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Brief reports

Melatonin is not stored in platelets

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ABSTRACT

Background: Melatonin is one of numerous biologically active compounds reported to be stored in platelets. As melatonin is increasingly linked to several diseases, we wanted to confirm the storage of melatonin in platelets using sensitive liquid chromatography in combination with isotope dilution tandem mass spectrometry (LC-MS/MS). The difference between melatonin levels analyzed in platelet-rich plasma (PRP) and platelet-poor plasma (PPP) served as proxy for platelet levels of melatonin.
Methods: Melatonin concentrations were analyzed in PRP and PPP from nineteen healthy volunteers by ELISA and LC-MS/MS. A Wilcoxon signed-rank test was performed to assess if the melatonin levels measured in PRP and PPP were different. Results for melatonin concentrations obtained by LC-MS/MS or ELISA were compared using Passing-Bablok regression.
Results: Comparison of the ELISA with the LC-MS/MS method showed poor agreement for melatonin concentrations in PRP and PPP. No indication was found for storage of melatonin in platelets by either LC-MS/MS or ELISA ($P = .89$ and $P = .53$ for the LC-MS/MS and ELISA analysis, respectively).
Conclusion: In this study we could find no evidence for melatonin storage in platelets.

1. Introduction

Over 300 bioactive components have been detected in platelet releasates [1,2]. Once secreted these bioactive components act in an autocrine or paracrine way to modulate cell signaling.

One of these bioactive components was considered to be melatonin [3]. This is of relevance given the increasing interest in the potential roles of melatonin in the human body. It is already known that melatonin is a regulator of the circadian rhythm. Deregulation of the circadian rhythm and of circulating melatonin levels are associated with increased incidence of cancer, neurological, cardiovascular and metabolic diseases, and epigenetic abnormalities [4,5].

The finding of platelet melatonin was based on an ELISA assay [3]. We developed a refined method for melatonin detection to facilitate detailed melatonin research [6]. Therefore, we also wanted to confirm the storage of melatonin in platelets using a sensitive liquid chromatography in combination with isotope dilution tandem mass spectrometry (LC-MS/MS). Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) served as proxy for platelet levels of melatonin, because isolation of platelets is prone to errors [7].

2. Material and methods

Twenty healthy volunteers over 18 years of age were included. All gave written informed consent. The study was waived by the Medical Ethical Review Committee of the University Medical Center Groningen. Blood (two 10 mL EDTA tubes per volunteer) was drawn at 8:00 AM using a butterfly needle. Blood was immediately processed to minimise platelet activation [7]. To obtain PRP, one EDTA tube per volunteer was centrifuged at 120*g* for 30 min at room temperature. Of this, 500 µL served to determine the platelet count with Sysmex XE-2100. The other EDTA tube was centrifuged at 2500*g* for 15 min at room temperature to obtain PPP. All samples were stored at −80 °C until analysis. As previously shown, the freezing process results in complete fragmentation of platelets, thereby liberating cell constituents such as serotonin from its storage granules.

Melatonin concentrations in PRP and PPP from all participants were analyzed using LC-MS/MS and ELISA [6]. The LC-MS/MS assay was performed as previously described [6]. In short, 200 µL of PRP of PPP was pipetted into a 2.0 mL 96-deep well plate. To this, 50 µL of internal standard solution was added, and the plate was vortex mixed for 1 min. Proteins were precipitated by adding 200 µL of 0.3 M zinc sulfate in methanol (1:5 *v*/v) and the plate was vortexed again for 1 min. Water

