Liquid Chromatographic Determination of Serotonin in Homogenized Dog Intestine and Rat Brain Tissue Using a 2 mm i.d. PEEK Column

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Serotonin in homogenized dog intestine and rat brain tissue was determined by liquid chromatography with a 100 x 2 mm ODS 3 µm column. The column tube, frits and fittings were fabricated with PEEK. High efficiency was obtained due to the smoothness and inertness of the inside wall of the column. This 2 mm PEEK column proved to be suitable for tissue samples. The column lifetime and concentration detection limit were very favorable. The relationship between internal diameter and detection limits (both in amount and in concentration) are discussed for columns with internal diameters of 0.32, 1.0, and 2.0 mm. Serotonin degradation was studied under different conditions, including the effects of pH, temperature, sample vial material, and metal ions on sample stability.

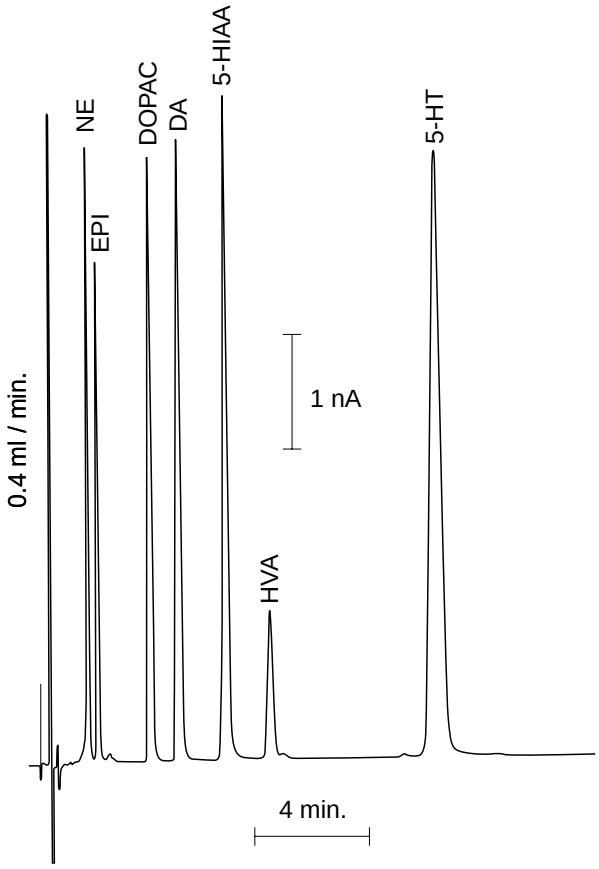
Serotonin, 5-hydroxytryptamine (5-HT), is an important neurotransmitter. It has been implicated in a broad range of behavioral disorders affecting sleep, eating, sex, and mood (1). In order to obtain low detection limits for small volume samples, microbore 1 mm i.d. columns have been widely used (2-5). An LC system equipped with a 1 mm microbore column can easily provide detection limits of 1 fmole/5 µL. This system enables basal striatal serotonin to be released and measured without the addition of a serotonin uptake inhibitor to the perfusion medium during microdialysis (6). However, care should be taken when analyzing tissue samples. Clogging problems may be encountered since the column ends are so small. When used for tissue samples, 2 mm columns are more rugged than 1 mm columns because their cross sectional area is four-times larger.

Microbore columns have higher mass sensitivity compared to conventional columns. Detection limits (by amount) are lower. Compared to a 3.2 mm i.d. column, a 1 mm i.d. column provides a nine times lower detection limit (by amount) (7); however, the peak will broaden if the sample volume is too large. For those samples with a low concentration and a large volume, the detection limit by concentration may be worse with a smaller i.d. column. Our study illustrates that a smaller column provides lower detection limits in amount, but the detection limit in concentration might not be lower due to the smaller injection volume required. If a sample has low concentration and adequate volume, it will generally be beneficial to choose a larger column and inject a larger volume for best detection. The question of optimum diameter is a complex one, because active and inactive detector volume and pump performance at different volume flow rates also enter into the picture. There are, for example, many UV and fluorescence detectors which simply can not be used with small (< 2 mm i.d.) columns due to their cell volumes.

