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Microfluidic Separation and Electrochemical Detection of Serotonin Using a Portable Lab-on-a-Chip Device

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Abstract- Using a lab-on-a-chip device, an analytical method was developed and validated for the quantitative determination of serotonin. Serotonin is a neurotransmitter that has a number of roles in biological processes. It is essential to be able to detect and quantify serotonin in different biological fluids because it can be used to evaluate and diagnose several disorders and diseases including depression, anxiety, Parkinson's disease and Alzheimer's. It is also beneficial to study and research the physiological roles that this neurotransmitter is involved in. Many of the conventional analytical methods require expensive instrumentation and reagents, time consuming pretreatment and derivation processes, and long analysis time. The developed method utilized microfluidic electrophoresis separation coupled with electrochemical detection. The limit of detection and limit of quantitation of serotonin were found to be 1.57 μM and 5.22 μM, respectively. The calibration curve showed a linear range between 25 μM and 500μM with a correlation coefficient of 0.9851. Accuracy was evaluated at low (50 μM), middle (200 μM), and high (500 μM) concentrations; achieving over 93% recovery with good reproducibility (%RSD>6.6%). Lastly, robustness was determined for each of the experimental conditions such as separation voltage, injection voltage, detection potential, and pH of the buffer. This novel analytical technique for serotonin detection offers many advantages including high speed analysis, great versatility, low cost, portability for on-site detection, negligible consumption of reagents/samples, and negligible waste generation. Furthermore, with modifications this method could be applied to detect serotonin in different biological fluids.

Keywords- Serotonin, Microfluidics, Microchip Electrophoresis, Neurotransmitters, Lab-on-a-Chip

1. INTRODUCTION

Serotonin, also known as 5-hydroxytryptamine (5-HT), is a biogenic monoamine that plays a crucial role in many biological processes [1]. It is primarily found in the central nervous system (CNS), enterochromaffin cells of the gastrointestinal tract, and the platelets [1]. Over 90% of serotonin in the body can be found in the enterochromaffin and platelets; the platelets are the major storage [2]. It was originally isolated and studied for its vasoconstrictor properties that were found to cause contractions of the smooth muscle. However, its most recognized role is as a neurotransmitter in the CNS [2]. Neurotransmitters act as primary chemical messengers released by the pre-synaptic nerves that relay, amplify, and transmit the signals to post-synaptic cells and play a vital role in neuronal communication of CNS [3].

Serotonin has an important role in different psychological and behavioral disorders and corresponding pharmaceutical treatments. It has been established that decreased availability of serotonin in the CNS is associated with psychological disorders such as depression, mania, bipolar, and anxiety [3]. In addition, serotonin has been linked to late-onset neurodegenerative diseases such as Parkinson's and Alzheimer's diseases [3]. The serotonergic system is specifically known for its diverse physiological roles in regulating temperature, appetite, sleep cycles, emesis, and sexual behavior [3]. Other biological roles of importance include regulation of gastrointestinal motility, vascular tone such as vasoconstriction and vasodilation, and platelet aggregation. It has also been implicated to have an effect on migraines, irritable bowel syndrome (IBS), hypotension and hypertension [2]. These are only some of the various ways that quantitation of serotonin can be of significant use in research. One specific illustration, not mentioned previously, is platelet-related bleeding disorders such as Hermansky-Pudlak syndrome. Serotonin on platelets can serve as a marker on platelet dense granules. Its deficiency can be used to diagnose disorders of dense granule deficiency.Without the means to quickly and accurately diagnose these diseases, the patients suffer and lack beneficial clinical intervention [4]. It is essential to be able to measure serotonin concentration in different biological fluids because it can be used as a diagnostic tool for many diseases. Further clinical relevance is to investigate drug action to the specific pathways involved which is important for pharmacotherapy.

Several techniques have been developed to quantify neurotransmitters taking advantage of their electrochemical properties and their important physiological roles. Past and current methods include high performance liquid chromatography (HPLC), fluorometric detection, capillary electrophoresis, coulometry, mass spectrometry, spectrophotometric technique, and flow injection analysis [3,5-6]. Although many of these analytical methods have low detection limits and good reproducibility, they have several drawbacks as well. Many of these conventional methods require expensive instrumentation and reagents, time consuming pretreatment and derivation processes, labor, and long analysis time [3,5]. Therefore, many

developments of several different analytic methods have been made to lower costs, labor, and analysis time.

