Journal of Chromatography B, 895–896 (2012) 182–185

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Short communication

Reverse injection capillary electrophoresis UV detection for serotonin quantification in human whole blood

Angelo Zinellu[a,*], Salvatore Sotgia[a], Luca Deiana[a,b], Ciriaco Carru[a,b,*]

[a] Dept. Biomedical Sciences, University of Sassari, Italy
[b] Medicina di Laboratorio – Azienda Ospedaliero Universitaria, Sassari, Italy

ARTICLE INFO

Article history:
Received 7 December 2011
Accepted 19 March 2012
Available online 26 March 2012

Keywords:
Serotonin
Capillary electrophoresis
Reverse injection
Tris phosphate

ABSTRACT

We describe the first capillary electrophoresis UV detection method to measure serotonin in human whole blood (WB). Procedural parameters such as concentration and pH of run buffer and injection mode were investigated. The reverse injection allows to decrease the analysis time by injecting samples at the outlet end of the silica capillary close to the detection window, so reducing the migration distance. Thus, when a capillary with an effective length of 10 cm and a 400 mmol/L Tris phosphate as background electrolyte at pH 3.25 was used, the migration time of the serotonin peak was 2.6 min. These conditions gave a good reproducibility of migration times (CV, 0.77%) and peak areas (CV, 2.44%). Intra- and inter-assay CV were 3.85% and 7.32%, respectively, and the analytical recovery was between 96.8% and 99.4%.

1. Introduction

Serotonin (5-hydroxytryptamine, 5-HT) is an established neurotransmitter and vasoconstrictor mainly located in the enterochromaffin cells of the intestine, where acts as a regulator of the intestinal movements [1]. All the blood 5-HT is found in platelets which do not synthesize it, but take it from plasma where it is released by enterochromaffin cells [2]. A minor amount of 5-HT is synthesized in the serotoninergic neurons of the brain, where 5-HT is involved in the elaboration of an adapted response of the central nervous system to external media [3]. Serotonin is secreted from platelets at sites of endothelial injury [4] where causes local vasoconstriction [1] and promotes thrombogenesis, mitogenesis, and proliferation of smooth muscle cells [5]. The serotonin system contributes to complex traits, including cognition, affect, endocrine regulation, neurotrophic effects, pain, appetite, emesis, sex, sleep, aggression, perception, sensory-motor function, and vascular and gastrointestinal regulation [6]. Elevated serotonin levels may indicate the presence of a carcinoid tumor [7]. Even within normal limits, variations of serotonin levels have been associated with various psychiatric conditions including anorexia, anxiety, depression, schizophrenia and others [8]. The first consideration when designing a protocol to measure serotonin is the specific blood fraction to be assayed. Matrix choices include whole blood, platelets, platelet-rich plasma (PRP centrifuged at low g), and platelet-poor plasma (PPP centrifuged at high g). More than 95% of serotonin is found in the platelets [2], but its release into plasma occurs easily [9]. The measurement of the much smaller pool of platelet-poor plasma has been proven much more difficult, with a wide range of reference values reported [9]. The main difficulties are due to the extremely low concentrations of serotonin present in PPP and in preparing PPP without contamination from platelet-derived serotonin. Therefore, many researchers have focused on the measurement of serotonin in whole blood because it is simpler than preparing blood fractions and also because this procedure allows to reduce the above mentioned pitfalls. WB serotonin was measured principally by HPLC coupled with either electrochemical [10–12] or fluorimetric detection [7,13] while literature lacks of a capillary electrophoresis method for the human whole blood serotonin measurement. At now, in fact, few CE methods have been described for the quantification of 5-HT in plasma by using complex detection systems [14–16]. By this work we have aimed to develop the first CE-UV assay with reverse injection mode for a rapid measurement of WB serotonin levels.

2. Materials and methods

The blood samples from 20 healthy volunteers were collected by venipuncture into EDTA-containing tubes and immediately processed. 200 μL of whole blood were mixed with 400 μL of cold water and the samples were sonicated for 10 min in a ultrasonic water bath in the dark. 1.2 mL of cold acetonitrile was added and

Abbreviations: CE, capillary electrophoresis; 5-HT, 5-hydroxytryptamine; WB, whole blood.