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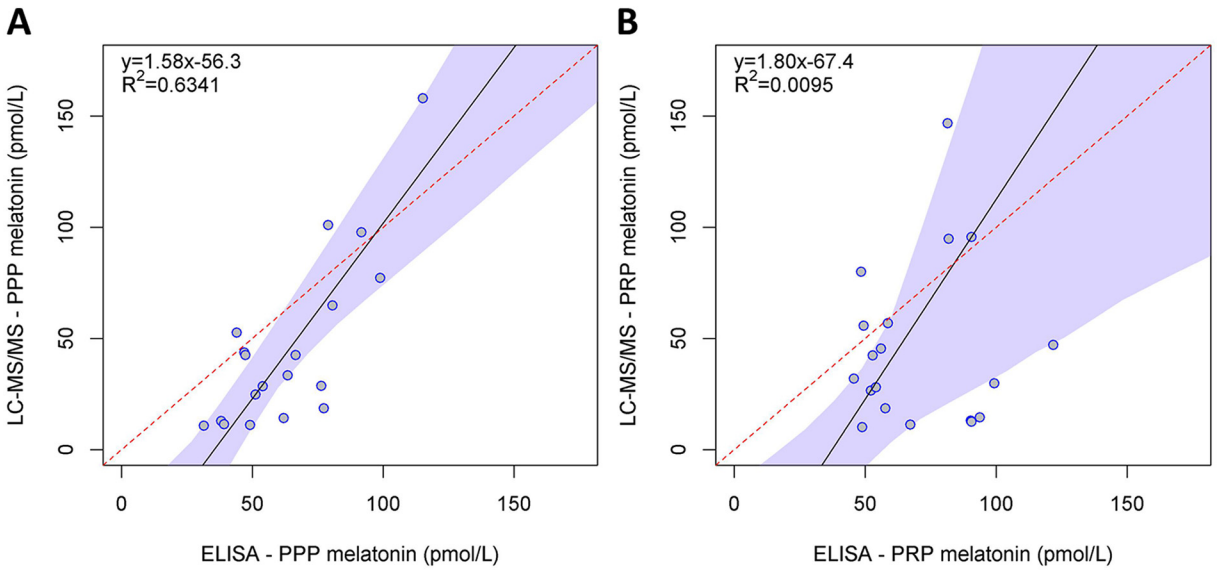

Fig. 1. Scatter plots for the method comparison of platelet poor plasma (PPP) melatonin concentrations analyzed with enzyme-linked immunosorbent assay (ELISA) on the x-axis versus analyzed with isotope dilution liquid chromatography combined with mass spectrometry (LC-MS/MS) on the y-axis (A) and platelet rich plasma (PRP) melatonin concentrations analyzed with ELISA on the x-axis versus analyzed with LC-MS/MS on the y-axis (B). Solid line represents the Passing-Bablok regression line. Dashed lines represent line of identity. Shaded area is the 95% confidence interval.