Microchip electrophoresis (MCE) is a novel analytical technique that employs capillary electrophoresis (CE) with the use of the growing field of microfluidics. It is considered to be one of the first strides towards a “true”Lab-on-a-Chip (LOC), which integrates all the steps of an analytical process on a single compact microfluidic device [5]. These devices offer several advancements such as high speed, great versatility, high throughput, low cost, performance of parallel assays, portability and miniaturization, automation, and negligible consumption of reagents/sample and waste generation. However, these microdevices require a miniature and sensitive detection system. Electrochemical detection (ED) in conjunction with these capillary electrophoresis microchips has been proven to be more effective, in comparison to other detection techniques, due to characteristics such as inherent miniaturization, sensitivity, low cost, portability, short measurement time, and compatibility with other microfabrication technology [5]. Using ED is the simplest proposed detection method because the neurotransmitter can be detected directly, without derivation, due to their intrinsic electrochemical behavior [7]. The most widely employed electrochemical technique for MCE has been amperometric detection, since it was first reported in 1998 by Wolley et al [8]. The precision and sensitivity of the proposed microfluidic platform is comparable to previous reports using other conventional methods based on CE and HPLC [5]. Detection limits cited with these devices have typically been in the nM to μM range [8].

The electrochemical detector for the SU-8/Pyrex microchip is integrated at the end of the separation channels. The ED system is based on three platinum electrodes: working, reference and auxiliary in respect to position at the outlet of the separation channel. The electrochemical technique is based on oxidation and reduction reactions (electron transfer reactions) which take place on the working electrode surface. The electrochemical oxidation that is responsible for the electrochemical response of serotonin at the working electrode is shown in Fig. 1.

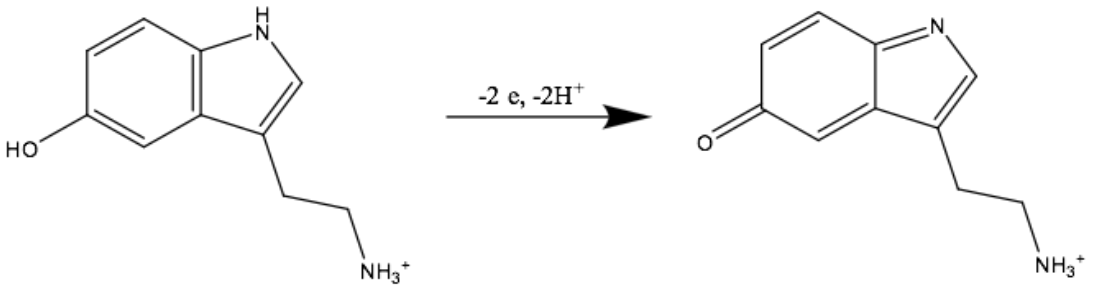

Fig. 1. Electrochemical oxidation of serotonin

2. EXPERIMENTAL

2.1. Chemicals

Serotonin HCl, 4-Aminophenol (p-Aminophenol), and 2-(N-Morpholino)-ethanesulfonic acid hydrate (MES hydrate) were all purchased from Sigma-Aldrich (St. Louis, USA). Serotonin HCl and p-Aminophenol (pAP) stock solutions were prepared with 0.1 N hydrochloric acid with concentrations of 5 mM and 10 mM , respectively. The 20 mM MES running buffer was prepared with ultra-pure water (Millipore Corporation, Billerica, MA, USA). The pH of the buffer was adjusted to 6.0 using standard NaOH. Serotonin HCl and pAP solutions were prepared daily in the running buffer. All solutions were filtered using 0.2 μm syringe filters (Fishers Scientific, Waltham, MA) before use in the microchip to ensure the removal of small particles which can block the microchannels. Stock solutions were stored in a refrigeration unit and covered with aluminum foil to prevent decomposition.