* Corresponding authors at: Dept. Biomedical Sciences, University of Sassari, Viale San Pietro 43/B – 07100 Sassari, Italy. Fax: +39 079228275.
E-mail addresses: azinellu@uniss.it (A. Zinellu), carru@uniss.it (C. Carru).

1570-0232/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2012.03.024

Table 1
Comparison between the electrophoretical parameters obtained by normal and reverse injection mode. Analysis was performed with standard serotonin 2.5 μmol/L.

	Migration time (min)	Peak height (AU)	Efficiency (N/m)	Peak width (min)	Peak asymmetry
Normal injection	7.53	5278	96,510	0.253	1.064
Reverse injection	2.63	7494	24,579	0.158	0.969

the samples were vortex-mixed and then centrifuged. 1.5 mL of supernatant was evaporated under vacuum and the residue was redissolved with 50 μL of water. The sample was then injected in CE. A MDQ capillary electrophoresis system equipped with a diode array detector was used (Beckman instruments, CA, USA). The system was fitted with a 30 kV power supply with a current limit of 300 μA. The analysis was performed in an uncoated fused-silica capillary, 40 cm long × 75 μm I.D., 30 cm (normal injection) or 10 cm (reverse injection), injecting 90 nL of sample (0.5 psi × 10 s) at the capillary end. The separation was carried out in a 400 mmol/L Tris as background electrolyte (BGE) titrated with 1 mol/L phosphoric acid to the pH 3.25, 20 °C and 14 kV (250 μA) at reverse polarity. After each run, the capillary was rinsed with, 0.5 min (at 0.5 psi) of 0.5 mmol/L NaOH, 0.5 min (at 0.5 psi) of water, 0.5 min (at 0.5 psi) of 0.1 mmol/L HCl and equilibrated with run buffer for 1 min.

All data are expressed as mean ± SD.

3. Results and discussion

In order to optimize the electrophoretic separation of serotonin in WB, preliminary experiments were carried out by using a capillary with an effective length of 30 cm. We started from the electrophoretic conditions of our previous CE assay for the measurement of small basic analytes in which good separations were reached by using the Tris phosphate as effective BGE [17]. Background electrolyte at different concentrations (from 50 to 400 mmol/L) and different pH values (from 2 to 4 pH units) was employed. Moreover, we tested the effect of cartridge temperatures by increments of 5 °C from 20 to 45 °C. A good separation between serotonin and contaminant peaks was obtained by the use of 400 mmol/L Tris phosphate BGE, pH 3.25, 14 kV and 20 °C. As shown in Fig. 1A, under these conditions, with a normal injection configuration, the long distance to cross towards the cathodic detection window caused extended analysis time, so that the serotonin standard was detected in 7.5 min. To reduce the migration distance as much as possible, we injected the serotonin at the end of the capillary, near to the detection window, under the same electrophoretic conditions (polarity of the electrodes was obviously switched over). In this case, the migration distance for serotonin was reduced to about 10 cm and migration occurred in less than 3 min (Fig. 1B). We compared the analysis time, the peak height, the efficiency and the asymmetry of peaks obtained in reverse and in normal injection mode (Table 1). A higher efficiency could be observed by using the normal injection, but the migration time for serotonin decreased from 7.5 to 2.6 min and peak height rose, giving an increase of 42% in sensitivity when the reverse injection mode was employed. To evaluate the best wavelength for the detection we performed an absorbance spectra between 190 and 400 nm in the selected run buffer for all the analytes. As reported in the inset of Fig. 1B, a maximum absorbance was observed at 198, 220 and 271 nm. 220 nm wavelength was set for the following experiments since it allowed a good absorbance for serotonin peak and lower signal intensity for contaminant peaks, if compared with traces at 198 nm. The signal of serotonin peak at 271 was too low to allow its quantification in whole blood.

