Quantitative Determination and Validation of Serotonin in Human Plasma and Human Cerebrospinal fluid

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Introduction:

Serotonin (5-hydroxytryptamine, 5-HT) is a neurotransmitter involved in many physiological functions (sleep, behavior regulation, hemostasis, and gastrointestinal motility). It also has a role in pathological conditions that include cancer, neurological disorders, and cardiovascular disease. Depending on the condition, certain therapies are designed to either promote serotonin availability or to inhibit it.

Selective serotonin reuptake inhibitors (SSRIs) are commonly used as antidepressants, but additional indications for use are increasing. This class of drug increases the amount of interstitial serotonin available after synaptic release. Effects of this process can be seen in both CSF and plasma. Pathological conditions related to tumor growth and smooth muscle contraction can also lead to observable changes in serotonin in these matrices. Monitoring plasma and cerebral spinal fluid (CSF) serotonin can be a useful biomarker for a wide variety of applications.

A wide range of serotonin levels for plasma and CSF have been reported, depending on population, method of collection, and method of analysis. Reported here are two methods for serotonin analysis in human K2-EDTA plasma and human CSF. The quantitative ranges for each were based on concentrations found in otherwise normal individuals.

Methodology:

	Human Plasma	Hu
Calibration Range	0.05 to 25 ng/mL	5 to 250 pg/mL
	Sample Extraction	
Sample Volume	0.200 mL	0.100 mL
Internal standard solution added	20 µL of serotonin-D4 (200 ng/L)	10 μL of serotonin-D4 (12.5
Reagent added	0.200 mL 6% ammonium hydroxide solution	0.250 mL 6% ammonium hy
Liquid extraction	Isolute SLE+ 400 μ L extraction plate with 4 × 0.4 mL ethyl acetate	Liquid-liquid extraction with
Organic fraction treatment	 0.200 mL acetic anhydride 10 μL pyridine heat 60°C, 30 min. evaporate to dryness 	 0.125 mL propionic anhydric 15µL pyridine heat 60°C, 30 min. evaporate to dryness
Reconstitute for injection	0.200 mL mobile phase A	0.125 mL mobile phase A
	LC Analysis	
Column (temp.)	Phenomenex Luna C18 3 μ , 100 × 2.0 mm (40°C)	Phenomenex Gemini-NX C
Mobile phase A	Water/formic acid/ammonium hydroxide (1000:2.0:0.4)	Water/formic acid/ammoniu (1000:2.0:0.4)
Mobile phase B	Methanol/formic acide/ ammonium hydroxide (1000:2.0:0.4)	Acetonitrile/formic acid/ami (1000:2.0:0.2)
Gradient conditions	30% to 60% solvent B over 4.5 minutes, linear gradient, 0.3 mL/min	30% solvent B isocratic for 3
Injection volume	5 μL	10 µL
	MS-MS	
Instrument	Sciex 4000	Sciex 5000
Ion transitions	$261 \rightarrow 160$ Serotonin $265 \rightarrow 164$ Serotonin-D4	$289 \rightarrow 160 \text{ Serotonin}$ $293 \rightarrow 164 \text{ Serotonin-D4}$

Two Elimination Methods Used:

In plasma, endogenous serotonin was removed in order to generate blank control matrix. This procedure utilized sodium hypochlorite as a strong oxidizer to attack the sensitive indole substructure of serotonin. For CSF, this procedure left significant residual serotonin compared to the target LLOQ peak. An alternative method using hydrogen peroxide yielded a "cleaner" control matrix for the lower detection limit.

Results:

Examples of chromatography for each method are presented in figures 1 through 10. One factor in moving from analysis in plasma to analysis in CSF is the need for a much lower LLOQ in human CSF. Using the mass transitions of the diacetylserotonin on a Sciex 5000 instrument yielded approximately a 2-fold improvement in sensitivity, however preliminary screening of human CSF samples suggested baseline endogenous levels would be in the range of 10 to 20 pg/mL. In addition, availability of sample volume in CSF is more restricted than with plasma, so increasing the sample aliquot volume for extraction to improve sensitivity was not an option. Changing the ion transition setting by utilizing a larger derivative proved beneficial to sensitivity to where background noise was reduced and the detection limit improved.











Human Plasma	Human CSF
mL plasma + 0.4 mL ium hypochlorite (4%)	40 mL CSF + 1.4 mL hydrogen peroxide (30%)
eat at 60°C overnight	Heat at 60°C overnight
store frozen until use	Add 0.8 mL sodium bisulfate (40%)
	Store frozen until use

Validation Data:

Pla	asma Pre	ecision a	nd Accur	racy		CS	CSF Precision and Accuracy							
	LLOQ (0.0500 ng/ mL)	Low (0.150 ng/mL)	Mid (5.00 ng/mL)	High (20.0 ng/mL)	5× Dilution (125 ng/mL)		LLOQ (5.00 pg/ mL)	Low (15.0 pg/ mL)	Mid (50.0 pg/ mL)	High (200 pg/mL)				
	0.0499	0.155	4.49	17.4	126		4.91	13.2	48.3	185				
	0.0481	0.156	4.65	16.6	123		5.87	13.9	47.4	188				
n 1	0.0482	0.155	4.56	18.1	127	n 1	6.53	15.4	44.3	187				
Ru	0.0513	0.143	4.61	17.3	125	Ru	5.90	13.4	43.7	181				
	0.0476	0.148	4.45	18.3	125		4.56	13.7	41.6	180				
	0.0474	0.139	4.52	17.5	126		5.49	13.2	46.6	196				
	0.0511	0.159	4.60	17.7		in 2	5.04	10.6	43.5	179				
	0.0507	0.147	4.66	18.2		Rı	4.21	11.6	45.3	188				
in 2	0.0491	0.151	4.48	18.1		un 3	6.65	14	45.4	186				
Rı	0.0496	0.150	4.67	17.5		<u>R</u> i	4.50	13.1	46.9	194				
	0.0523	0.154	4.75	15.8				%CV						
	0.0504	0.151	4.80	18.3			16.0 10.0 4.5		4.5	3.0				
	0.0524	0.151	4.81	18.1			Overall %Bias							
	0.0516	0.153	4.74	17.2			7.3	-11.9	-9.4	-6.8				
un 3	0.0522	0.144	4.71	18.0										
Rı	0.0531	0.165	4.67	17.4		Plas	Plasma stability testing was dor							
	0.0556	0.160	4.57	18.0		scre	ened to	find ind	ividual	oatches th				
	0.0485	0.158	4.64	18.0		and	high set	rotonin	level. T	hese two s				
		(Overall %CV			over	the cou	urse of tw	wo runs	to calcula				
	4.3	4.3	2.2	3.3	1.1	high stability pool had a mean value								
		0	verall %Bias			poo	I had a r	nean va	lue of 0	./48 ng/n				
	1.0	2.0	-7.0	-11.5	0.0									

PI	'lasma Stability																		
<u> </u>	High Stability (20.0 ng/mL)	%Bias	Low Stability (0.748 ng/mL)	%Bias	haw	High Stability (20.0 ng/mL)	%Bias	Low Stability (0.748 ng/mL)	%Bias	0°C	High Stability (20.0 ng/mL)	%Bias	Low Stability (0.748 ng/mL)	%Bias	stab.	High Stability (20.0 ng/mL)	%Bias	Low Stability (0.748 ng/mL)	%Bias
s @ R	18.3	-8.5%	0.760	1.6%	reeze/t	18.1	-9.5%	0.762	1.9%	s @ -2	18.4	-8.0%	0.759	1.5%	xtract	18.5	-7.5%	0.783	4.7%
24 hr	19.4	-3.0%	0.721	-3.6%	cycle f	18.3	-8.5%	0.757	1.2%	24 day	17.9	-10.5%	0.773	3.3%	9 hrs. e	18.4	-8.0%	0.798	6.7%
	17.9	-10.5%	0.755	0.9%	S	17.7	-11.5%	0.782	4.5%	1	18.4	-8.0%	0.771	3.1%	8	18.6	-7.0%	0.758	1.3%

Stability testing results in human CSF are not available at this time. However, all indications from pre-validation tests support similar stability to that found in human plasma.

Matrix effects in plasma and CSF were tested by measuring endogenous levels in six different lots of unaltered matrix. These same lots were fortified with additional serotonin by spiking an aliquot of each with a standard solution. A target value was calculated for each matrix lot and compared to the found value. These results are presented below.

	Matrix Effe	ect in Unal	tered Huma	an Plasma		Matrix Effect in Unaltered Human CSF						
Plasma Lot #	Endogenous Amount Found (ng/mL)	Amount Added (ng/ mL)	Total Amount Expect- ed (ng/mL)	Total Amount Found (ng/mL)	% Bias	Lot #	Endogenous Amount Found (pg/mL)	Amount Added (pg/ mL)	Total Amount Expect- ed (pg/mL)	Total Amount Found (pg/mL)	% Bias	
1	0.345	1.00	1.34	1.32	-1.9%	1	31.5	50.0	81.5	76.9	-1.9%	
2	3.22	1.00	4.22	4.43	5.0%	2	53.1	50.0	103	102	-1.1%	
3	0.564	1.00	1.56	1.54	-1.5%	3	9.86	50.0	59.9	62.4	4.2%	
4	0.628	1.00	1.63	1.64	0.74%	4	11.0	50.0	61.0	63.2	3.6%	
5	0.461	1.00	1.46	1.42	-2.8%	5	11.4	50.0	61.4	61.7	0.49%	
6	1.41	1.00	2.41	2.46	2.1%	6	23.5	50.0	73.5	73.0	-0.68%	
	·	•	•	·			•	•	•	`		

Endogenous Serotonir





Extraction recovery of serotonin from plasma was very good, ranging from 90% to 110%. Recovery from CSF was measured to be slightly lower, ranging from 61% to 74%.

Precision and accuracy data for both methods are given to the left. Currently, the plasma method has completed the entire validation process, and all data are presented. The CSF method validation has not been completed as of present, but the data that is available is presented here.

unaltered, authentic plasma samples. Plasma lots were hat had an appropriate concentration to represent a low selected lots (low and high) were originally analyzed ate mean anchor values for each prior to stressing. The e of 20.0 ng/mL with a CV of 3.8%. The low stability nL with a CV of 1.2%.

Twenty-two different unaltered plasma samples were tested in order to confirm an appropriate calibration range was chosen. Likewise, twelve different CSF lots were analyzed to test the calibration range. All samples produced measurable values within the range of quantitation.

Conclusion:

Serotonin is an important endogenous compound for neurological/physiological health. Its level in blood and CSF can be a useful biomarker for certain therapeutic applications. The low molecular weight, endogenous presence, and relatively low abundance of serotonin make it a challenging compound to measure in biological fluids. The approach taken with these two methods has demonstrated sensitivity, accuracy, and robustness in human plasma and CSF.