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Micellar electrokinetic chromatography with laser induced fluorescence detection shows increase of putrescine in erythrocytes of Parkinson's disease patients

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ABSTRACT

A highly sensitive method was developed to measure putrescine by micellar electrokinetic chromatography with laser induced fluorescence detection with excellent linearity in the 1 nM to 3 μM range. The technique was tested on a drop of blood from Parkinson's disease patients obtained by finger prick. The results showed a statistically significant increase of putrescine in the erythrocytes compared to controls and a non-significant increase in plasma. This high level of putrescine does not constitute by itself proof that putrescine and polyamines are directly related to Parkinson's disease. However, the present results and several others addressed in the discussion suggest that these compounds might be causally involved in the pathophysiology of Parkinson's disease. In addition, the analytical method reported here may help to find new biomarkers for many diseases including Parkinson's disease.

1. Introduction

Putrescine is a polyamine with many biological actions, including effects on cell proliferation, differentiation and signaling [1]. Moreover, polyamines are involved in several pathological processes very often as causative agents [2,3]. Due to their biological relevance, several techniques to measure putrescine have been developed, including thin layer chromatography [4,5], high performance liquid chromatography (HPLC) with fluorescence detection [6–21], HPLC with electrochemical detection [22], voltammetry [23], gas chromatography with nitrogen-phosphorus detection [24–26], gas chromatography with hydrogen flame ionization detection [27], HPLC with tandem mass spectrometry (HPLC-MS-MS) [28,29], and capillary zone electrophoresis (CZE) with either fluorescence [30,31], laser induced fluorescence (LIF) [32], conductometry [33], indirect photometry [34], chemiluminescence [35–37], amperometry [38] or mass spectrometry detection [39].

HPLC is most commonly used to resolve polyamines in complex biological matrices. However, methodological issues make quantification unreliable. Using HPLC as a separation technique for polyamines is not ideal since sometimes, due to its low peak capacity, it is difficult to separate some polyamines like agmatine and putrescine. Furthermore, prior studies using HPLC fluorescence have not reliably detected putrescine in red blood cells (RBC) [14,19,40,52]. In contrast, HPLC-MS-MS or flow injection analysis with atmospheric pressure chemical ionization mass spectrometry of dansylated polyamines have reported averages of 200 and 376 nM concentrations of putrescine in 1 μl of RBC assuming a red cell count of 5 million per μl of blood [28,41].

Capillary electrophoresis (CE) in both modalities, zone (CZE) or micellar electrokinetic chromatography (MEKC), has become very popular over the last 20 years. As a separation technique CE offers better peak capacity and resolution compared to HPLC. Furthermore, CZE or MEKC with laser induced fluorescence detection (LIFD) have

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reached the zeptomoles (10^{-21}) range [40,42,43] and recently it has been reported the yoctomoles (10^{-24}) range [44] of mass detection.

In medicine, the measurement of polyamines has gained importance as marker for both diagnostic and treatment outcome purposes in several diseases. These include a wide variety of cancers, neurodegenerative diseases such as Parkinson's disease (PD) and Alzheimer's disease (AD), diabetes mellitus, pancreatitis and other gastroenterological disorders, immunological disorders and infections (see [2,3] for review). Typically for the analysis of polyamines, blood samples are the preferred sample source. Analysis of RBC in PD patients has previously been performed, demonstrating an increase of spermine and spermidine in RBC. We focused on putrescine measurement because very often this polyamine goes undetected in RBC and only one study reports low levels of putrescine in RBC in PD patients [18]. Therefore, we derivatized putrescine with Fluorescein isothiocyanate Isomer 1 and measured the resultant Fluoresceinthiocarbamate putrescine (FTCP) by MEKC-LIFD in RBC and plasma in patients affected by PD and in age-matched controls and found significantly higher putrescine levels in RBC of PD patients.

2. Materials and methods

2.1. Reagents

Fluorescein isothiocyanate Isomer 1 (FITC), sodium carbonate, sodium bicarbonate, putrescine was obtained from Sigma Chemical Co, sodium dodecylsulphate and acetone from Fluka, sodium tetraborate from Merck KGaA, formic acid from Thermo-Fisher and acetonitrile HPLC grade from Fisher Scientific. All the reagents were prepared with 18 Megaohm-ultrapure water. Ringer solution was a 139 mM NaCl; 4 mM KCl; 1.2 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM $NaHCO_3$ in water solution.

