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TECHNICAL NOTE 21966

Clinical research method for the quantitative determination of free plasma serotonin and 5-hydroxyindoleacetic acid (5-HIAA) in human plasma by UHPLC-MS/MS

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Keywords
Acclaim Vanquish, serotonin, SSRIs, plasma, Polar Advantage II, SOLA SAX, bioanalysis, Vanquish Horizon UHPLC system, TSQ Quantiva, triple quad, MS/MS, solid phase extraction, reversed phase

Goal

To develop and assess a clinical research method for the quantitation of serotonin and 5-HIAA from human plasma for clinical research. Thermo Scientific™ SOLA™ SAX solid phase extraction (SPE) technology is used for sample clean-up of the plasma prior to analysis. A Thermo Scientific™ Acclaim™ Vanquish PA2 UHPLC 2.2 µm column is used on the Thermo Scientific™ Vanquish Horizon™ UHPLC system coupled with the Thermo Scientific™ TSQ Quantiva™ MS/MS.

Application benefits

- Novel and robust clean-up using SOLA SAX SPE technology vastly reduces matrix effects from human plasma samples
- Excellent potential for automation in high-throughput clinical research environments using the 96-well plate format
- Rapid quantitative analysis of serotonin and its major metabolite, 5-HIAA, in one injection
- Superior Gaussian peak shape and resolution from interferences at the lower limits of quantitation

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Introduction

The use of antidepressants, particularly selective serotonin reuptake inhibitors (SSRIs), is on the rise in our modern society due in part to a greater awareness of mental illness and increased research into such disorders. It is believed that these drugs act specifically on the body's ability to re-absorb serotonin into nerve cells, thereby initiating an effect via an increase in brain serotonin levels. However, serotonin toxicity can arise from the use of more than one of these SSRIs or in conjunction with a monoamine oxidase inhibitor (MAOi) and can result in hospitalization and death.

This technical note describes an accurate and robust analytical method for the measurement of plasma concentrations of serotonin and its major metabolite, 5-HIAA. Both structures are crucial for measuring changes in serotonin plasma concentrations. A novel and rapid clean-up analytical method has been developed using the SOLA SAX 96-well plate. With this solid phase extraction clean-up method in a 96-well plate format, major matrix components can be removed and the target compounds pre-concentrated before injection onto the LC-MS/MS system. A Vanquish Horizon UHPLC system was used and coupled to a TSQ Quantiva mass spectrometer. For the separation the Acclaim Vanquish PA2 UHPLC column (150 mm × 2.1 mm) was chosen, which allows excellent separation of the analytes of interest. Excellent separation from matrix interferences was also achieved when compared to a regular C18 column.

Experimental

Instrumentation

- Vanquish Horizon UHPLC system consisting of the following:
  - System Base Vanquish Horizon (P/N VH-S01-A)
  - Binary Pump H (P/N VH-P10-A)
  - Split Sampler HT (P/N VH-A10-A)
  - Column Compartment H (P/N VH-C10-A)
  - Active Pre-heater (P/N 6732.0110)
- TSQ Quantiva triple-stage quadrupole mass spectrometer

Chromatography consumables

- Acclaim Vanquish PA2 2.2 µm, 150 × 2.1 mm column (P/N 071401-V)
- SOLA SAX SPE 10 mg / 2 mL 96 well plate (P/N 60309-003)
- Thermo Scientific™ WebSeal™ 96 well non-coated plastic microplates, 50 pack (P/N 60180-P202)
- Thermo Scientific™ WebSeal™ Nonsterile Mats, blue silicone (pack of 5) (P/N 60180-M122)