2.2. Washing Procedure/Pretreatment

Before running the samples, the SU-8 pyrex microchip was rinsed using a high-voltage washing protocol [9]. The importance of washing the microchip is to clean the electrodes for a more sensitive and precise electrochemical detection. The three different solutions used for the procedure, in respect to the order that they are used, include 0.1 M NaOH, ultra-pure water, and the 20 mM MES running buffer solution pH=6.0. After washing, the baseline was achieved by applying the high voltage (1000 V) between A and B reservoirs (Fig. 2), and the detection potential at 0.8 V. The baseline was stabilized, signaling the instrument ready for experimentation.

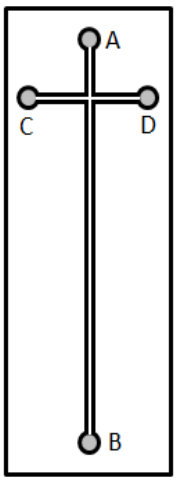

Fig. 2. Microchip Design

2.3. Instrument Testing

The MicruxHVStat portable instrument (high voltage power supply and potentiostat), microchip holder, and SU-8/pyrux microchips with integrated platinum electrodes, were evaluated for proper working conditions using pAP testing protocol procedure [9]. Phenolic compounds such as pAP are used because they have a well-known electrochemical behavior. After obtaining a stable baseline, the running buffer in reservoir C was vacuumed out and filled with a filtered solution of 500 μM pAP diluted with the running buffer. pAP analysis conditions included an injection voltage of 750 V between reservoir C and D with a short injection time of 3 seconds and separation voltage of 1000 V between reservoir A and B with a separation time of 70 seconds (Fig. 2). The amperometric mode was DC with a detection potential (WE1) of 0.8 V. The instrumentation was assumed to be in proper working condition when a peak was seen at a consistent migration time, after multiple runs, just before 30 seconds. Limit of detection (LOD) and limit of quantitation (LOQ) were also calculated for pAP to check the sensitivity.

2.4. Detection of Serotonin

A 250 μM serotonin solution diluted with the running buffer was analyzed using the procedures used to analyze pAP [9] as a starting point. The expectation was to find a peak with a consistent migration time. To further optimize the electrochemical detection of serotonin the buffer solution composition and pH, the concentration of the samples as well as the conditions for the injection, separation, and detection of the samples of serotonin were evaluated and adjusted accordingly. The optimal conditions were assumed to be those with peak parameters that had the largest peak area, largest peak height, smallest width and half width, and largest number of theoretical plates (N).

2.5. Method Validation

Method validation was done upon completion of optimization, which included: limit of detection (LOD), limit of quantitation (LOQ), linearity, accuracy or recovery at low, middle, high concentration levels, and robustness. To determine linearity, a calibration curve was constructed with serotonin solutions ranging in concentration from 25 μM to 500 μM. Recoveries were performed using three individual samples at three different concentration levels; the concentration levels include low concentration (50 μM), middle concentration (200 μM), and high concentration (500 μM). Each individual sample was injected and run three times; the areas of the last two runs were averaged and the concentration was calculated using the linear equation. This was then used to find the percent recovery of each sample within a concentration level, average percent recovery of the three samples for each concentration level, and the relative standard deviation. Lastly, robustness was determined

by changing the optimized conditions slightly above and below. Separation voltage and injection voltage were changed by ±10% of the optimized condition. Detection potential was changed by ±0.1 V and the pH of the buffer was adjusted within ±0.2 pH units.

3. RESULTS AND DISCUSSION

The MicruxHVStat portable instrument was tested with pAP. The testing conditions included: a buffer composition of 20 mM MES-NaOH pH 6.0, an injection voltage of 750 V (from reservoir C to D), a separation voltage of 1000 V (from reservoir A to B), and a detection potential of 0.8 V. A consistent peak for 500 μM sample of pAP was achieved and a calibration curve was constructed between 25 μM and 750 μM. The linearity yielded a correlation coefficient value of 0.9987. The LOD and LOQ of pAP were found to be 1.48 μM and 4.93 μM, respectively.

