

Optimization and Validation of a LC-MS/MS Method for Peripheral Serotonin (5-HT) as a Pharmacodynamic Biomarker of Treatment with Tryptophan Hydroxylase Inhibitors

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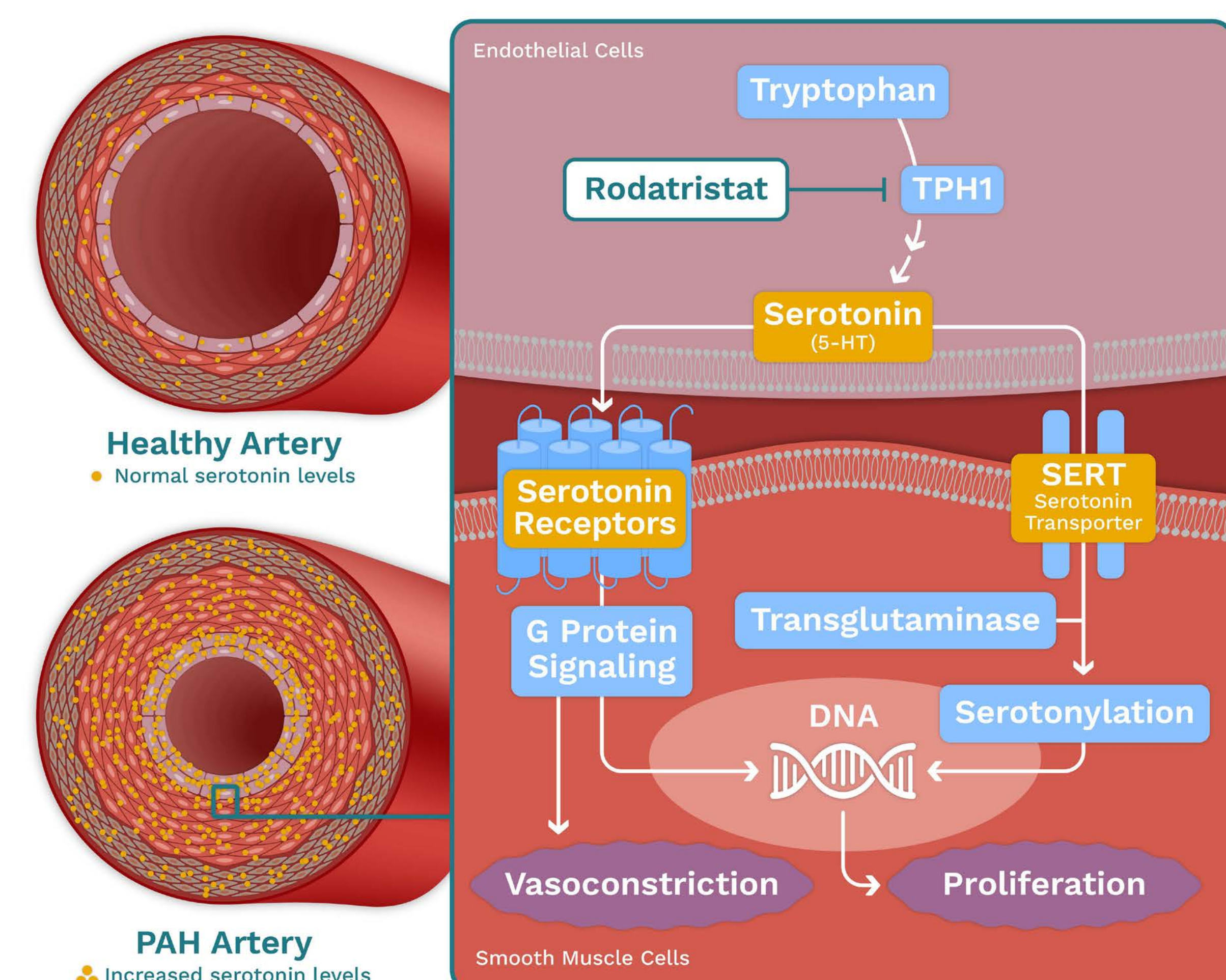
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Background

Pulmonary arterial hypertension (PAH) is a progressive disorder with a median survival <40% at 5 years in advanced patients and is characterized by increased pulmonary vascular resistance, remodeling and occlusion. While the etiology is unknown, nonclinical and clinical data implicate a causative role for serotonin (5-HT). While best known as a neurotransmitter, 5-HT is also produced outside of the central nervous system in the GI tract, lungs, blood vessels, and other organs. In the periphery 5-HT is produced from tryptophan via a biosynthetic pathway in which conversion of tryptophan to 5-hydroxytryptophan by tryptophan hydroxylase 1 (TPH1) is the rate-limiting step.

Role of Serotonin in Pulmonary Arterial Hypertension



Strong evidence that serotonin plays an important role in the development of PAH

Increased locally-produced and platelet-derived serotonin contributes to the development of PAH → Excess serotonin stimulates thickening and constriction of the pulmonary blood vessels by acting through serotonin receptors and transporters → Pulmonary arterial pressure increases and leads to stress on the right ventricle and ultimately heart failure