Table 1. Summary of assay

Analytes:	Serotonin	5-HIAA
Analytical matrix:	Human plasma, K2 EDTA	
Calibration range:	1–100 ng/mL	5–500 ng/mL
Lower limit of quantification (LLOQ):	1 ng/mL	5 ng/mL
Sample volume:	350 µL	
Calibration model:	Linear regression	Quadratic regression
Weighting factor:	$1/x^2$	$1/x^2$
Accuracy (bias) and precision:	-5.2–5.3% (CV% = 1.5–2.1)	-19.5–14.4% (CV% 2.2–10.5)
Carryover:	<20% of the LLOQ peak area after 2 double blanks	
Specificity:	<20% of the LLOQ peak area in 6 individual sources	
Recovery:	67.0%	58.1%
Matrix factor:	1.01 (CV% 1.1)	1.01 (CV% 0.7)

Reagents

- Thermo Scientific™ UHPLC-MS grade water (P/N W8-1)
- Thermo Scientific™ UHPLC-MS grade acetonitrile (P/N A956-1)
- Thermo Scientific™ UHPLC-MS grade methanol (P/N A456-1)
- Fisher Chemical™ Optima™ UHPLC-MS grade formic acid (P/N A117-50)
- Fisher Chemical™ Extra pure sodium hydroxide pellets (P/N S/4880)
- Fisher BioReagents™ PBS tablets (P/N BP2944-100)
- Albumin from chicken egg white, lyophilized powder, ≥98% (Sigma-Aldrich, P/N A5503-5G)

Sample preparation protocol

1. Bring all frozen human plasma to room temperature then centrifuge at 7000 rpm for 10 minutes, use this plasma for the quality control samples.
2. Add 350 µL of either the control plasma or 5% chicken egg albumin in PBS (w/v) to the bottom of the 96 well plate or matrix tubes for single (internal standard added but no analyte added) and double blank (no internal standard added and no analytes added) samples.
3. Add 350 µL of the calibration standards, QCs, and test samples to the 96 well plate or matrix tubes.
4. To the single blanks, calibration standards, QCs, and test samples add 50 µL of Internal Standard B (140 ng/mL).
5. To the double blanks and matrix effect over-spike samples add 50 µL of water and vortex.
6. Carefully add 600 µL of 1% sodium hydroxide to all samples.
7. Condition the SOLA SAX plate (10 mg/2 mL) with 450 µL of methanol, followed by 450 µL of 1% sodium hydroxide.
8. Load the samples onto the SOLA plate and allow to drip through under vacuum.
9. Wash with 450 µL of 1% sodium hydroxide.
10. Wash with 450 µL of a methanol/sodium hydroxide, 1% of 5 M solution v/v (25:75, v/v), drying at maximum vacuum for 30 seconds.
11. Elute with 2 × 250 µL of acetonitrile/formic acid (95:5, v/v) into a fresh 96-well plate.
12. Evaporate to dryness under N_2 at 60°C.
13. Reconstitute in 100 µL of water/formic acid (100:0.1, v/v), and vortex.
14. Reconstitute matrix effect over-spike samples using MEREC non-extract solution*.
15. Prior to injection, centrifuge samples for 5 minutes at 4000 rpm.

* Serotonin (300 ng/mL), serotonin-d4 (70 ng/mL), 5-HIAA (1400 ng/mL), 5-HIAA-d5 (350 ng/mL); all standards purchased from Toronto Research Chemicals.

Separation conditions

Table 2. UHPLC parameters

Mobile phase A:	Water/formic acid (100/0.1, v/v)
Mobile phase B:	Methanol/formic acid (100/0.1, v/v)
Flow rate:	0.5 mL/min
Column temp.:	60°C, still air mode
Injection volume:	20 µL
Run time:	8.5 min

MS/MS conditions

Table 3. MS/MS source parameters

Source:	Thermo Scientific™ Ion Max source with HESI-II probe
Polarity:	Serotonin (Positive), 5-HIAA (Negative)
Spray voltage:	+2500 V, -4500 V
Vaporizer temperature:	320°C
Sheath gas pressure:	50 Arb
Aux gas pressure:	15 Arb
Ion transfer tube temperature:	330°C
CID gas pressure:	1.5 mTorr

Table 4. Compound transition details

Compound	Polarity	Precursor (*m/z*)	Product (*m/z*)	Collision Energy (V)
Serotonin	Positive	177.1	160.0	10.253
Serotonin-d4	Positive	181.1	118.1	28.860
5-HIAA	Negative	190.1	146.2	10.253
5-HIAA-d5	Negative	195.1	148.1	22.084

Data processing

The Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS), version 7.2.9, was used for LC/MS system control, data acquisition, and data analysis.