In the analysis of new compounds, conditions such as the buffer composition and pH, separation voltage, injection voltage, and detection potential should all be evaluated and optimized for the proper methodology. Each of these parameters has a potential effect on the microchannels and detection system. The electrochemical response of the detection system of the Micrux SU-8/Pyrux microchip was studied using serotonin to develop a proposed methodology for serotonin specifically. This methodology was then validated using limit of detection (LOD), limit of quantitation (LOQ), linearity, accuracy or recovery at low, middle, high concentration levels, and robustness.

The first parameters evaluated and optimized were the pH of the 20 mM MES-NaOH buffer and the separation voltage. The buffer composition and pH influences the electroosmotic flow (EOF) and the charge of the analyte affecting the rest of the parameters for efficient separation and detection [5]. The buffer was studied at pH 4.0, 6.0, and 7.0. SU-8 presents the highest electro-osmotic mobility at pH≥7; therefore the pH was not studied beyond that [8]. The results for pH 4.0 were inconsistent and not reproducible and therefore were not reported. At pH 6.0, the electrochemical response was studied at separation voltages 500 V, 1000 V, and 1500 V. At pH 7.0, the electrochemical response was studied at separation voltages 500 V, 750 V, 1000V, 1250 V, and 1500 V. The optimized condition was chosen to be pH 7.0 with a separation voltage of 1000 V because it gave the best instrumental signal.

Using 20 mM MES-NaOH buffer pH 7.0 and a separation voltage of 1000 V, the injection voltage was evaluated at 500 V, 750 V, and 1000 V; all applied for 3 s. The greatest instrumental signal is displayed at 750 V. Lastly, the detection potential was evaluated for the new conditions by constructing a hydrodynamic voltammogram (HDV) shown in Fig. 3. The HDV was made for a 250 μM sample of serotonin with a separation voltage of 1000 V and an injection voltage of 750 V applied for 3 s. This curve was created by plotting the peak height (nA) given at each 0.2 V increment of the detection potential. The HDV showed the

highest point in peak height to be at 0.8 V and then a slight decrease after; therefore 0.8 V was applied as the optimal detection potential.

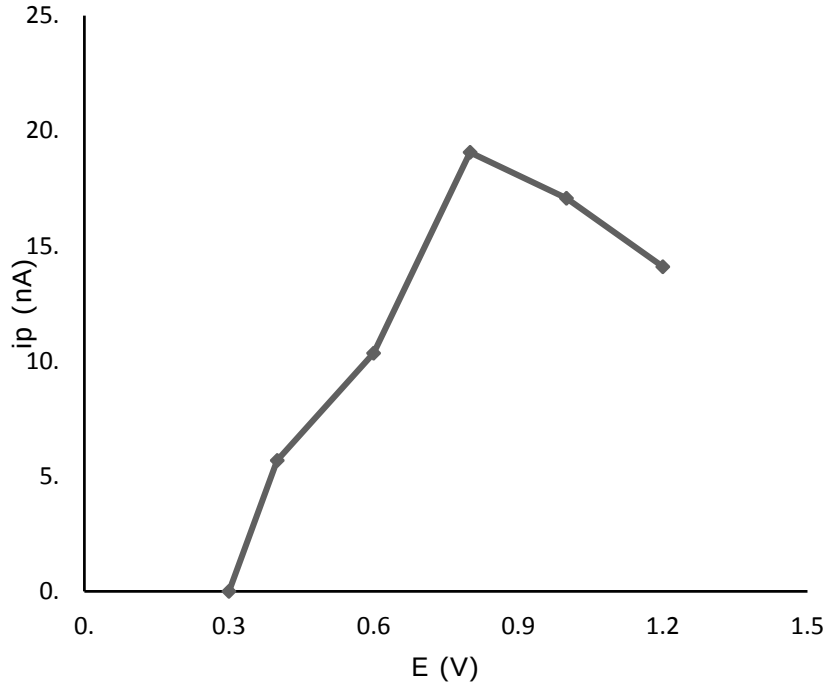

Fig. 3. Hydrodynamic voltammogram for 250 μM serotonin. Conditions: running buffer: 20 mM MES-NaOH pH=7.0; separation voltage=1000 V, and injection voltage=750 V for 3 s