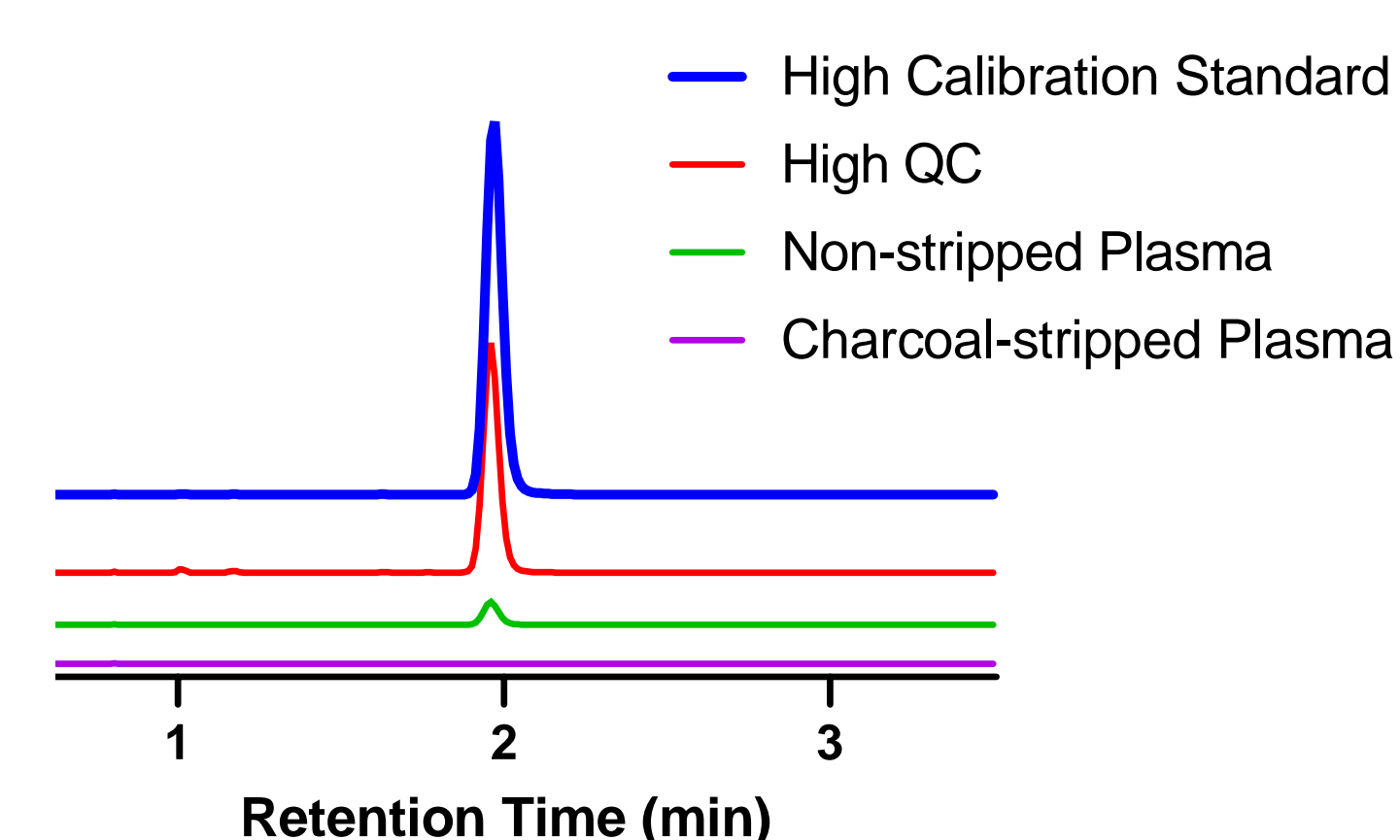
Altavant Sciences is currently developing rodatristat ethyl, an oral prodrug of rodatristat, as an inhibitor of tryptophan hydroxylase to reduce the peripheral concentration of 5-HT and impact the entire 5-HT system. Bioanalytical methods for quantifying 5-HT were required to investigate the role of 5-HT in disease progression and treatment, and to support nonclinical safety studies. Measurement of peripheral 5-HT production is complicated by analyte instability, partitioning between plasma and platelets, low endogenous concentrations in plasma, and platelet rupture during sample collection and processing.

Quantification of 5-HT in clinical blood samples is important for understanding the role of 5-HT in PAH disease progression and potential treatment response. Suitable methods for quantification of 5-HT in a clinical setting need to be developed.

Clinical Analytical Method

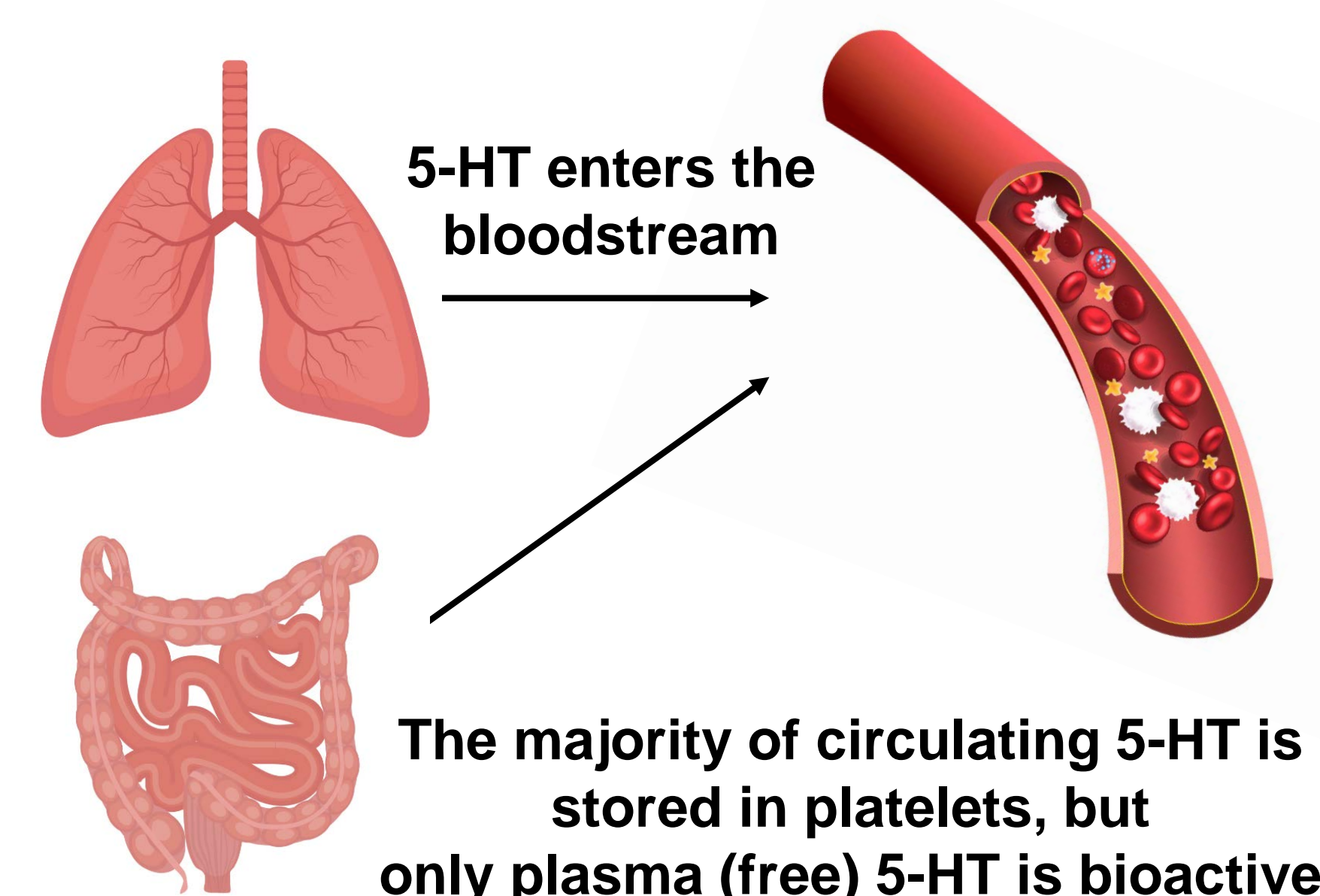
- This method was validated according to 21 CFR 58, GLPs for serotonin (5-HT) over the concentration range of 1-400 ng/mL in Na₂ EDTA/NaF human plasma.
- Plasma samples were treated with ascorbic acid solution preservative. Deuterated 5-HT internal standard was added before protein precipitation with 2M perchloric acid.
- Samples were analyzed on a Sciex™ API 6500 LC-MS/MS with positive-mode electrospray ionization (turboionspray for desolvation).
- Chromatography: ACE 3 C18-PFP, 50 x 4.6 mm, 3 μm column, Milli-Q water/methanol (85/15) with ammonium acetate and acetic acid mobile phase.
- Calibration standards were prepared in charcoal-stripped human plasma (NaF/Na₂ EDTA).
- To measure concentrations below endogenous levels, low level QCs were prepared in charcoal-stripped human plasma. Mid and high QCs were prepared in plasma with added 5-HT.