Results and discussion

Calibration model and range

The calibration model for serotonin was assessed to be a linear regression with a $1/x^2$ weighting, while 5-HIAA used a quadratic regression with $1/x^2$ weighting, providing the best fit for the data. Eight calibration standards were freshly prepared in 5% chicken egg albumin in phosphate buffered saline; this was done to allow quantitation of endogenous concentrations of the analyte in plasma that can then be added onto the expected QC levels to account for each analyte's endogenous amount. The calibration standards were analyzed in duplicate to give 16 points for each calibration line. The linear calibration line for serotonin is shown in Figure 1. Serotonin had a coefficient of determination (slope) of 0.999, while 5-HIAA was 0.997.

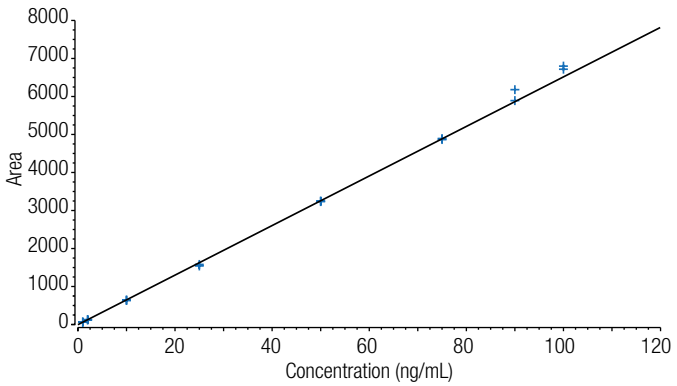

Figure 1. Calibration line for serotonin

Accuracy and precision

The accuracy and precision of the assay were determined by analysis of six replicates of quality control (QC) plasma samples spiked at four concentrations within the calibration range tested (1–100 ng/mL for serotonin and 5–500 ng/mL for 5-HIAA).

Therefore, for serotonin and 5-HIAA the tested concentrations were spiked at 1.00 ng/mL and 5.00 ng/mL for the LLOQ; 2.5 ng/mL and 12.5 ng/mL for the Low QC; 20 ng/mL and 100 ng/mL for the Mid QC; and 80 ng/mL and 400 ng/mL for the High QC, respectively. The actual QC concentrations are higher than the spike levels because of endogenous amounts in the plasma, and this is reflected in the calculated concentrations used to determine QC accuracy and precision. Example data for accuracy and precision is shown in Table 5 and example chromatograms in Figure 2.

Table 5. Accuracy and precision data for low QC samples

	Low QC	
	Serotonin (5.31 ng/mL)	5-HIAA (16.2 ng/mL)
Mean (n=6):	5.13	14.2
Bias %:	-3.30	-12.2
CV %:	3.60	7.50

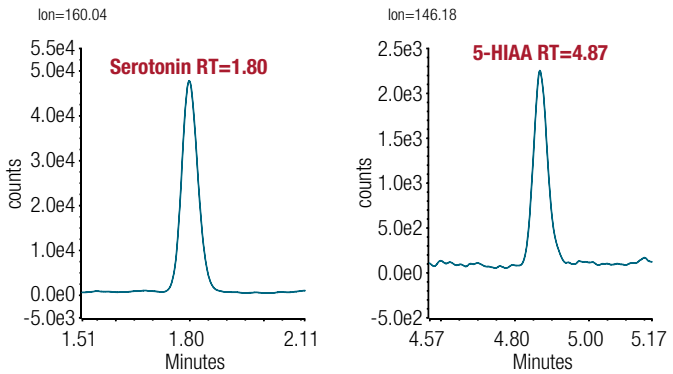

Figure 2. Representative chromatograms of serotonin (left) and 5-HIAA (right) at low QC level

Determination of assay carryover

The assay carryover was assessed by injection of two blank samples after the ULOQ with no IS; no measurable carryover was observed.