After the optimized conditions were proposed for the analysis of serotonin, the methodology was validated. A calibration curve for serotonin was constructed for the peak area and the different concentrations of serotonin. The electropherograms for different concentrations of serotonin can be seen in Fig. 4. The results showed good linear relationship between 25 μM and 500 μM with a correlation coefficient of 0.9851 obtained for serotonin (Fig. 5). The LOD and LOQ of serotonin were found to be 1.57 μM and 5.22 μM, respectively. Accuracy was evaluated at low (50 μM), middle (200 μM), and high (500 μM) concentrations. Over 93% recovery was achieved with good reproducibility at low, middle, and high concentrations. The percent recovery and reproducibility did slightly decrease with increased concentrations. Lastly, robustness was determined for each of the conditions that were evaluated for the proposed methodology. The optimized conditions still yielded decent results, recovering at least 83% of the original peak area for all evaluated conditions.

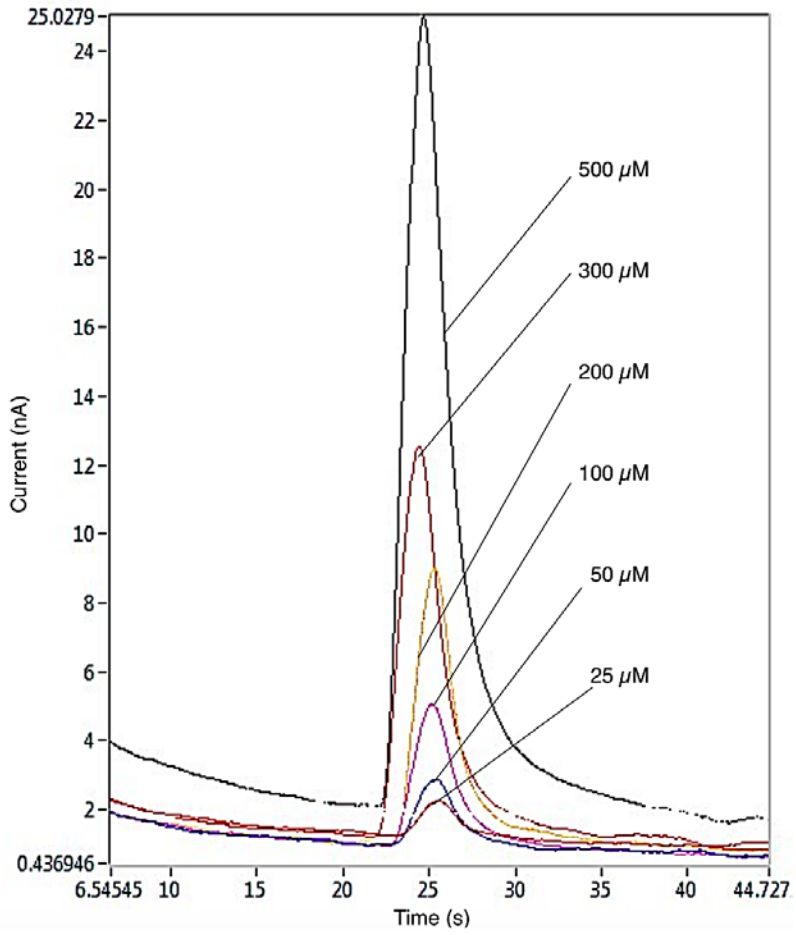

Fig. 4. Electropherograms for different concentrations of Serotonin

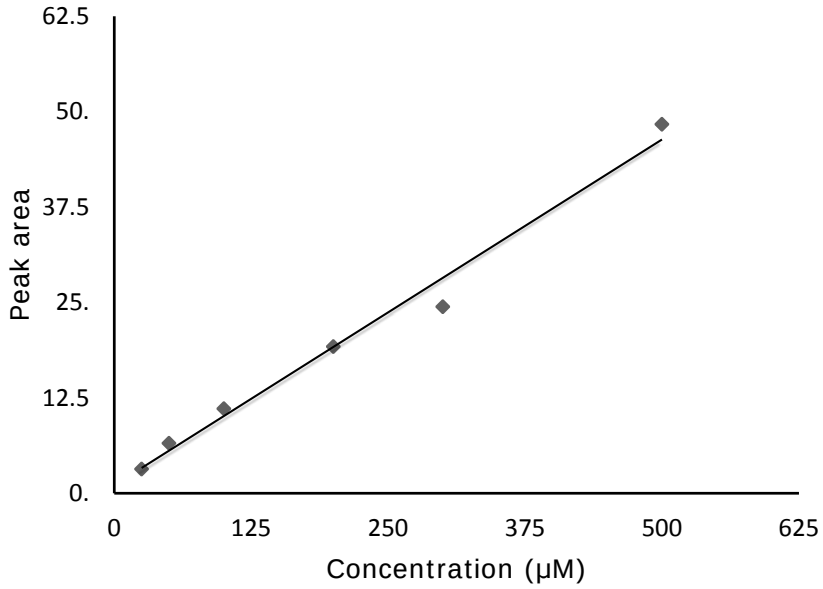

Fig. 5. Calibration curve (y=0.0906x+1.0512) for serotonin with a correlation coefficient (R^2) of 0.9851

Table 1. Recoveries & Reproducibilities of Serotonin at Low, Middle, and High Concentrations

Serotonin Concentration Level	Sample Solution #	Percent Recovery (%)	Average Recovery (%)	Relative Standard Deviation (%)
Low Concentration (50 μM)	1	95.8	99.7	3.4
	2	101.9		
	3	101.4		
Middle Concentation (200 μM)	1	104.1	98.5	5.9
	2	98.9		
	3	92.5		
High Concentration (500 μM)	1	99.9	93.4	6.6
	2	92.8		
	3	87.6		

Table 2. Robustness results. (The bolded conditions are the proposed condition)

Parameter Optimized	Change in Parameter	Migration time (s)	Area of the Original Condition (%)
Separation Voltage (V)	900	24.10	99.3
	1000	21.68	**100**
	1100	19.45	89.1
Detection Potential (V)	0.7	21.18	83.2
	0.8	25.47	**100**
	0.9	24.28	95.3
Injection Voltage (V)	675	18.43	93.4
	750	19.73	**100**
	825	18.51	93.8
pH of the Buffer	6.8	22.06	87.9
	7.0	25.09	**100**
	7.2	28.31	88.6

Table 3. Comparisons of the proposed method for the detection of serotonin with other available methods