The inertness and smoothness of the column inside wall are well known to be very important to column performance. Glass is a good material for both inertness and smoothness. When used to line the inside of a stainless steel tube, high performance was obtained (8); however, glass alone is too delicate and not strong enough for high pressure. On the other hand, stainless steel is very strong, but the inside wall of microbore stainless steel tubes is very difficult to polish as smooth as glass. Fused silica is a suitable lining for stainless steel to form a microbore column. PEEK is also a good material for a column interior. Although not as strong as stainless steel, it is more favorable

F1

Separation of tryptophan and tyrosine metabolites. Column: PEEK 3 µm ODS, 100 x 2 mm. Flow rate: 400 µL/min., mobile phase B.



than glass, but at this point it is not used below 2 mm i.d. Supported by an aluminum outer sleeve, a PEEK tube can withstand up to 10,000 psi (680 bar) pressure. In our study the inertness and the smoothness of the PEEK body provided 20% more column plates when conditions remained the same as for stainless steel.

Serotonin is well recognized as an unstable compound. It decomposes quickly if treated improperly. Since the 5-HT concentration in many biosamples is very low, decomposition should be minimized. In our study, we found that serotonin was quite unstable in some circumstances, but stable in others. While frequently treated in the same manner as for catecholamines, serotonin acts quite differently. Temperature, pH, and some metal ions affected its degradation. Proper sample preparation and storage condition were important for accurate results.

Experimental

A BAS PM-80 pump was used in this study. Flow rate was set at 400 µL/min. for a BAS UniJet PEEK 3 µm ODS column, 100 x 2 mm (MF-8957), and 100 µL/min. for a BAS UniJet 3 µm ODS column, 100 x 1 mm. No flow splitter was used. When a BAS UniJet 3 µm ODS fused silica-lined column (0.32 x 150 mm) was used, a flow rate of 9 µL/min. was obtained by using a flow splitter. The BAS PEEK column was fabricated with a 100 x 2 mm i.d PEEK tube cladded with aluminum for strength. Both column ends had PEEK frits and PEEK fittings. Stationary phase (ODS, 3 µm 80 Å) was slurry packed into the column.

The column back pressure at 400 µL/min. was 2200 psi. A BAS LC-26A degasser was on-line for degassing the mobile phase. A refrigerated Sample Sentinel autosampler was on-line between the pump and a Rheodyne injection valve. A 1/16 inch o.d. 0.005 inch i.d. PEEK tubing was used to connect the two injection valves. The manual Rheodyne injection valve was in the "load" position when the autosampler was being used. The total system back pressure was about 2600 psi when the 2 mm column was run at a flow rate of 400 µL/min. The BAS LC-4C detector gain was set between 10 and 0.2 nA full scale. A 3 mm glassy carbon electrode was used in a cross flow thin-layer cell for detection of serotonin at +650 mV *vs.* Ag/AgCl. BAS ChromGraph® Data Acquisition and Data Processing software was used.

Mobile phase A was used for separation of serotonin in tissue samples. The buffer contained 0.1 M NaH_2PO_4, 0.5 mM EDTA, and 0.15 g/L sodium octyl sulfate. The buffer was adjusted to pH 5.0 and mixed with acetonitrile in the ratio of 100:7. Mobile phase B was used for separation of the metabolites of tyrosine and tryptophan. The buffer contained 25 mM NaH_2PO_4, 50 mM Na-citrate, 0.03 mM EDTA, 10 mM diethylamine HCl, and 2.2 mM octylsulfonic acid/sodium salt. The buffer was adjusted to pH 3.2. One liter of the buffer was mixed with 30 mL methanol and 22 mL dimethylacetamide.