Determination of internal standard normalized matrix factor

The presence of matrix effects was assessed in this assay by extracting three replicate double blank samples of each of the six matrices. These were then reconstituted in a solution made up to the expected concentration of the High QC standard. By comparing the post-spiked extracts with the non-extracted (NE) standard solution, the matrix effects (ME) from each individual source can be determined. The pooled source (High ME 1) of plasma showed the highest amount of matrix effects but was corrected for by internal standard as presented in Table 6. Chromatograms showing the excellent resolution of both serotonin and 5-HIAA from matrix components in human plasma are shown in Figure 3.

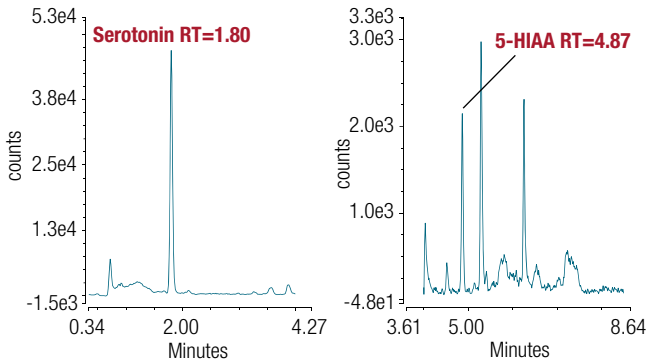

Figure 3. Representative chromatogram of matrix components around serotonin and 5-HIAA in human plasma at low QC level; serotonin on the left and 5-HIAA on the right

Determination of assay specificity

The assay specificity was assessed with blank matrix from five individual sources and one pooled source. The pooled source was used for the QC samples Low QC, Mid QC and High QC. Each source had three replicate injections of single blanks and one double blank. This was then used to back-calculate the endogenous concentration of serotonin and 5-HIAA in each source. The average concentration determined from the single blanks was added onto the set QC levels to account for the endogenous concentrations of each analyte.

For the calibration line and LLOQ QC, an analogue matrix of 5% chicken egg albumin in PBS was used because it contained no endogenous serotonin or 5-HIAA, unlike the alternative bovine serum albumin used initially. At the LLOQ level, the variation in endogenous analyte would have been unacceptable in human plasma against the assay pass criteria. No interfering peaks were detected in the 5% chicken egg albumin used and so the calibration line demonstrated acceptable specificity for both analytes and internal standards. Figure 4 shows the single blank of QC plasma used and Figure 5 shows the double blank of the QC plasma used.

Determination of recovery

The recovery of both analytes was assessed by comparing the analyte peak area of the high QC over-spike samples (High ME 1) to the HQC samples analyte peak area. Using this data, it was determined that a high yield of 67.0% could be achieved for serotonin and 58.1% for 5-HIAA. It should be noted that increasing the elution strength of the solvent in the elution step in order to increase the yield may increase unwanted matrix effects. The current yield already provides excellent sensitivity for the concentrations assessed in this technical note.