Detection Method	Linear range, μM	LOD, μM	Retention Time, min	Recovery, %	Ref.
Square-wave adsorptive stripping voltammetric determination of serotonin at glassy carbon electrode modified with safranine O.	0.03-10	0.005	_	_	[10]
Selective determination of serotonin on poly(3,4-ethylenedioxy pyrrole)-single-walled carbon nanotube-modified glassy carbon electrodes	0.1-10	0.005	_	_	[11]
Electrochemical determination of serotonin on glassy carbon electrode modified with various graphene nanomaterials	1-100	0.005	_	_	[12]
HPLC-amperometric detection of serotonin in urine	5-500	0.02	12.3	78.2 at 5.67E-7 M	[13]
Microfluidic electrochemical detection of serotonin using a portable lab-on-a-chip device	25-500	1.57	0.5	93.4 at 5.00E-4 M	This work

4. CONCLUSION

This study presents the development and validation of a microfluidic microchip electrophoresis methodology to separate and detect serotonin using a SU-8 pyrex microchip. Linearity was achieved between 25 μM and 500 μM with detection limits of 1.57 μM. According Table 3, this method shows larger linear range (25-500 μM), fast separation (~30 s), and good recovery (93.4%) compared to the other available methods for serotonin analysis. Further work needs to be done with real biological samples and mixtures before this technique can be applied for detecting and quantifying serotonin in the biochemical/biomedical field. This is a relatively new method and its promising applications can bring great advantages in improving clinical, pharmacological, forensic, environmental, and other industrial analysis. Due to the simplicity of the samples, results from this study can be useful in the pharmaceutical field. Also, we tested blank sample of synthetic urine which showed no signs of any matrix effects. This means the compounds presents in urine does not affect the serotonin detection by this method. The proposed method can detect serotonin in less than 70 seconds and requires a small amount of a particular sample per run. Also, the microchips are relatively inexpensive. Thus, the complete platform which consists of the

potentiostat, the reusable holder, and the microchips has been proven to be very economical in sample analysis.

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