Sample Preparation

The dog intestines were stored at -80° C until analysis. The tissue was thawed and diced into pieces, roughly 5 mm x 5 mm. 0.5 g of the tissue was added to a pre-cooled test tube (4° C). 10 mL of 2% (by weight) pre-cooled (4° C) trichloroacetic acid solution (TCA) was added and the tissue was homogenized. 4 mL 0.33 M NaH_2PO_4 (pH 6) solution containing 0.5 mM EDTA was added to the homogenate and vortexed. This slurry was centrifuged for 5 minutes. 200 µL of the supernatant was filtered using 0.45 µm Nylon-66 filter disk membrane. The filtered supernatant (5

T1

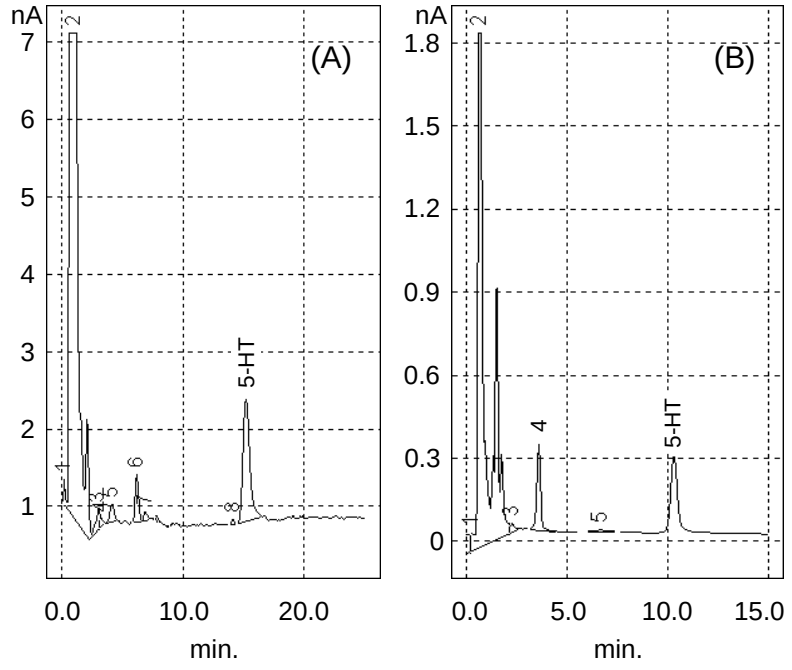
Serotonin detection limit comparison using 0.32, 1, and 2 mm i.d. microbore columns.

Column Size	0.32 mm	1 mm	2 mm
Flow Rate	9 µL/min. (flow split used)	100 µL/min.	400 µL/min.
Pump Frequency	10 piston strokes/min.	1 piston stroke/min.	4 piston strokes/min.
Linear Flow Velocity	113 mm/min.	127 mm/min.	127 mm/min.
Detection Limit (0.5 µL injected)	0.04 pg (80 fg/µL)	0.15 pg (300 fg/µL)	0.4 pg (800 fg/µL)
Detection Limit (5 µL injected)	0.05 pg (10 fg/µL), dispersed, interference by large void peaks	0.15 pg (30 fg/µL)	0.4 pg (80 fg/µL)
Detection Limit (50 µL injected)	peak was not detected, covered by huge void peaks	0.6 pg (12 fg/µL), dispersed, interference by large void peaks	0.4 pg (8 fg/µL)

F2

(A) Chromatogram of serotonin in homogenized dog intestines. Column: PEEK 3 µm ODS, 100 x 2 mm. Flow rate: 400 µL/min., mobile phase B.

(B) Chromatogram of serotonin in homogenized rat cortex. Column: PEEK 3 µm ODS, 100 x 2 mm. Flow rate: 400 µL/min., mobile phase A.



µL) was injected into the 2 mm PEEK column.

The rat cortex (CTX) was removed from the brain immediately following decapitation and kept at -80° C until analysis. The rat CTX was thawed, weighed and placed in a pre-cooled test tube (4° C). 5 mL of the 2 % TCA solution (pre-cooled to 4° C) was added to the tissue and then homogenized. 2 mL 0.33 M NaH2PO4 (pH 6) solution containing 0.5 mM EDTA was added to the homogenate and vortexed. This slurry was then centrifuged for 5 minutes. 200 µL of the supernatant was filtered using 0.45 µm Nylon-66 filter disk membrane. 5 µL of the filtered supernatant was injected into the 2 mm PEEK column. All procedures were done at room temperature (21° C).