Measured Concentration of 5-HT (ng/mL)				
	LLLQC 1.00 ng/mL	QC1 3.00 ng/mL	QC2 200.00 ng/mL	QC3 300.00 ng/mL
N	18	18	18	18
Mean	1.014	3.011	198.894	298.156
SD (±)	0.0450	0.0892	5.2044	5.1855
CV (%)	3.45	2.96	2.62	1.74
% Bias	1.39	0.37	-0.55	-0.61

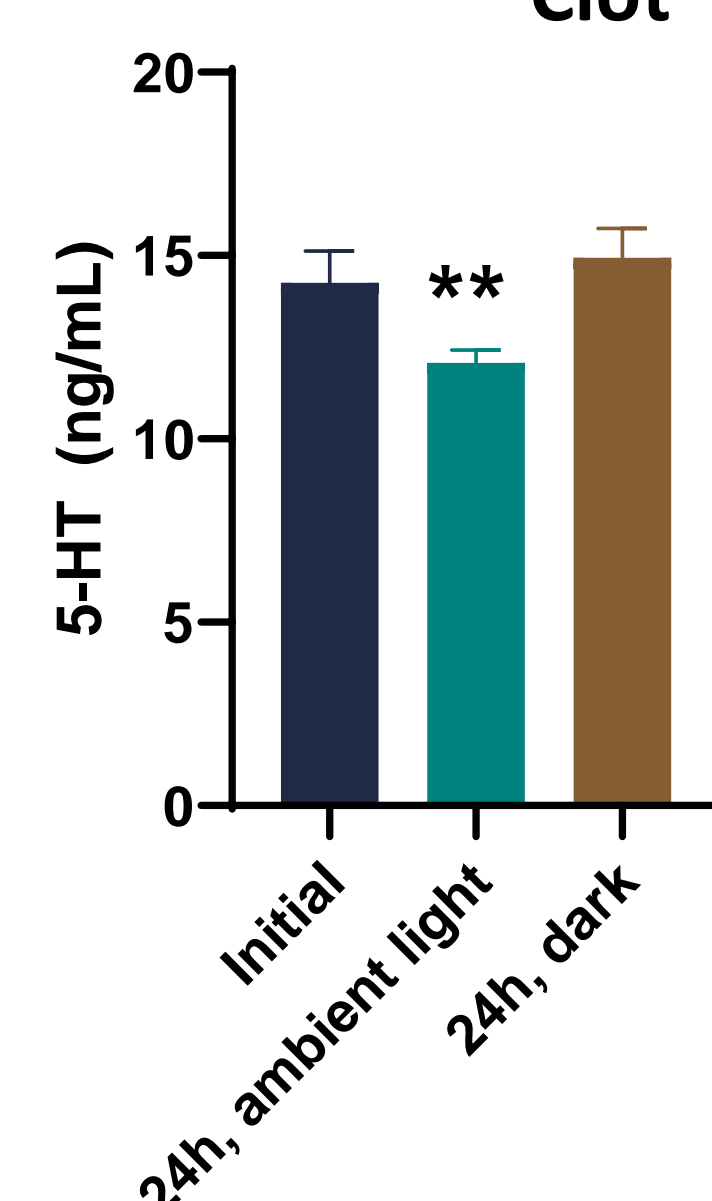


Challenge of Platelet Contamination in 5-HT Measurement

- Peripheral serotonin (5-HT) is produced primarily in enterochromaffin cells in the GI tract.
- Circulating 5-HT is rapidly taken up by and stored in platelets. Plasma concentrations of free 5-HT are comparatively low.
- 5-HT is released from platelets upon activation to regulate processes such as blood clotting and immune responses.