Since the sample analysis needed protein elimination we evaluated the best condition for the sample processing. Direct sample filtration with Microcon centrifugal filters was not recommended due to the elevated costs, while acidic precipitants like trichloroacetic or metaphosphoric acid were avoided because their presence in the sample matrix prevented the possibility to inject large volumes onto the capillary since they might provoke voltage leak during the CE run. Moreover, the decrease of peak efficiency after acidic precipitation reduced the peak signal thus further lowering the assay sensitivity. Hence, we decided to perform protein precipitation with acetonitrile. Starting from 200 μL of whole blood after precipitation and evaporation, we redissolved the dried residue in 50 μL of water thus increasing sensitivity of about 4 fold. Fig. 2 shows the electropherogram obtained from a WB sample in the adopted electrophoretical conditions. For

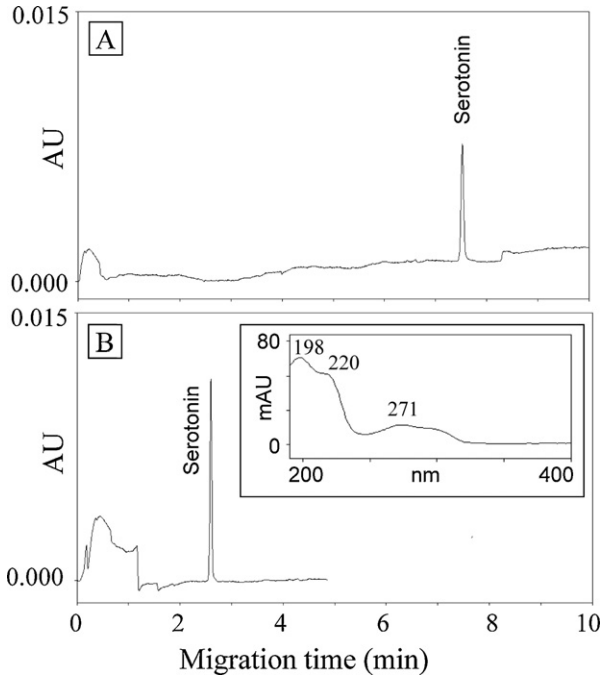

Fig. 1. Electropherograms of standard serotonin using (A) normal injection and (B) reverse injection mode (serotonin concentration: 10 μmol/L). Electrophoretic conditions: fused-silica capillary, 40 cm total length, 30 cm effective length for (A) and 10 cm for (B); background electrolyte, 400 mmol/L Tris phosphate, pH 3.25; applied voltage, 14 kV, temperature, 20 °C; detection, 220 nm. Inset shows the serotonin UV spectra between 190 and 400 nm.

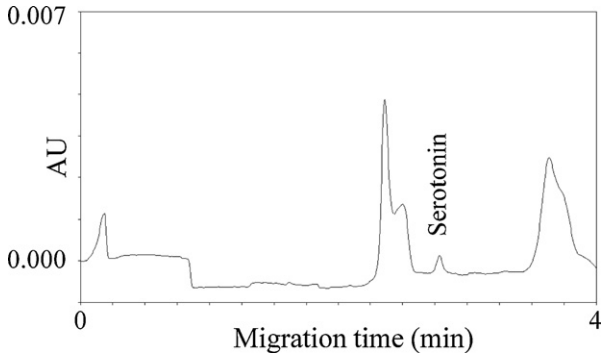

Fig. 2. Electropherogram of WB sample obtained by CE with reverse injection configuration mode (serotonin concentration: 635 nmol/L).

identity check, the UV/VIS spectrum of the analyte peak was compared to the spectrum of reference standard.

To minimize the potential bias due to sample matrix effects, calibration curves were prepared by spiking to 0.2 mL of WB increasing amounts of standard serotonin (between 0.25 and 2 μmol/L).

The calibration curve $Y = 612X - 14$ showed a linear response over the concentration tested with a regression coefficient $R^2 = 0.999$. The injection reproducibility was calculated by injecting ten times consecutively the same sample. The within-run precision (intra-assay) of the method was evaluated by measuring the same biological sample 10 times in the same sample set, while the between-run (inter-assay) precision was determined by measuring the same biological sample on 5 consecutive days. The precision tests indicate a good repeatability of our method, both for migration times (CV = 0.77%) and areas (CV = 2.44%). Moreover, a good reproducibility of intra- and inter-assay tests was obtained (CV = 3.85% and CV = 7.31% respectively). For the assessment of the analytical recovery, WB was spiked with standard solutions of serotonin at three different concentrations (0.25, 0.5, 1 μmol/L), and the mean of recovery, evaluated by five different experiments, was between 96.8% and 99.4%. The lowest limit of quantification (LOQ) for a S/N of 10, determined on a real WB sample prepared as above described (after four fold concentration), was 100 nmol/L while the LOQ for the standard serotonin directly injected was 350 nmol/L.

