quality. Though prior calibration can partially compensate for these effects, sample components and the bio-matrix can also affect enzyme activity and by this means ATP quantification. In addition, luminescence detection is highly susceptible to substances quenching or absorbing the emitted light. Furthermore, loss of nucleotide due to degradation or re-uptake by the platelet may distort the results. Analogously to the quantification of serotonin with a radioactive tracer, also pre-loading of platelets with ^{14}C -adenine has been used for determination of nucleotide secretion [35]. Loading of platelets with appropriate tracers is, however, not only time-consuming but can also affect significantly platelet responsiveness due to effects on receptor functionality, and the tracer may itself affect platelet signal transduction. Serotonin and adenine can both act on platelet receptors or signaling pathways, either activating or desensitizing receptors, or modulating platelet responses to agonists.

HPLC separation is frequently used for nucleotide detection from various biomatrices [36, 37]. We have adapted the

Table 4. Comparison of detection methods for total platelet nucleotide content^a

Analyte	Method					
	HPLC/PuDP		¹⁴ C [39]		luciferase [18]	
	mean	% CV	mean	% CV	mean	% CV
ATP	8.39 ± 0.99	11.7	7.53 ± 3.03	27	5.80 ± 0.882	14.1
ADP	4.85 ± 1.68	34.7	4.69 ± 1.25	26.7	2.80 ± 0.50	17.9
AMP	0.64 ± 0.31	48.5	1.91 ± 0.84	44.0	n.d.	n.d.

^aConcentrations for HPLC analysis are given as means (n = 5) in nmol/10⁸ platelets ± standard deviation and coefficient of variance (% CV). Data from ¹⁴C-adenine tracer analysis are taken from [39], luciferase data from [18].

HPLC analysis of nucleotides to platelet secretion analysis, accounting for interferences by plasma components or drugs and potential re-uptake or degradation of nucleotides. By separating cells and cell debris from the medium through a diffusion barrier, any effect of cells or cellular components on the nucleotides in the medium is prevented. The addition of 6-Cl-PuDP, a biologically inactive compound with similar properties as the analyte, as internal standard enables reliable, absolute quantification (supplemental table 4). The method can compensate for reduced yield due to sample preparation and interferences by components or properties of the biomatrix. Drugs or other substances like metal ions which can strongly affect luminescence measurement and/or enzymatic activity do not interfere with the separation. The method can also be employed to determine nucleotide effects in presence of endogenous nucleotides by using biologically active ADP or ATP derivatives (fig. 4). By reducing the sample volume prior to the analysis, the assay sensitivity can be significantly increased. Furthermore, the small sample volume needed permits analysis even if only a minimum amount of blood is available, e.g., in animal experiments. The possibility to detect all kinds of nucleotides in parallel offers the option for additional application to platelet functional analysis. The method presented can provide all data necessary for the characterization of dense granule secretion. Quantification of total nucleotide content in relation to the amount of released nucleotide can provide evidence for either storage deficiency or impaired release. Time-resolved sampling with different agonists can hint specific signal transduction defects (fig. 3). Compared to published data, the method appears to work reliably and performs comparably good as radiotracer techniques (table 4).

The detection of functional defects in transgenic animals is increasingly relevant to improve our understanding of the role of a particular protein in signal transduction and the interaction of pathways. The low sample consumption of HPLC analysis allows a quantitative determination of nucleotide secretion from an individual animal, even repetitively (fig. 5, table 3).

Shortcomings and Enhancements

Though pre-analytical procedures have been limited to a minimum, still several pre-analytical steps have to be performed to make a sample fit for use in HPLC. By using more advanced equipment, the HPLC separation can be optimized with regard to time and efficiency. Using a derivatization reaction for nucleotides as described [38] could improve the sensitivity of the method significantly; however, with the disadvantage of losing the advantage of an internal standard. The use of WB samples has been shown [37]; however, with the drawback of additional sample treatment. Adaption of our technique to WB as matrix without elaborate pre-analytical procedures remains a challenge.

Acknowledgments

This work was supported by a grant of the German Ministry for Science and Education BMBF (MedSys project SARA 315395D).

Disclosure Statement

The authors declare no conflict of interest. The authors did not receive any financial support in addition to the project grant mentioned.