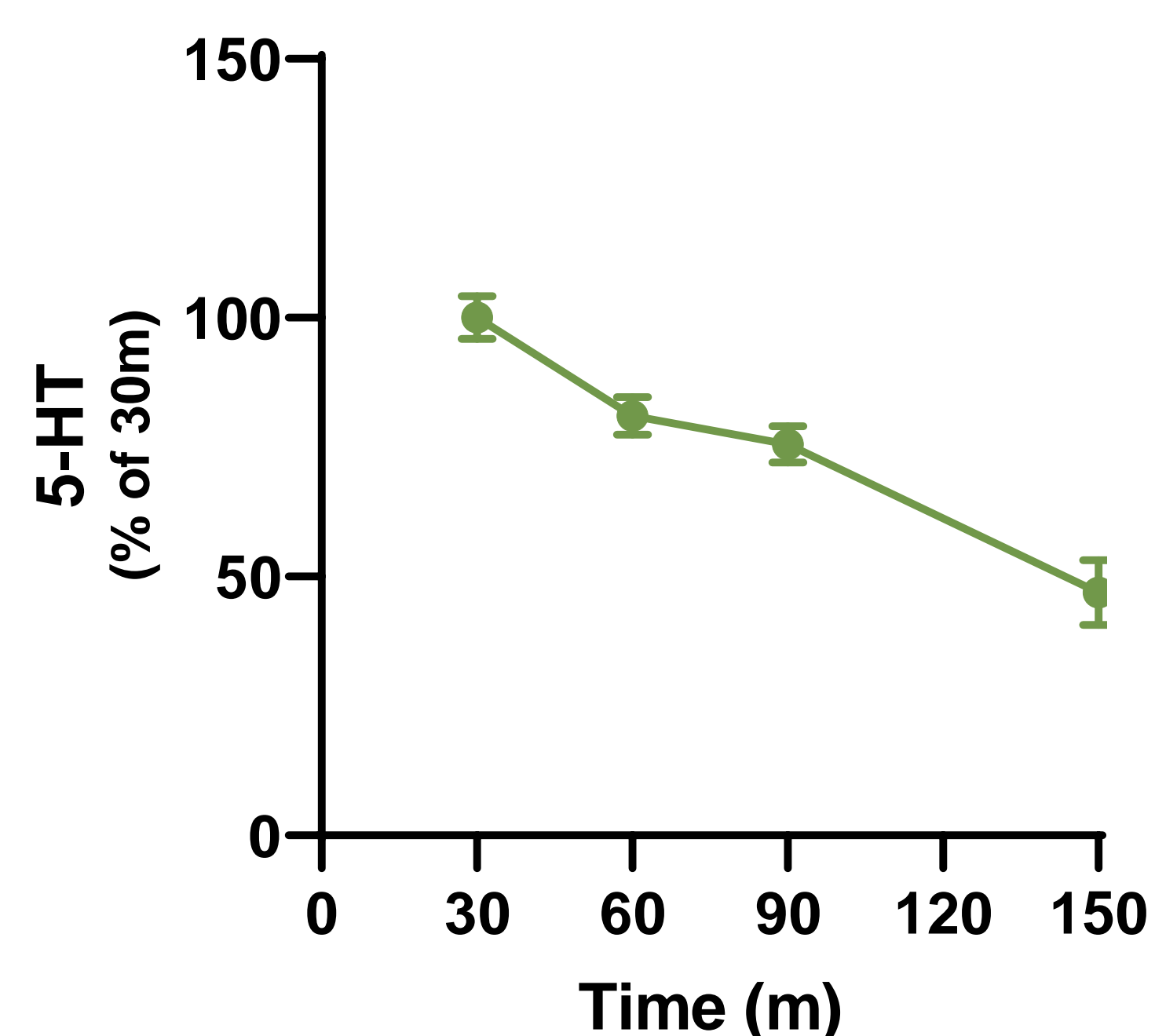


Serum 5-HT Stability in Serum without Blood Clot



- Freshly collected blood was allowed to clot on the benchtop for 60 minutes, centrifuged, and the serum was separated from the clot for immediate assay and stored overnight before re-assay.
- 5-HT in serum collected this way was stable for 24 hours when stored protected from light at room temperature. Under ambient laboratory lighting, 5-HT concentrations decreased approximately 15% ($P < .005$, two-tailed student t-test).

5-HT Stability in Serum with Blood Clot

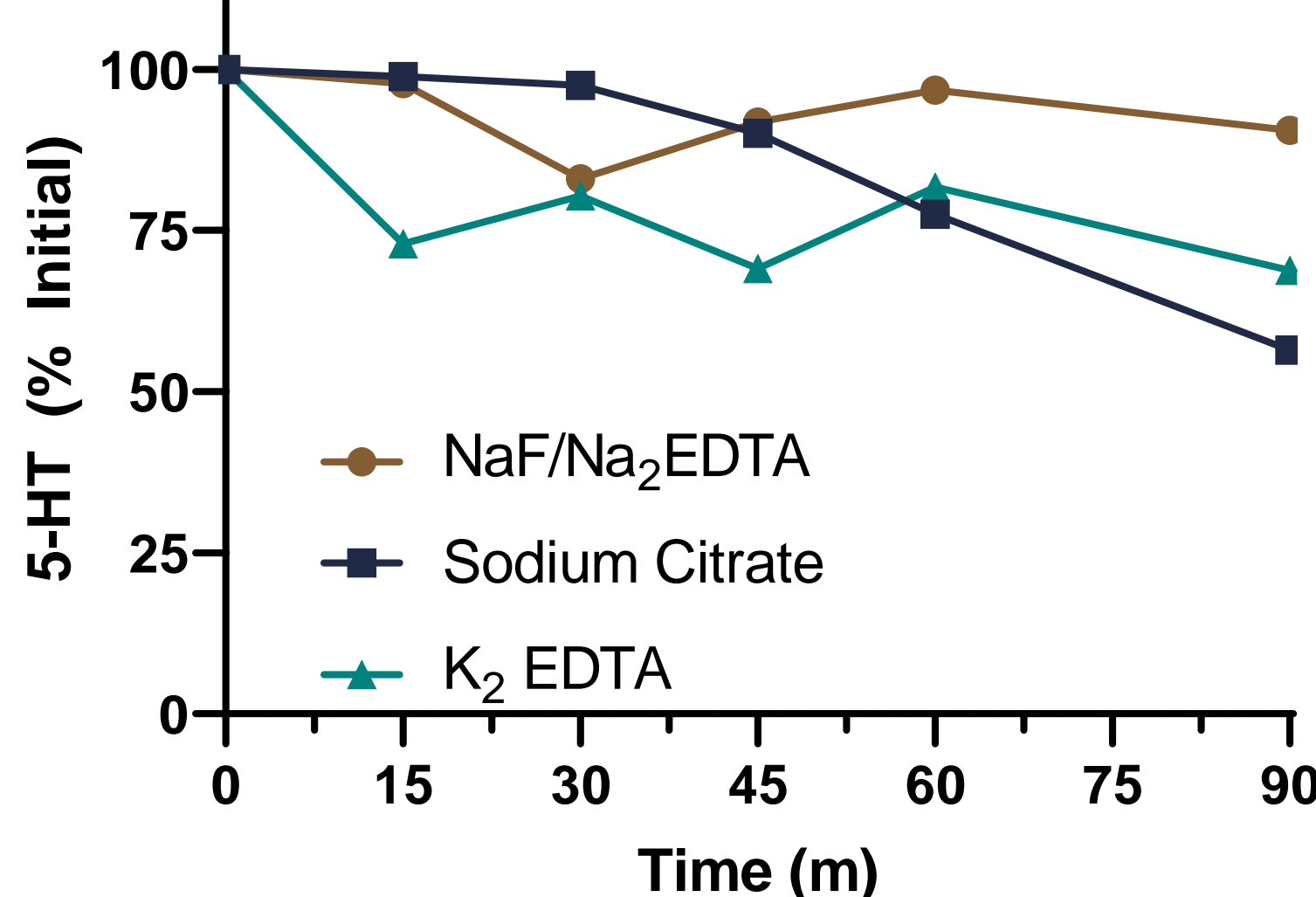


- Freshly drawn whole blood was aliquoted into serum tubes and allowed to clot for 30, 60, 90 or 150 minutes at room temperature prior to centrifugation and analysis.
- Measured 5-HT concentrations decreased as coagulation time increased. Data have been normalized to the initial timepoint (30 minutes of coagulation).
- This instability makes serum a poor choice of matrix for 5-HT measurement in a clinical setting where coagulation times may vary.
- The cause of this instability is likely oxidation mediated by oxyhemoglobin.