Table 6. Internal standard (IS) matrix factor assessment for serotonin

	Serotonin				
Sample Name	**Mean Analyte Peak Area (n=3)**	**Analyte Matrix Factor**	**Mean IS Peak Area (n=3)**	**IS Matrix Factor**	**IS Normalized Matrix Factor**
High ME 1	3199717.3	0.777	104526.0	0.771	1.01
High ME 2	3799983.3	0.923	124175.0	0.916	1.01
High ME 3	3917323.3	0.951	124248.0	0.916	1.04
High ME 4	3795574.7	0.922	124485.3	0.918	1.00
High ME 5	3823281.3	0.929	125489.7	0.925	1.00
High ME 6	3759005.3	0.913	124849.3	0.921	0.99
High NE	4117051.0		135615.3		**Mean** 1.01
					CV % 1.5

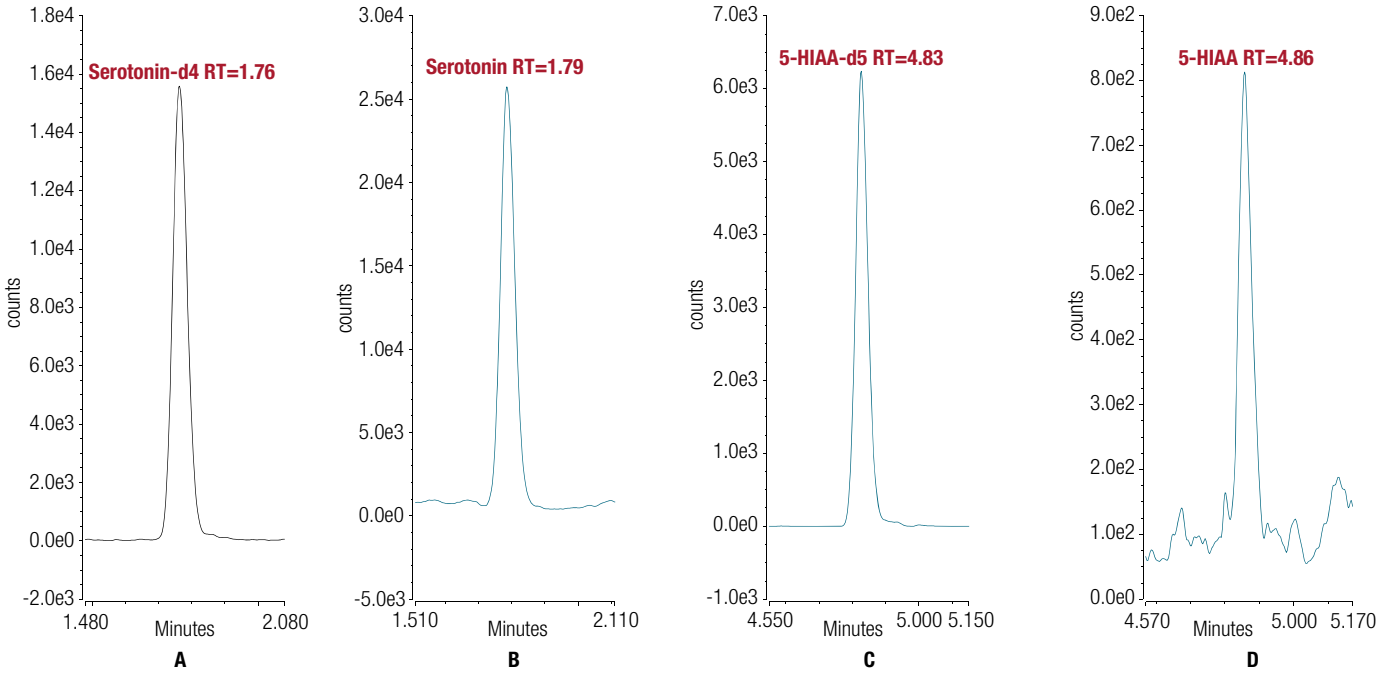

Figure 4. Representative chromatograms of single blank of QC plasma; serotonin-d4 (A), serotonin (B), 5-HIAA-d5 (C), 5-HIAA (D)