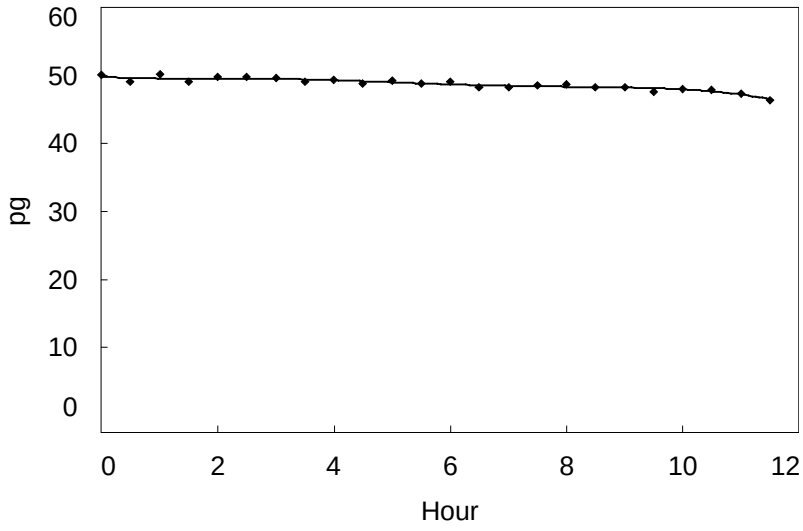
Results and Discussion

The 2 mm i.d. PEEK column exhibited high efficiency (narrow peaks for a given retention time). A 10 cm long column had 13,500 plates (equivalent to 135,000 plates/m) when methyl benzoate was used as a test compound. The column easily separated tryptophan and tyrosine metabolites (***F1***). Typical chromatograms of 5-HT in homogenized dog intestines and rat cortex samples are shown in ***F2A*** and ***F2B***. There were no peaks interfering with 5-HT in these samples. In order to confirm the identity of the 5-HT peak in samples, mobile phase A and mobile phase B were run through the PEEK column. Both a 5-HT standard solution and a tissue sample were injected. The identification of a serotonin peak in the tissue samples was confirmed by comparing the retention of the serotonin for both mobile phases. The 5-HT concentration in homogenized rat cortex was 28.8 ng/g and 450 ng/g in dog intestines. In both cases, the concentration was far above the detection limit.

Compared with 1 mm i.d. columns, the cross sectional area of 2 mm i.d. column is four-times greater, so we can theoretically expect a fourfold lower peak height with the 2 mm column if other conditions are kept the same. On the other hand, larger columns withstand larger injection volumes with less dispersion. Thus a lower concentration of interest can be accurately determined if a larger volume is injected. ***T1*** reports a comparison of three different diameter microbore columns: 0.32, 1, and 2 mm i.d. columns. The detection limit (signal to noise ratio of 5) can be expressed in two ways: detection limit in amount or detection limit in concentration. When a 0.32 mm column was used, the detection limit in amount was 40 fg while the sample concentration limit was 80 fg/µL for 0.5 µL injection. When the 2 mm column was used, the de-

F3

Stability of serotonin in pH 6 Ringer's solution Temperature: 35° C.



tection limit in amount was 400 fg while the concentration was only 8 fg/µL with 50 µL injected. The smallest column gave the lowest detection limit in amount, while the largest column gave the best detection limit in concentration if adequate sample was available. Dispersion on the 2 mm column was much less than for the 1 mm column when 50 µL of 5-HT solution was injected. The three different size columns have their own advantages. Microbore 0.32 and 1 mm columns are generally suitable for small volume samples such as microdialysates. The 2 mm column is generally suitable for large volume samples with low concentrations such as biosample extraction supernatant. For best column performance (minimum peak dispersion), an injection volume greater than 5 µL is usually not recommended for a 1 mm column, although in some cases 20 µL or larger of a very hydrophobic sample can be loaded with little peak dispersion due to a trace enrichment effect on the top of the column (9). For samples with large volumes and low concentrations, the 2 mm column is a good choice.