While 5-HT is stable for 24 hours in serum that has been separated from the blood clot and protected from light, changes in serum 5-HT concentration during clotting make serum a poor choice of matrix for 5-HT measurements in a clinical setting.

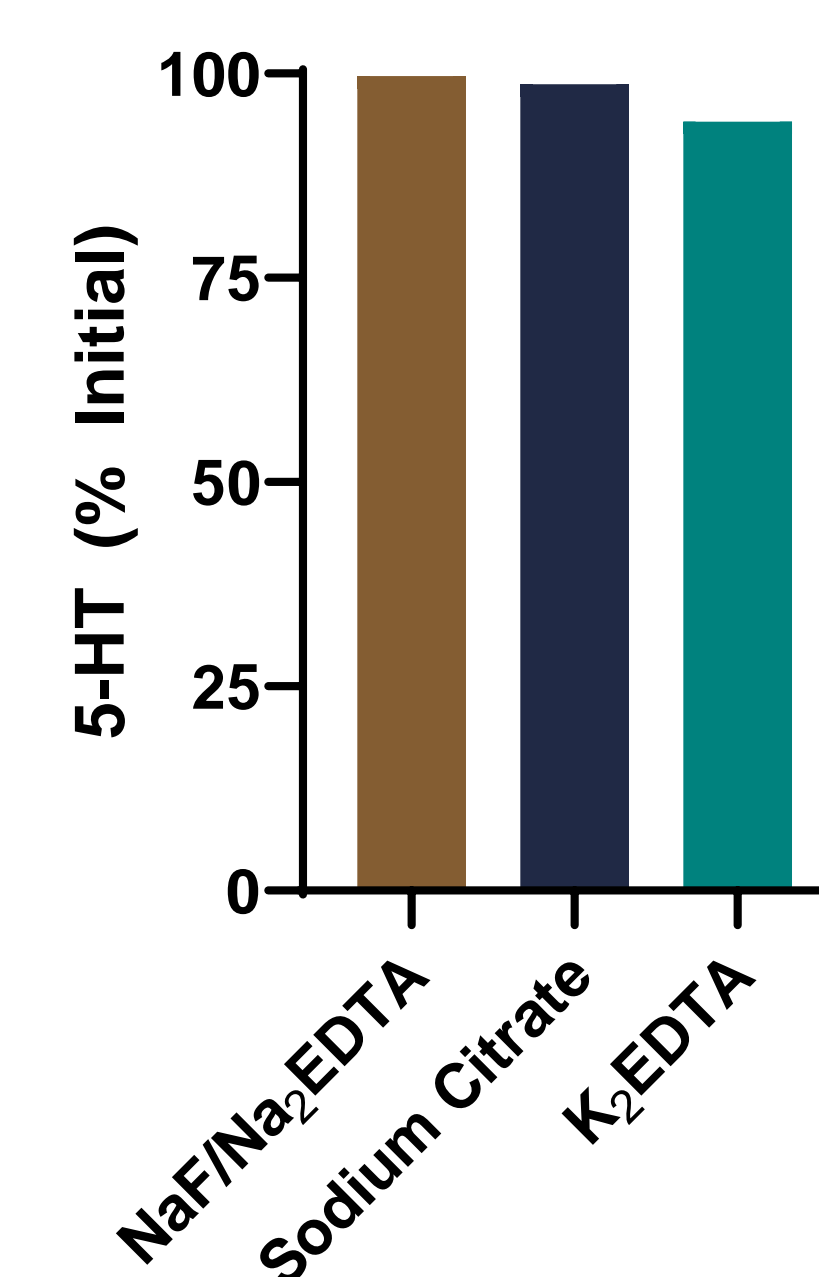
Plasma and the role of anticoagulant

5-HT Stability in Whole Blood



- 5-HT stability in whole blood was investigated to determine if samples could be stabilized prior to centrifugation.
- Freshly drawn whole blood was kept on ice for 15, 30, 24, 60, or 90 minutes before being quenched with acetonitrile and analyzed for 5-HT.
- NaF/Na₂EDTA treated blood was most stable with 90% 5-HT remaining at 90 min.