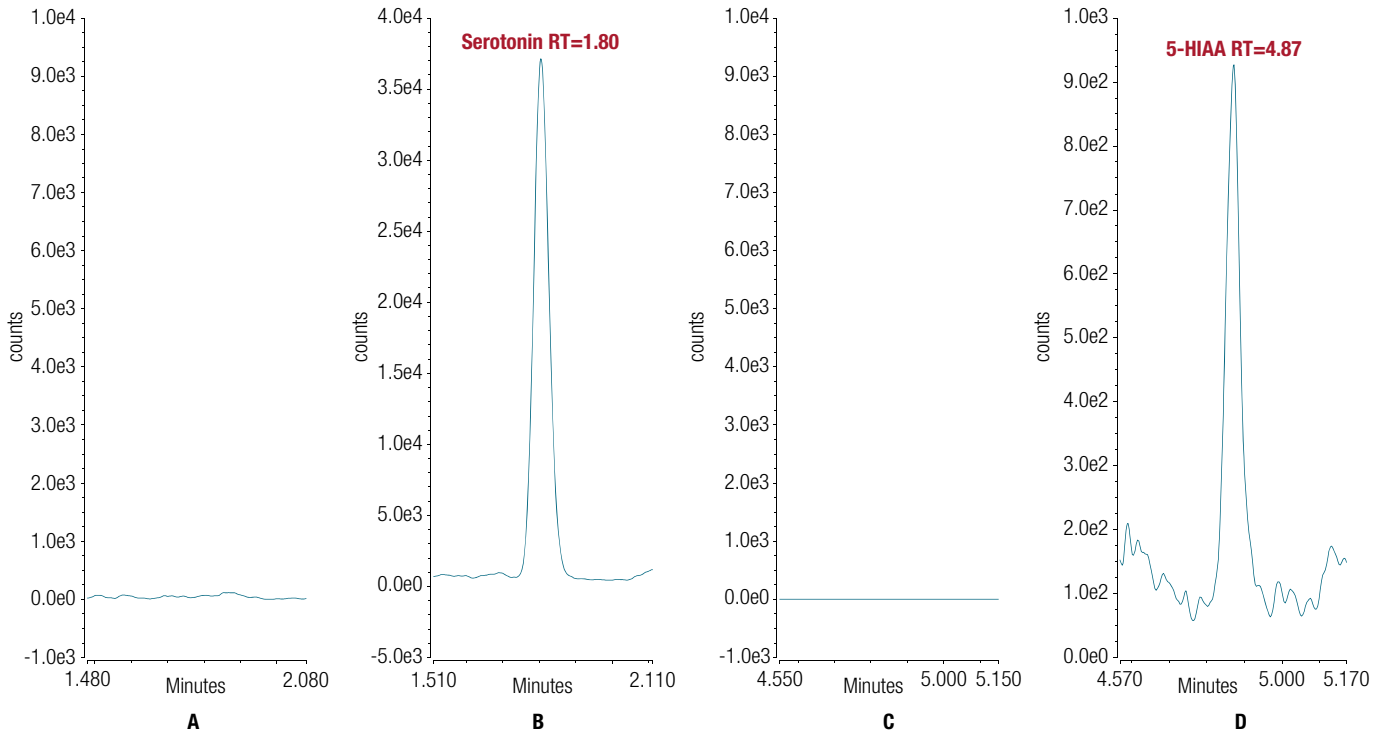

Figure 5. Representative chromatograms of double blank of QC plasma; serotonin-d4 (A), serotonin (B), 5-HIAA-d5 (C), 5-HIAA (D)

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Conclusion

- Novel and robust clean-up analytical method for clinical research using SOLA SAX SPE technology balances achieving high recovery of the analytes while also vastly reducing matrix effects from human plasma samples
- Excellent potential for automation in high-throughput clinical research environments using the 96 well plate format
- Exceptional separation provided by the Acclaim Vanquish PA2 column, moving additional matrix interferences away from area of interest. Provided by a combination of the exemplary performance from the Vanquish Horizon UHPLC system with the selectivity of the Acclaim Vanquish PA2 UHPLC column provides a rapid analysis of serotonin and its metabolite in one injection.
- Superior gaussian peak shape and resolution from the baseline at the lower limits of quantitation

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