A 400 µL/min. flow rate was used most often for the 2 mm column. At this flow rate the mobile phase velocity through the 2 mm column was equivalent to 100 µL/min. through a 1 mm microbore column. The retention and the back pressure of the two columns were comparable; however, the pump piston moves four times faster to reach the 400 µL/min. flow rate. Faster piston movement increases the moving frequency and increases the "noise" frequency, which can be more easily dampened hydraulically and filtered electronically. In our experiment, the baseline noise at 400 µL/min. was 30% lower compared with 100 µL/min. Although the cross sectional area of the 2 mm column is four-times larger than that of the 1 mm column, the detection limit in amount was not four-times higher because of the lower noise level and higher column performance (***T1***). The detection limit for 5-HT was 0.4 pg for each injection for a 2 mm column and 0.15 pg for a 1 mm column (5 µL was injected). Serotonin in homogenized dog intestines and rat brains was easily determined using this 2 mm PEEK column.

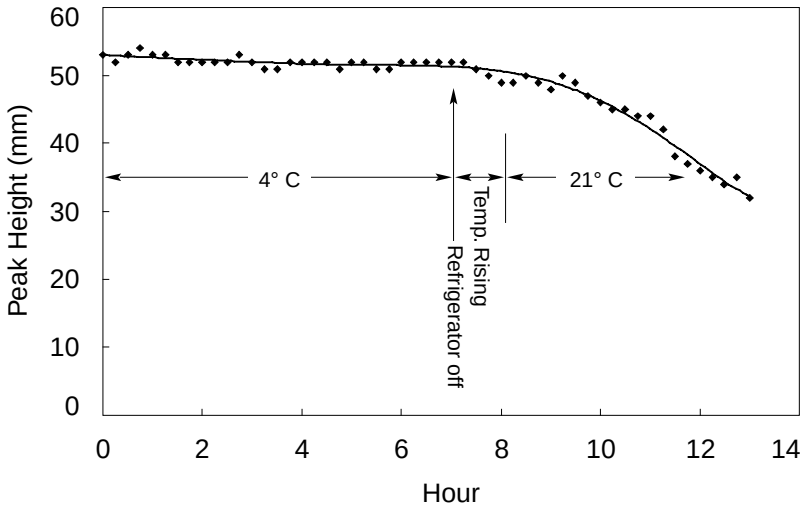
It is well known that smaller microbore columns have the advantage of higher mass sensitivity, but they also have the practical disadvantage of being susceptible to clogging. These columns are ideal for microdialysates where the membrane filters out particulates. The column end is so small that clogging is not uncommon if used for tissue or blood samples. A 2 mm column, with the larger cross sectional area, is less likely to be clogged when used for tissue homogenates. If the linear velocity through the two columns is the same, their clogging probability due to particles present in the mobile phase should be the same. However, the clogging probability due to insoluble material present in samples is not the same. If insoluble particles or proteins exist in a dirty sample, they will be injected into the columns. Thus the four-times smaller column will be four-times easier to clog with the same amount of injected particles. The longevity of a 2 mm column will be roughly four-times longer than the 1 mm column if such dirty samples are injected. In this study, a 2 mm PEEK column was used for homogenized tissue samples for six months without clogging. The column back pressure remained at about 2300 psi at 400 µL/min., and no significant back pressure increase was noticed. Needless to say, a filtered mobile phase and an efficient sample preparation are also very important. Homogenized samples should be thoroughly denatured, centrifuged and filtered. Otherwise particulates or protein will quickly clog columns no matter how large they are.

The degradation of 5-HT was studied. Standard solutions containing norepinephrine (NE), epinephrine (EPI), 3,4-hydroxyphenyl acetic acid (DOPAC), dopamine (DA), 5-hydroxyindole-3-acetic acid (5-HIAA), homovanillic acid (HVA), and serotonin were prepared. We repeatedly injected 5 µL of the standard solution containing 20 to 50 pg of each of the above compounds under different conditions for 12 hours and measured the peak heights to evaluate their degradation. These results showed that serotonin stability is influenced by many factors. Temperature, pH, sample matrix, vial material, and some metal ions play very important roles in the degradation of all of these compounds.

In our study serotonin was very stable both in Ringer's solution (pH

F4

Temperature effect on serotonin stability at pH 0.85.



T2

Conditions related to serotonin stability.