5-HT Stability in Plasma

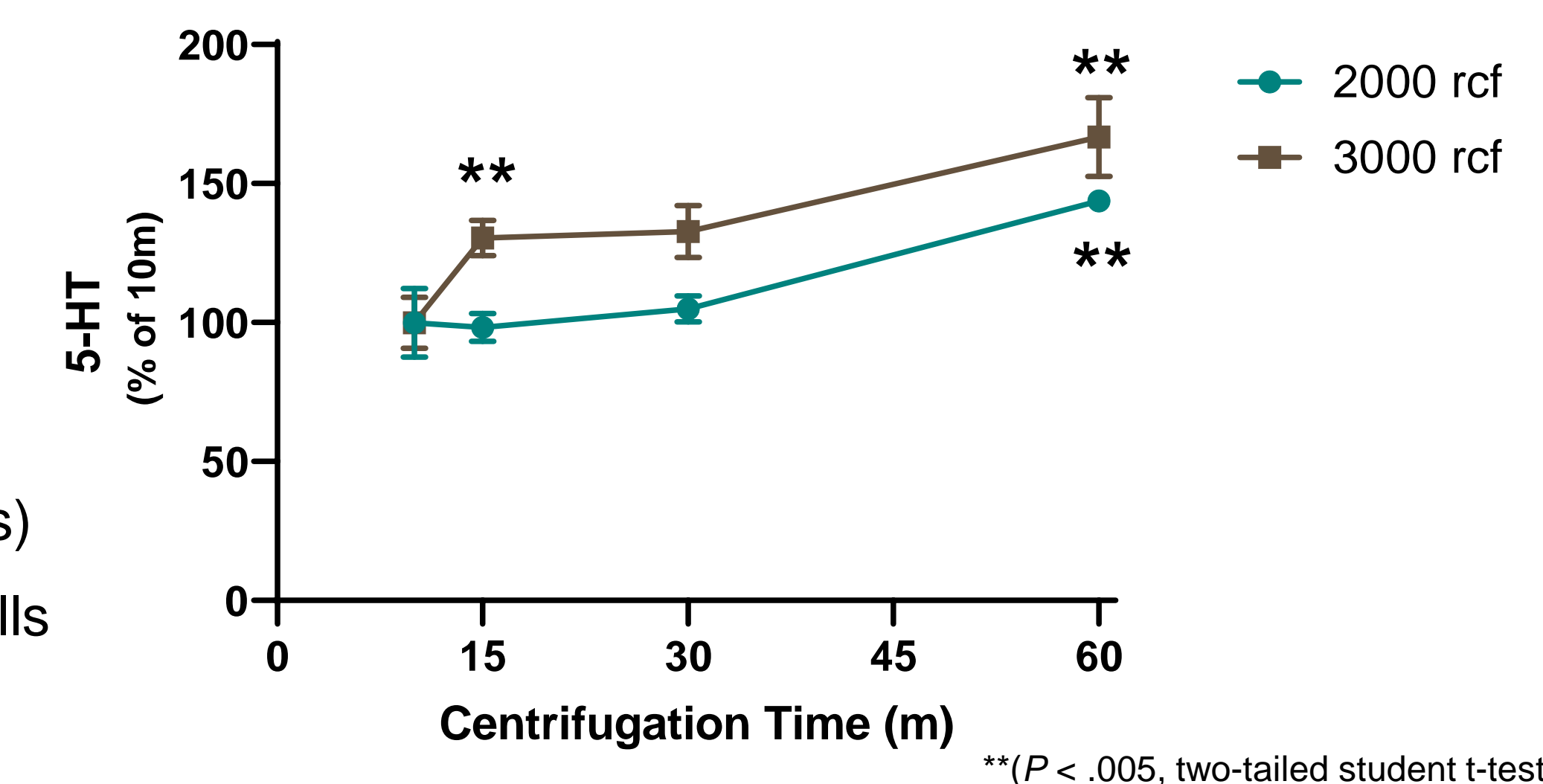
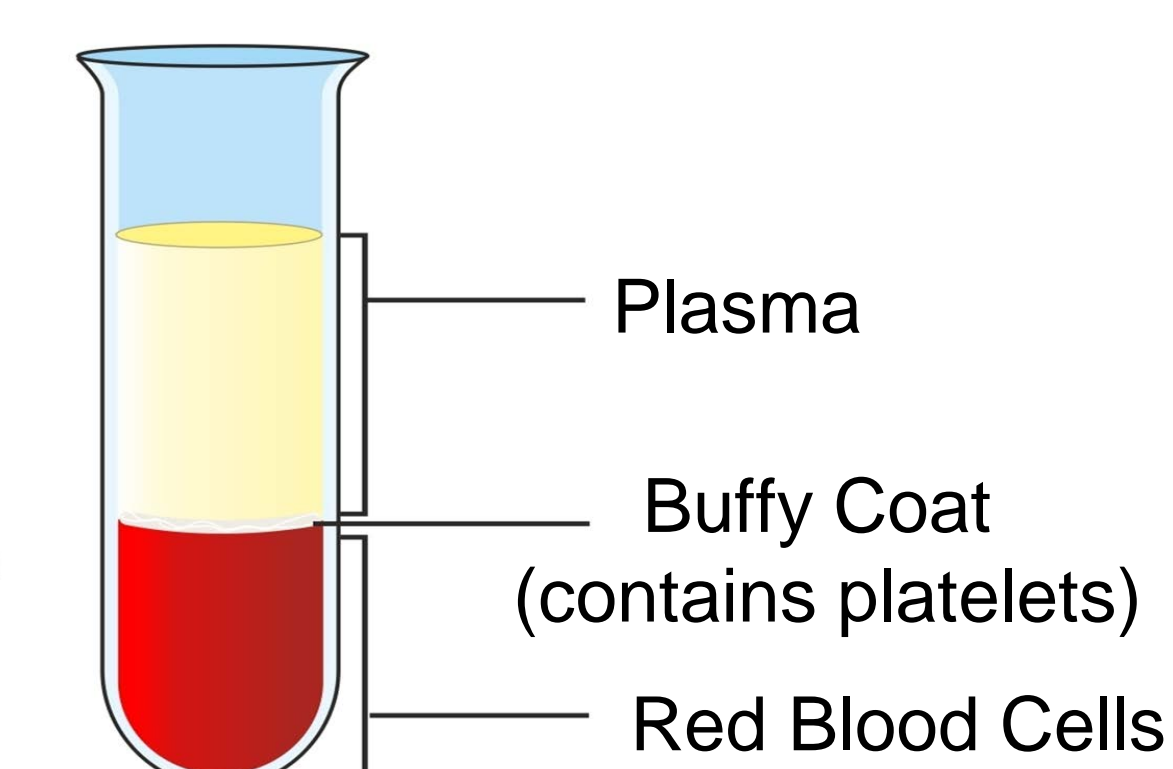


- 5-HT stability was determined in plasma prepared immediately after blood collection and following 18 hours of storage at room temp while protected from light
- 5-HT appeared to be stable in all anticoagulants under these conditions.

NaF/Na₂EDTA anticoagulant improves stability of 5-HT in whole blood. 5-HT is stable in plasma and serum when protected from light.

Role of Centrifugation on Platelet Stability and Cell Count in Plasma

Centrifugation speed, time, and temperature all impact the cell count and stability of platelets in plasma. Longer and higher spin speeds separate platelets more efficiently, generating more accurate quantification of free-plasma serotonin (5-HT). However, centrifugation may activate platelets, causing them to release 5-HT into plasma, thereby artefactually increasing free-plasma 5-HT measurements.