The suitability of the method was tested by measuring 5-HT levels in 20 healthy volunteers (12 females, 8 males; mean age 57 ± 18 years). The obtained values of 722 ± 312 nmol/L were similar to those recently reported in literature by Hara et al. (713 ± 287 nmol/L) [18] and by Hirowatari et al. (774 ± 249 nmol/L) [19]. To evaluate the assay accuracy and selectivity we compared WB serotonin levels obtained when analyzing samples using reverse or normal injection. The data were then evaluated by specific statistical methods for the measurement comparison. The Sotgia regression was performed in order to evaluate the agreement between the two procedures [20]. The output of this test is displayed in Fig. 3A, which reports graphically the difference in magnitude between the two analytical methods and provides an agreement degree expressed as determination coefficient (R^2) of 0.99. Moreover the Bland–Altman test, obtained by plotting the difference between the analyte concentrations measured by the two procedures, against the average of the two values, demonstrated the absence of bias (3B). The Passing and Bablok regression analysis showed a close agreement of both slope and intercept, with the target values of 1 and 0 within the ±95% confidence limits. Moreover, the Cusum test for linearity showed no significant deviation from linearity ($p > 0.10$) for the analyte tested (data not shown). In summary, the statistical comparison demonstrated that the data obtained by the two methods were equivalent thus suggesting that the reverse injection does not lead to co-migration of unwanted endogenous compounds.

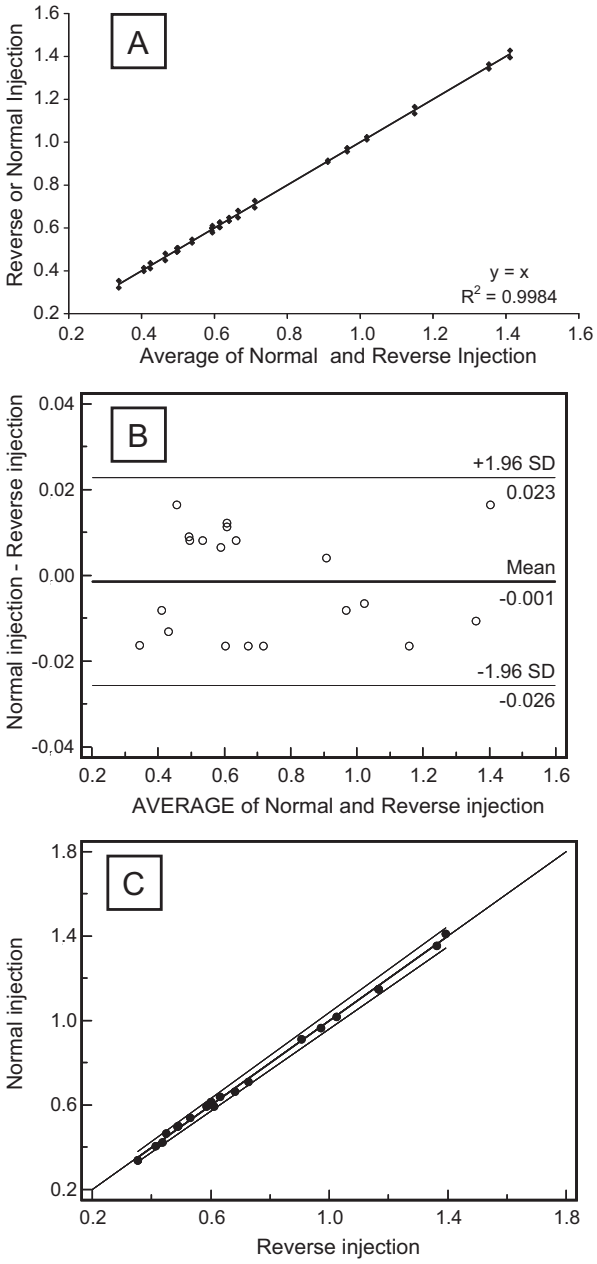

Fig. 3. Sotgia plot (A), Bland–Altman test (B) and Passing–Bablock regression (C) of data obtained by reverse injection and normal injection.