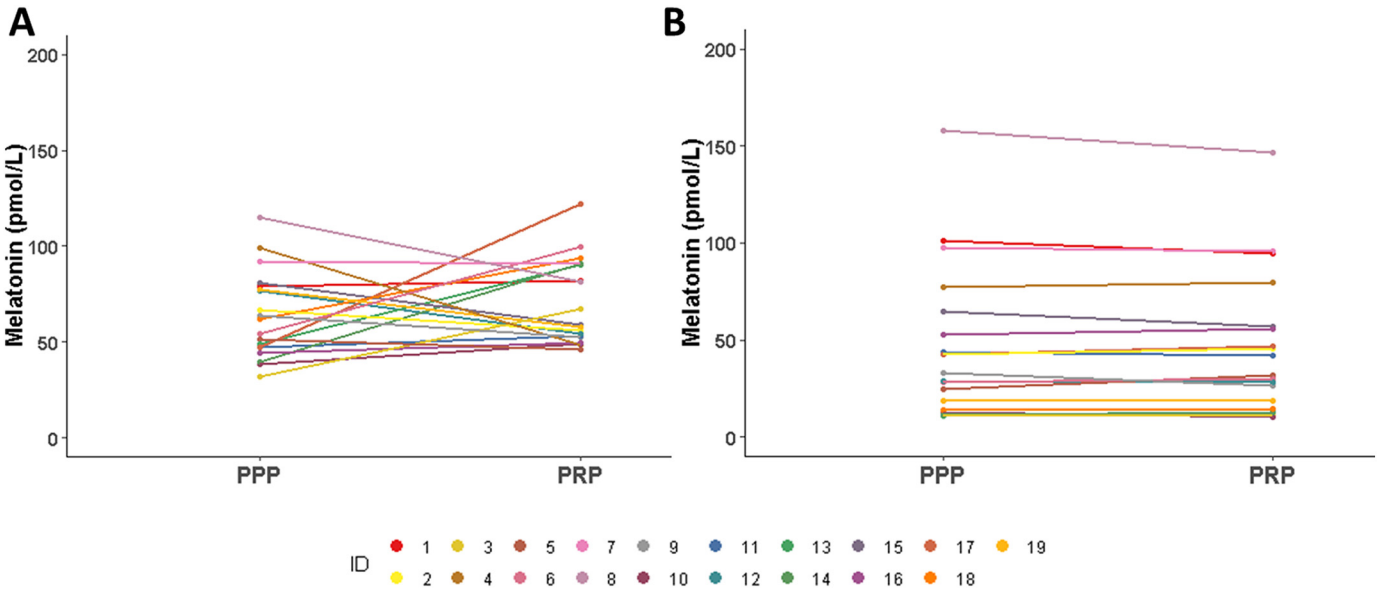

Fig. 2. Pairwise comparison of melatonin levels in platelet poor plasma (PPP) and platelet rich plasma (PRP) in 19 participants measured by ELISA (A) and LC-MS/MS (B).

was added to fill up to 1.0 mL and the plate was sealed and centrifuged for 15 min at 2500 ×*g*. Following centrifugation, the plate was transferred to the autosampler and 100 µL of the supernatant was injected for online solid phase extraction (SPE) and LC-MS/MS analysis. The ELISA was performed with extraction (in duplicate performed) as used in the original report (catalogue number RE54021, IBL International, Hamburg, Germany). The only difference with the original report was that we used solid phase extraction (SPE) instead of chloroform extraction [3]. The SPE procedure for the ELISA was performed with a vacuum manifold as follows: SPE columns were conditioned with 1.0 of methanol, equilibrated with 1.0 mL of destilled water, followed by application of 0.5 mL of sample and standards. Subsequently, 0.5 mL of destilled water was applied. The columns were was washed by 2 × 1.0 mL of 10% methanol in destilled water (*v*/v). Elution of melatonin was performed with 1.0 mL of methanol. Extract was evaporated under nitrogen and reconstituted with 0.15 mL of destilled water. The tubes were vortexed for one minute and 50 µL of the extracted samples and standards were pipetted into the respective wells of the microtiter plate and the ELISA procedure as described by the vendor was followed. *N*-acetylserotonin in PRP and PPP was analyzed by LC-MS/MS, using n-acetlyserotonin-D4 (SynInnova, Edmonton, Canada) as stable isotope labeled internal standard, essentially as described for dopamine [8]. The limit of detection for melatonin by LC-MS/MS was 3.3 pmol/L, for *N*-acetylserotonin 1.3 pmol/L. LOQ's were 10 pmol/L and 4 pmol/L, respectively) The melatonin ELISA reported a limit of detection of 7 pmol/L. If melatonin is stored in platelets, melatonin levels in PRP are expected to be higher than melatonin levels in PPP. A Wilcoxon signed-rank test served to evaluate if the melatonin levels in PRP and PPP were different, when measured by LC-MS/MS or ELISA. The relationship between melatonin concentrations in PRP and PPP for both detection

Table 1
Characteristics of 19 healthy individuals and their melatonin levels in PPP and PRP.