References

- King SM, McNamee RA, Hounig AK, Patel R, Brands M, Reed GL: Platelet dense-granule secretion plays a critical role in thrombosis and subsequent vascular remodeling in atherosclerotic mice. *Circulation* 2009;120:785–791.
- Ren Q, Ye S, Whiteheart SW: The platelet release reaction: just when you thought platelet secretion was simple. *Curr Opin Hematol* 2008;15:537–541.
- Blair P, Flaumenhaft R: Platelet alpha-granules: basic biology and clinical correlates. *Blood Rev* 2009;23:177–189.
- Morrissey JH: Polyphosphate multi-tasks. *J Thromb Haemost* 2012;10:2313–2314.
- Nurden AT, Freson K, Seligsohn U: Inherited platelet disorders. *Haemophilia* 2012;18(suppl 4): 154–160.
- Nurden A, Nurden P: Advances in our understanding of the molecular basis of disorders of platelet function. *J Thromb Haemost* 2011;9(suppl 1):76–91.
- Masliyah-Planchon J, Darnige L, Bellucci S: Molecular determinants of platelet delta storage pool deficiencies: an update. *Br J Haematol* 2013;160:5–11.

- 8 Podda G, Femia EA, Pugliano M, Cattaneo M: Congenital defects of platelet function. *Platelets* 2012;23:552–563.
- 9 Mezzano D, Quiroga T, Pereira J: The level of laboratory testing required for diagnosis or exclusion of a platelet function disorder using platelet aggregation and secretion assays. *Semin Thromb Hemost* 2009;35:242–254.
- 10 Tohgi H, Suzuki H, Tamura K, Kimura B: Platelet volume, aggregation, and adenosine triphosphate release in cerebral thrombosis. *Stroke* 1991;22:17–21.
- 11 van Ommen CH, Peters M: The bleeding child. Part I: primary hemostatic disorders. *Eur J Pediatr* 2012;171:1–10.
- 12 Freson K, Labarque V, Thys C, Wittevrongel C, Geet CV: What's new in using platelet research? To unravel thrombopathies and other human disorders. *Eur J Pediatr* 2007;166:1203–1210.
- 13 Koike K, Rao AK, Holmsen H, Mueller PS: Platelet secretion defect in patients with the attention deficit disorder and easy bruising. *Blood* 1984;63:427–433.
- 14 D'Andrea G, Hasselmark L, Alecci M, Cananzi A, Perini F, Welch KM: Platelet secretion from dense and alpha-granules in vitro in migraine with or without aura. *J Neurol Neurosurg Psychiatry* 1994;57:557–561.
- 15 Kappelmayer J, Nagy B Jr, Miszt-Biasius K, Hevessy Z, Setiadi H: The emerging value of P-selectin as a disease marker. *Clin Chem Lab Med* 2004;42:475–486.
- 16 Gachet C: P2 receptors, platelet function and pharmacological implications. *Thromb Haemost* 2008;99:466–472.
- 17 Murugappan S, Kunapuli SP: The role of ADP receptors in platelet function. *Front Biosci* 2006;11:1977–1986.
- 18 Cattaneo M, Canciani MT, Lecchi A, Kinlough-Rathbone RL, Packham MA, Mannucci PM, Mustard JF: Released adenosine diphosphate stabilizes thrombin-induced human platelet aggregates. *Blood* 1990;75:1081–1086.
- 19 Geiger J, Teichmann L, Grossmann R, Aktas B, Steigerwald U, Walter U, Schinzel R: Monitoring of clopidogrel action: comparison of methods. *Clin Chem* 2005;51:957–965.
- 20 Watson S, Daly M, Dawood B, Gissen P, Makris M, Mundell S, Wilde J, Mumford A: Phenotypic approaches to gene mapping in platelet function disorders – identification of new variant of P2Y12, TxA2 and GPVI receptors. *Haemostaseologie* 2010;30:29–38.
- 21 Mindukshev I, Gambaryan S, Kehrer L, Schuetz C, Kobsar A, Rukoyatkina N, Nikolaev VO, Krivchenko A, Watson SP, Walter U, Geiger J: Low angle light scattering analysis: a novel quantitative method for functional characterization of human and murine platelet receptors. *Clin Chem Lab Med* 2012;50:1253–1262.