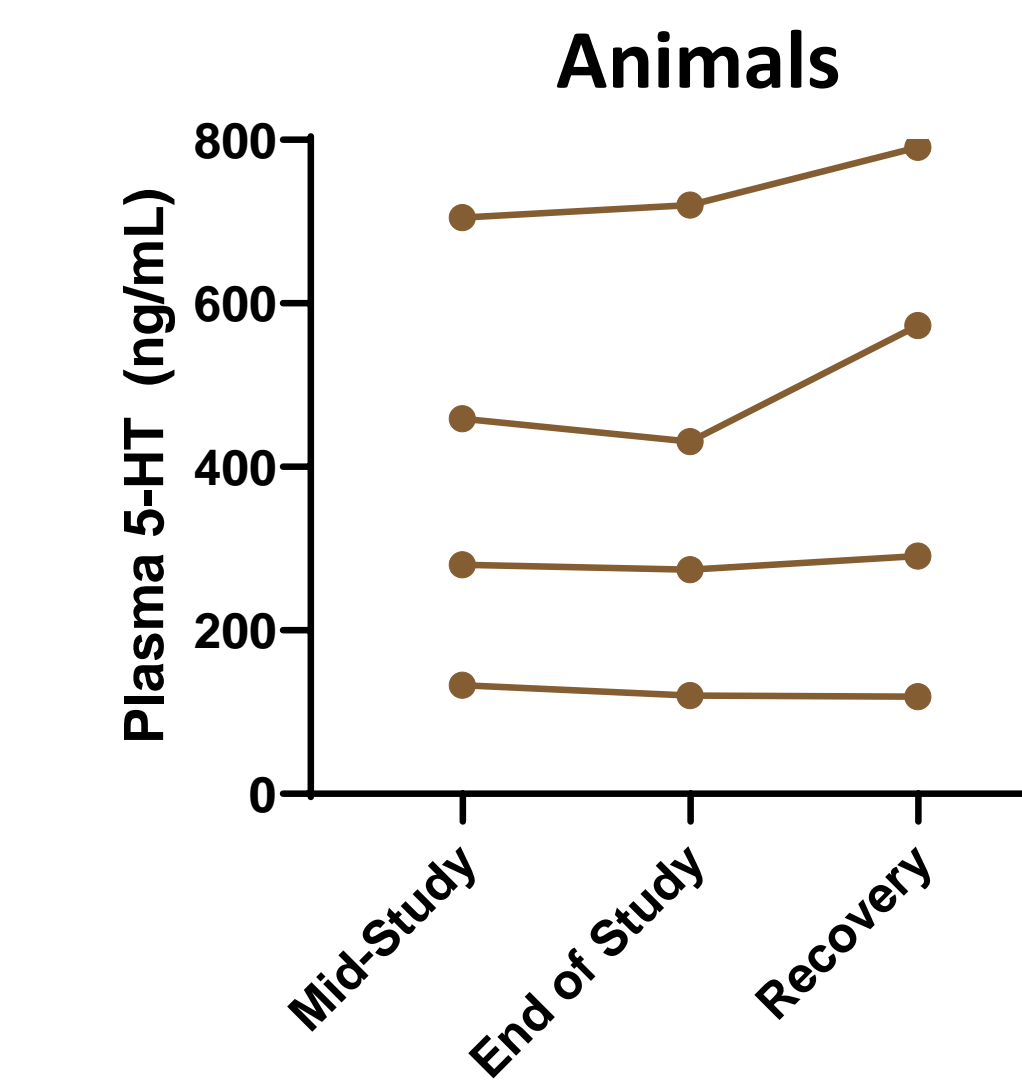


- Ultimately, a two-step centrifugation was needed to generate plasma and then platelet poor plasma to effectively remove platelets.

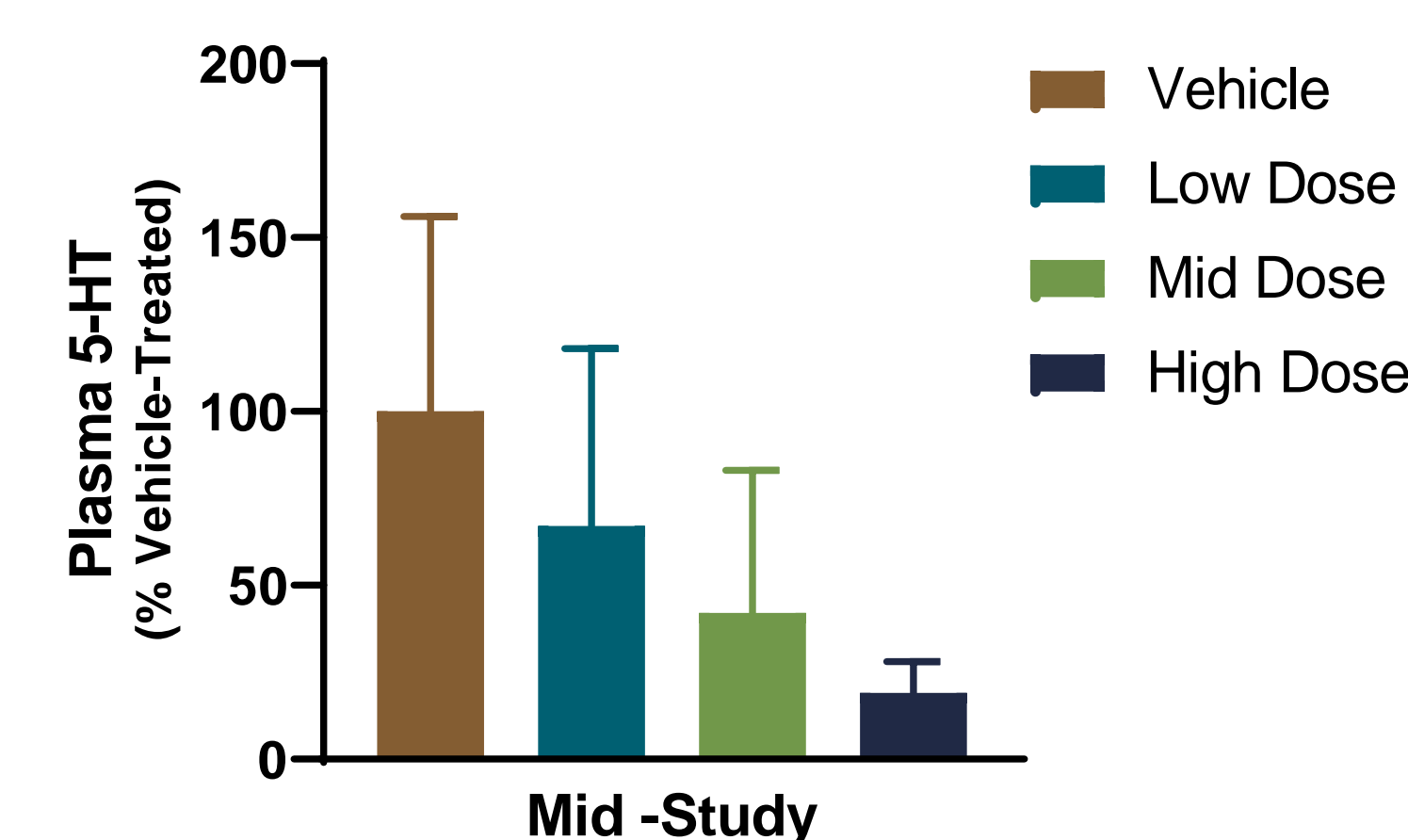
Contamination of plasma with platelets greatly impacts 5-HT measurements. Proper centrifugation plasma is essential to study success.