ID	Age (years)	Gender (F/M)	Platelets (10^9/L)	Melatonin* LC-MS/MS		Melatonin* ELISA		N-Acetylserotonin* LC-MS/MS	
				PPP	PRP	PPP	PRP	PPP	PRP
1	60	F	483	101	94.8	79.1	81.9	20.0	25.7
2	61	M	435	42.4	45.4	66.7	56.1	106.0	112.8
3	62	M	410	10.6	11.2	31.6	67.3	8.0	11.4
4	28	F	555	77.1	79.9	99.0	48.5	16.0	15.7
5	36	M	447	24.7	31.9	51.3	45.8	10.0	11.2
6	30	F	588	28.4	29.8	54.0	99.4	29.0	41.8
7	30	F	612	97.7	95.7	91.8	90.6	20.0	11.7
8	24	M	525	158	147	115	81.6	14.0	13.9
9	29	M	549	33.3	26.6	63.6	52.2	21.0	20.9
10	58	F	502	12.8	10.1	38.2	48.9	< 4.0	< 4.0
11	53	F	617	43.6	42.2	47.1	52.9	59.0	52.4
12	33	M	510	28.6	28.0	76.4	54.2	76.0	66.5
13	59	M	430	11.0	12.9	49.2	90.4	26.0	22.3
14	40	F	463	11.3	12.4	39.4	90.6	6.0	< 4.0
15	27	F	693	64.7	56.8	80.7	58.7	29.0	23.5
16	26	F	458	52.5	55.6	44.1	49.5	4.0	9.7
17	54	M	455	42.4	47.0	47.4	121.9	7.0	7.6
18	58	M	443	14.1	14.5	62.1	93.8	< 4.0	5.7
19	39	F	452	18.6	18.5	77.4	57.8	15.0	14.5
Median [IQR]	39 [29, 58]		483 [447, 555]	33 [14, 65]	32 [15, 57]	62 [47, 79]	59 [52, 91]	16 [7, 29]	14.5 [10, 26]

* Concentration in pmol/L. ELISA, enzyme-linked immunosorbent assay; IQR, interquartile range; PPP, platelet-poor plasma; PRP, platelet-rich plasma; LC-MS/MS, liquid chromatography tandem mass spectrometry.

techniques as well as the comparison of LC-MS/MS with ELISA was evaluated using Passing-Bablok regression. Graphpad Prism 5 (GraphPad Software, CA, USA) and Analyse-it (Analyse-it Software, Ltd., Leeds, United Kingdom) were used for statistical analyses. $P < .05$ was considered statistically significant. Sufficient sample was available of 19 participants,10 females with mean age 39.1 years, and 9 males, mean age 46.2 years.

3. Results

Comparison of the LC-MS/MS and the ELISA method showed poor agreement for melatonin concentrations in PRP (slope = 2.35 [0.65 to18]; 95% CI, intercept = −100 [−1015 to −7.3]; 95% CI) and in PPP (slope = 1.68 [1.04–2.71]; 95% CI, intercept = −61 [−123 to −27]; 95% CI) (Fig. 1).

With both techniques, we found no evidence for storage of melatonin in platelets, as no difference between PRP and PPP was detected using the Wilcoxon signed rank test, $P = .89$ and $P = .53$ for the LC-MS/MS and ELISA analysis, respectively (Fig. 2A, B, and Table 1). The Passing-Bablok regression analysis showed discordant results for the two methods: the comparison of the melatonin concentrations in PRP and PPP analyzed with LC-MS/MS showed excellent agreement (slope = 0.95 [0.91 to 1.1]; 95% CI; intercept = 1.1 [−0.88 to 3.3]; 95% CI), whereas the ELISA results showed considerable scatter (slope = 0.71 [0.27 to 3.8]; 95% confidence interval (CI); intercept = 20 [−132 to 46]; 95% CI) (Fig. 3). There was no difference between *n*-acetylserotonin levels measured in PRP or PPP using the Wilcoxon signed rank test, $P = .977$ (Table 1).

4. Discussion and conclusion

These results show that indeed plasma contains melatonin, but melatonin is not stored in platelets.

Our results are in contrast with a study where the same ELISA was used to demonstrate presence of melatonin in platelets of only three healthy individuals [3]. This difference can be explained by the following differences, first we studied a larger group of twenty subjects. Secondly, we used the difference between PRP and PPP as proxy for platelets. Isolation of platelets is prone to errors, as it is known that platelets are easily activated, potentially leading to (partial) degranulation [7]. In previous studies mRNA of melatonin synthesizing enzymes was found in platelets [9,10]. These studies from the same research group using the same radiometric enzyme assays, showed the presence of melatonin synthesizing enzyme activities, as well as *n*-acetylserotonin in platelets [9,10]. As radiometric enzyme assays can suffer from cross-reactivity, this might explain the observed discrepancy to our finding that no *n*-acetylserotonin is present in platelets [11]. Our observation is strengthened by proteome and transcriptome studies indicating that melatonin synthesizing enzymes are not present in platelets [12].