4. Conclusion

The present study shows the possibility to use capillary electrophoresis UV detection with reverse injection configuration for rapid analysis of serotonin in WB samples. We demonstrate the advantages of the reverse injection mode that allows both an improvement in the analyses times and sensitivity. The migration time of serotonin is of about 2.6 min, and each sample analysis needs about 5 min. Acetonitrile precipitation allows a quantitative recovery of 5-HT and after evaporation the dried samples should be concentrated thus improving the sensitivity of four times. Therefore, the relative short time of both sample pre-treatment and analysis time, the good sensitivity and the low analysis costs make this novel assay suitable for routine analyses both in research and in clinical applications.

Acknowledgements

This study was supported by the "Fondazione Banco di Sardegna – Sassari – Italy"and by the "Ministero dell'Università e della Ricerca" Italy. Angelo Zinellu was supported by Regione Autonoma della Sardegna "Ricerca cofinanziata PROGRAMMA OPERATIVO FSE SARDEGNA 2007-2013-L.R.7/2007 – *Promozione della ricerca scientifica e dell'innovazione tecnologica in Sardegna*". The manuscript language revision by Mrs. Maria Antonietta Meloni is greatly appreciated.

References

[1] M.D. Gershon, Aliment. Pharmarcol. Ther. 13 (1999) 15.

[2] M. Da Prada, J.P. Tranzer, A. Pletscher, Experientia 28 (1972) 1328.
[3] B. Grimaldi, A. Bonnin, M.P. Fillion, N. Prudhomme, G. Fillio, Neuroscience 93 (1999) 1223.
[4] D. Losco, M. Fineschi, C. Pierli, A. Fiaschi, N. Ferrara, S. Bianco, G. Longobardi, E. Pisani, A. Bravi, F. Rengo, Am. J. Cardiol. 84 (1999) 1317.
[5] S.T. Crowley, E.C. Dempsey, K.B. Horwitz, L.D. Horwitz, Circulation 90 (1994) 1908.
[6] G.R. Heninger, Proc. Natl. Acad. Sci. USA 94 (1997) 4823.
[7] I.P. Kema, A.M. Schellings, C.J. Hoppenbrouwers, H.M. Rutgers, E.G. de Vries, F.A. Muskiet, Clin. Chim. Acta 30 (1993) 143.
[8] M.A. Saracino, G. Gerra, L. Somaini, M. Colombati, M.A. Raggi, J. Chromatogr. A 1217 (2010) 4808.
[9] T. Brand, G.M. Anderson, Clin. Chem. 57 (2011) 1376.
[10] E.F. Marshall, W.N. Kennedy, D. Eccleston, Biochem. Med. Metab. Biol. 37 (1987) 81.
[11] P.C. Tagari, D.J. Boullin, C.L. Davies, Clin. Chem. 30 (1984) 131.
[12] M. Picard, D. Olichon, J. Gombert, J. Chromatogr. 341 (1985) 445.
[13] C.P. Bearcroft, M.J. Farthing, D. Perrett, Biomed. Chromatogr. 9 (1995) 23.
[14] Z.D. Peterson, M.L. Lee, S.W. Graves, J. Chromatogr. B 810 (2004) 101.
[15] M.D. Li, W.L. Tseng, T.L. Cheng, J. Chromatogr. A 1216 (2009) 6451.
[16] Y. Hu, W. Xu, J. Li, L. Li, Luminescence 27 (2012) 63.
[17] S. Sotgia, C. Carru, F. Franconi, P.B. Fiori, S. Manca, S. Pettinato, S. Magliona, R. Ginanneschi, L. Deiana, A. Zinellu, J. Chromatogr. A 1185 (2008) 145.
[18] K. Hara, Y. Hirowatari, Y. Shimura, H. Takahashi, Diabetes Res. Clin. Pract. 94 (2011) 167.
[19] Y. Hirowatari, K. Hara, Y. Shimura, H. Takahashi, J. Atheroscler. Thromb. 18 (2011) 874.
[20] S. Sotgia, A. Zinellu, G.A. Pinna, L. Deiana, C. Carru, Clin. Chem. Lab. Med. 46 (2008) 1046.