Evaluation of 5-HT in Dogs Treated with Rodatristat Ethyl

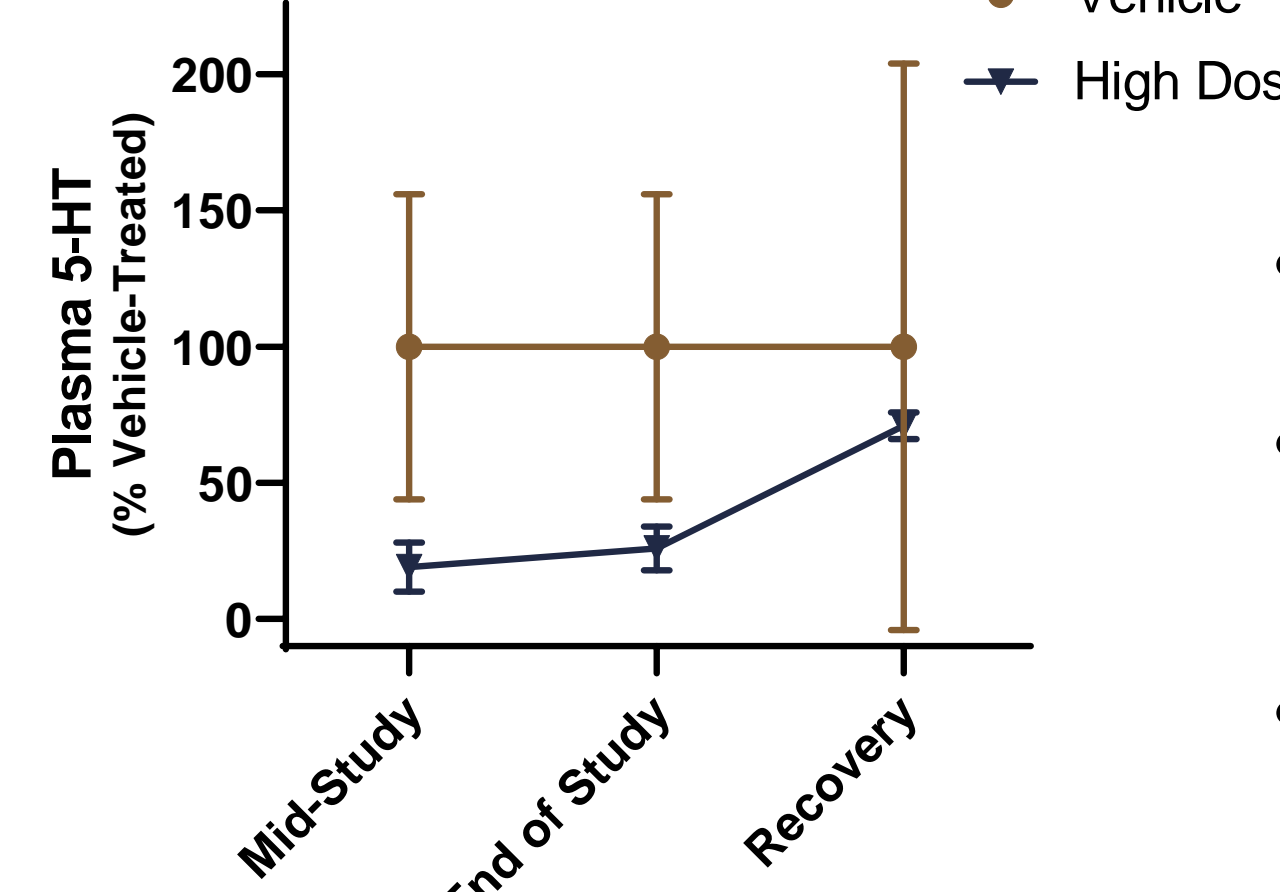
5-HT in Individual Vehicle-Treated Animals



Effect of Treatment on Plasma 5-HT



Recovery of Plasma 5-HT after Treatment



- Serotonin (5-HT) concentrations varied between vehicle treated animals, although intra-animal data were consistent throughout treatment (top left).
- Dogs were fed the same diet and blood samples were taken at the same time every day to reduce variations caused by diet or circadian effects. Data suggest inter-animal variability in dog.
- Rodatristat ethyl treated animals demonstrated a dose-dependent decrease in plasma 5-HT levels.
- In the high-dose group, plasma 5-HT samples returned to concentrations similar to the vehicle group after an off-dose period of 4 weeks at the end of the study.
- Double centrifugation was used to prepare platelet poor plasma from whole blood treated with NaF/Na₂EDTA for this study.

Treatment with rodatristat ethyl induced a dose-dependent and reversible decrease in plasma 5-HT in dogs.

Summary and conclusions

- Methods for detecting serotonin (5-HT) as a biomarker are necessary to understand disease development and treatment efficacy in 5-HT driven diseases such as pulmonary arterial hypertension.
- Unlike traditional small molecule analysis, method development required extensive study of sample collection and processing procedures to ensure data reliability.
- Measurements of serum 5-HT may be impacted by the duration of clotting. To eliminate this error, plasma measurements are preferred.
- Anticoagulants that prevent 5-HT degradation and reduce platelet activation provide more reliable analysis.
- Centrifugation must reliably remove platelets while preventing platelet activation.
- This method was used to measure dose-dependent reductions of plasma 5-HT in dogs treated with rodatristat ethyl. It is currently being used to analyze plasma 5-HT during the clinical development of rodatristat ethyl by Altavant Sciences, Inc.

Acknowledgements

- The authors would like to acknowledge Tami Zmetra and Dave Brigham (Charles River Laboratories, Worcester, MA) for developing the method for 5-HT analysis in dog plasma and analyzing the samples.

References

- For a review on the role of 5-HT in PAH please visit www.altavant.com/science
- For a review on challenges while measuring 5-HT, please see: Brand T., Anderson GM. *Clin Chem.* 2011 Oct; 57(10):1376-86.