Condition	Effect
pH	Decomposed fast in acidic solution (pH 3 or lower at room temperature. Stable at pH 6 to 7.4 even at room temperature.
Temperature	Stable at low temperature (4° C or lower), even in acidic condition. Stable at high temperature (35° C or lower) if in pH 6.
Degassing Sample	No effect
Fe^{+++}, 10 µg/mL, pH 6	Decomposed very fast
Fe^{++}, 10 µL/mL, pH 6	Decomposition increased slightly
Mg^{++}, 0.2 M, pH 7.4	Peak distorted
Cu^{++}, 10 µg/mL, pH 6	No significant effect
Polyethylene or glass vials	No effect
EDTA	Chelated the metal ions and reduced decomposition.

6) and artificial CSF solution (pH 7.4). A standard solution of 8 pg/µL serotonin was prepared using pH 6 Ringer's solution and kept at 35° C. Repeatedly injecting this warm solution for 12 hours gave the results shown in ***F3***. During this 12 hour period, the serotonin concentration declined only 7%, which was equivalent to 0.6% per hour. Unlike catecholamines, serotonin does not favor acidic conditions. If serotonin was kept in a pH 2 solution, it decomposed rapidly at room temperature (21° C). However, it was still relatively stable at low temperature (4° C) even at this low pH. ***F4*** illustrates a test of serotonin stability in 5% trichloroacetic acid solution at pH 0.85. For the first seven hours, the solution was in the refrigerated autosampler at 4° C. 5-HT was stable at this low temperature. Only 2% of the solution decomposed over this seven hour period (0.3% per hour). After the Peltier cooling was turned off and the temperature increased, the serotonin concentration dropped very quickly. The decomposition rate increased up to 8% per hour. These results confirm that low temperature is important when collecting samples such as microdialysates. In many microdialysis applications, catecholamines and serotonin are measured simultaneously. For catecholamines, samples should be pre-acidified to pH 2 or lower for stabilization, but at this low pH serotonin is not stable. According to the results of ***F3***, if the vials are kept in a cool environment, 5-HT will decompose slowly and both catecholamines and serotonin can be measured correctly. In the dog intestine and rat brain homogenizing procedures, which were performed at room temperature, all reagents and test tubes were pre-cooled to 4° C. Trichloroacetic acid solution was added for denaturing the samples. After denaturing and homogenizing, a pH 6 phosphate buffer was added to adjust the pH to 6 and stabilize 5-HT in the following centrifuge and filter procedures. Some articles recommend preparation of serotonin solution with 0.2 M perchloric acid (10). According to our study, pH 6 phosphate buffer protected serotonin better and was suitable for preparing the stock solution. The 0.2 M acid was good only for preparing catecholamine standard solutions.

The conditions which influenced the degradation are illustrated in ***T2***. In addition to pH and temperature, Fe^{+++} dramatically increased the degradation and Fe^{++} slightly increased the degradation. Cu^{++} did not show a significant influence on 5-HT, although it significantly speeded up the degradation of NE, EPI, DA, DOPAC, and 5-HIAA. The metal ion concentration was 10 µg/mL. Mg^{++} at 0.48 mg/mL did not influence serotonin degradation, but if the concentration increased to 4.8 mg/mL, the serotonin peak was distorted while the DA, 5-HIAA, DOPAC and HVA peak shapes did not change. Other metal ions were not tested, but there may be more metal ions accelerating the serotonin degradation. Metal ion contamination of serotonin samples should be avoided.

The following two methods reduced metal-accelerated degradation: reducing the pH of the solution to pH 2 or lower, or adding EDTA to chelate the metal ions. Since low pH was not good for 5-HT stability, the other method of adding EDTA was acceptable for stabilizing serotonin. EDTA concentration of 10 times higher than the metal concentration was adequate for chelating. Usually 0.5 mM EDTA should be enough to protect 5-HT in samples.

Degassing the sample solution was useless for stabilizing 5-HT. Ringer's solution was bubbled with helium for 10 minutes for degassing and then used for preparing serotonin standard solutions. There was no difference between using degassed and non-degassed solution for preparation of serotonin solution. Rigorous deoxygenation would probably be of great help; however, this is not easily done with autosamplers where oxygen diffusion through septa is always a concern. Sample vial material was also tested. DOPAC decomposed faster in polyethylene vials than in glass vials, but polyethylene did not influence 5-HT degradation.

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