Melatonin concentrations measured were different between ELISA and LC-MS/MS. Immunoassays can be hampered by interferences like cross-reactivity, matrix effects and differences in binding protein concentrations, predominantly for low molecular weight analytes like melatonin [13,14]. As biogenic amines are stored in platelets in high concentrations, a low percentage of cross-reactivity can have a large effect on melatonin concentrations measured. Immunoassays are more prone to suffer from matrix differences, e.g. PRP versus PPP, than LC-MS/MS assays, as the latter use stable isotope labeled internal standards and extensive sample clean-up. The ELISA used in the original report and in this study was specifically designed for analysis of plasma (PPP) and/or serum, and not for PRP or platelets. The results here obtained show that when immunoassays are used in matrices different than they were designed for, critical appraisal of the results is necessary. In this study we show that the risk of interferences mentioned for immunoassays can be reduced by using mass spectrometry based methods, which are currently being introduced in the routine clinical laboratory for the analysis of biogenic amines, steroids, and vitamins [15,16].

In conclusion, we could find no evidence for melatonin storage in platelets. This observation is of importance, as it changes assumptions regarding platelet melatonin concentrations fundamentally. For future studies concerning analysis of melatonin, it must be ensured that well validated methods are used.

Author contributions

Study concept and design IK, SO, EdV, acquisition, analysis, and interpretation of data MvF, MP; drafting and critical revision of the manuscript MvF, MP, AW, EdV, SO, IK.

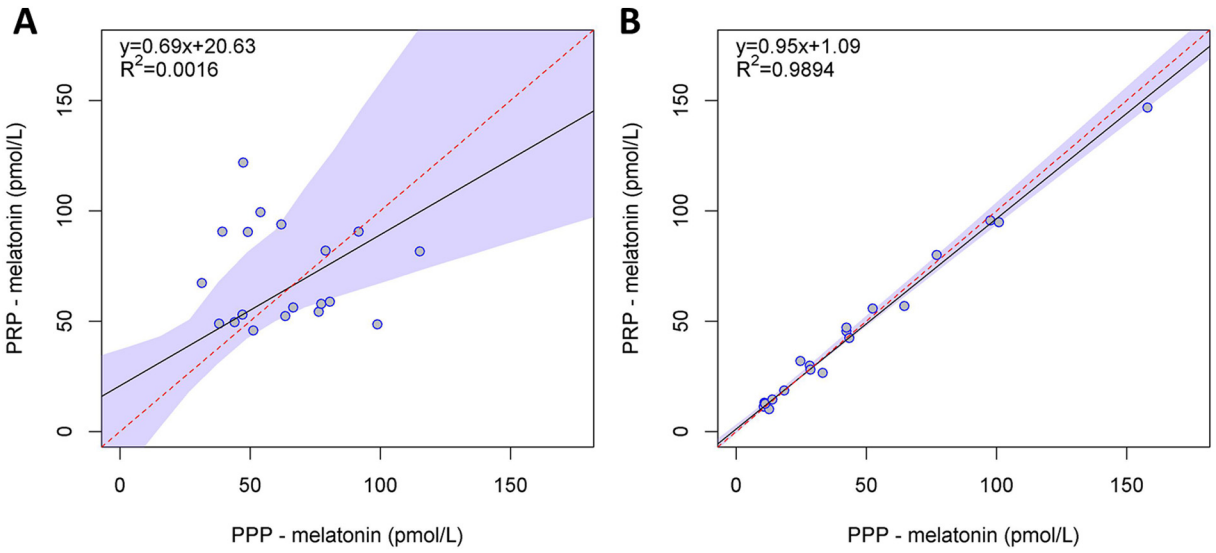

Fig. 3. Scatterplot of the comparison of melatonin levels in PPP (x-axis) versus melatonin levels in PRP (y-axis) analyzed by ELISA (A) or LC-MS/MS (B). Solid line represents the Passing-Bablok regression line, and the dashed line represents the line of identity. Shaded area is the 95% confidence interval.

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