- 22 Holmsen H, Dangelmaier CA: Measurement of secretion of serotonin. *Methods Enzymol* 1989;169:205–210.
- 23 Aktas B, Utz A, Hoenig-Liedl P, Walter U, Geiger J: Dipyridamole enhances NO/cGMP-mediated vasodilator-stimulated phosphoprotein phosphorylation and signaling in human platelets: in vitro and in vivo/ex vivo studies. *Stroke* 2003;34:764–769.
- 24 Geiger J, Brandmann T, Hubertus K, Tjahjedi B, Schinzel R, Walter U: A protein phosphorylation-based assay for screening and monitoring of drugs modulating cyclic nucleotide pathways. *Anal Biochem* 2010;407:261–269.
- 25 Rukoyatkina N, Walter U, Friebe A, Gambaryan S: Differentiation of cGMP-dependent and -independent nitric oxide effects on platelet apoptosis and reactive oxygen species production using platelets lacking soluble guanylyl cyclase. *Thromb Haemost* 2011;106:922–933.
- 26 Geiger J, Hönig-Liedl P, Schanzenbächer P, Walter U: Ligand specificity and ticlopidine effects distinguish three human platelet ADP receptors. *Eur J Pharmacol* 1998;351:235–246.
- 27 Meyers KM, Holmsen H, Seachord CL: Comparative study of platelet dense granule constituents. *Am J Physiol* 1982;243:R454–461.
- 28 Schmitt A, Guichard J, Massé JM, Debili N, Cramer EM: Of mice and men: comparison of the ultrastructure of megakaryocytes and platelets. *Exp Hematol* 2001;29:1295–1302.
- 29 Jones CI, Barrett NE, Moraes LA, Gibbins JM, Jackson DE: Endogenous inhibitory mechanisms and the regulation of platelet function. *Methods Mol Biol* 2012;788:341–366.
- 30 Israels S J, McNicol A, Robertson C, Gerrard JM: Platelet storage pool deficiency: diagnosis in patients with prolonged bleeding times and normal platelet aggregation. *Br J Haematol* 1990;75:118–121.
- 31 Kumar AM, Kumar M, Deepika K, Fernandez JB, Eisdorfer C: A modified HPLC technique for simultaneous measurement of 5-hydroxytryptamine and 5-hydroxyindoleacetic acid in cerebrospinal fluid, platelet and plasma. *Life Sci* 1990;47:1751–1759.
- 32 Koch CA, Lasho TL, Tefferi A: Platelet-rich plasma serotonin levels in chronic myeloproliferative disorders: evaluation of diagnostic use and comparison with the neutrophil PRV-1 assay. *Br J Haematol* 2004;127:34–39.
- 33 Wall JE, Buijs-Wilts M, Arnold JT, Wang W, White MM, Jennings LK, Jackson CW: A flow cytometric assay using mepacrine for study of uptake and release of platelet dense granule contents. *Br J Haematol* 1995;89:380–385.
- 34 Higashi T, Isomoto A, Tyuma I, Kakishita E, Uomoto M, Nagai K: Quantitative and continuous analysis of ATP release from blood platelets with firefly luciferase luminescence. *Thromb Haemost* 1985;53:65–69.
- 35 Edenbrandt CM, Murphy S: Adenine and guanine nucleotide metabolism during platelet storage at 22 degrees C. *Blood* 1990;76:1884–1892.
- 36 Childs KF, Ning XH, Bolling SF: Simultaneous detection of nucleotides, nucleosides and oxidative metabolites in myocardial biopsies. *J Chromatogr B Biomed Appl* 1996;678:181–186.
- 37 Coolen EJ, Arts IC, Swennen EL, Bast A, Stuart MA, Dagnelie PC: Simultaneous determination of adenosine triphosphate and its metabolites in human whole blood by RP-HPLC and UV-detection. *J Chromatogr B Analyt Technol Biomed Life Sci* 2008;864:43–51.
- 38 Bhatt DP, Chen X, Geiger JD, Rosenberger TA: A sensitive HPLC-based method to quantify adenine nucleotides in primary astrocyte cell cultures. *J Chromatogr B Analyt Technol Biomed Life Sci* 2012;889–890:110–115.
- 39 Bayer SM, McCoy EE: A comparison of the serotonin and ATP content in platelets from subjects with Down's syndrome. *Biochem Med* 1974;9:225–232.