2.2. Derivatization procedure

One mg of FITC was dissolved in 1 ml of Acetone. A twenty mM carbonate buffer (20CB) at pH 10 was prepared by diluting 5 ml of 200 mM $NaHCO_3$ and 5 ml of 200 mM Na_2CO_3 in 90 ml of nanopure water. The 1 ml of 20CB was mixed with the 1 mg/ml FITC in acetone to obtain a 1.28 mM FITC derivatizing solution. A known amount of this solution was added to standards and samples and protected from light. The mixtures were allowed to react for 16 h in the darkness before injecting using them for MS or MEKC.

2.3. Mass spectrometry

To validate the presence of the derivatized form of putrescine (FTCP), we conducted Mass Spectrometry. The spectra were observed in real time as parameters were adjusted including nozzle, capillary, and fragmentor voltages as well as gas temperature and the methods were saved when ideal response and background signals were achieved. Standards of putrescine and putrescine-FITC reaction mix were prepared by 10× serial dilution of 1 mg/ml putrescine stock and 100 μg/ml reaction mix in 20CB. Standards of 1140, 114, 11.4, 1.4, and 0.114 μmolar putrescine alone or putrescine derivatized with FITC were diluted 50× in 0.1% formic acid in 50% Acetonitrile/Water (1:1, v/v) for analysis. Samples were infused at 40 μl/min by a Harvard Apparatus PHD2100 infusion pump to an Agilent 6540 QTOF mass spectrometer with a Jet Stream Electrospray Ionization source (ESI). The optimal ESI source conditions for putrescine were as follows: Drying Gas Temp: 100 °C; Sheath Gas Temp: 100 °C; Drying and Sheath Gas Flow: 10 l/min; Nebulizer: 30 psig; VCap: 3000 V, Nozzle: 600 V, Fragmentor: 125 V; Skimmer: 65 V, OCT 1 RF Vpp: 250 V. The optimal ESI source conditions for putrescine-FITC reaction mix were as follows: Drying Gas Temp: 250 °C; Sheath Gas Temp: 250 °C; Drying and Sheath Gas Flow: 12 l/min; Nebulizer: 30 psig; VCap: 3000 V, Nozzle: 2000 V, Fragmentor; 150 V; Skimmer: 65 V, OCT 1 RF Vpp: 250 V. The acquisitions were performed in triplicate by full scan MS from *m/z* 50 to *m/z* 1000 for 1 min. Data was analyzed using Agilent Masshunter Qualitative Analysis software version B.06.00. Compounds were identified and confirmed utilizing the Molecular Feature Extractor (MFE) with a database filter. The personal compound database and library (PCDL) database consisted of the target compounds- putrescine, FITC, putrescine-FITC single label, and putrescine-FITC double label. Total ion counts for *m/z* 87.1073 (putrescine), 390.0431 (FITC), and 478.1431 (putrescine-FITC) were extracted and recorded from 0.12 to 0.99 min for each replicate. Linear regression analysis was performed using Microsoft Excel 2010.

2.4. Linearity of analysis

To test the linearity of the MEKC-LIFD method one milligram of putrescine was dissolved in 1 ml of 20CB at pH 10. This solution was dissolved 1:10 v/v in 20CB. Five μl of derivatizing solution was added to 1 ml of the 0.1 mg/ml putrescine solution in 20CB. This procedure produced a 6.38×10^{-6} M solution of FTCP. This solution was diluted in Ringer in steps of 2 to obtain the following μM solutions: 6380, 3190, 1595, 797, 398, 199, 99, 48, 24, 12, 6 and 3 nM. A blank was prepared by mixing 1 ml of Ringer with 5 μl of derivatizing solution. A 3 nM solution was prepared as described above. Both, the blank and the 3 nM solution were injected and analyzed 10 times each to calculate the Limit of Blank detection (LOB), the limit of detection (LOD) and the Limit of Quantitation (LOQ) and the Relative Standard Deviation (RSD). To test the linearity of the labelling chemistry a serial dilution of putrescine in human serum was prepared at the following concentrations: 15,500, 7700, 3800, 1900, 900, 480, 240, 120, 60, 30 and 15 nM. Then they were derivatized by mixing with derivatizing solution 1:1 v/v.

2.5. MEKC-LIFD instrument

The MEKC-LIFD instrument was built in-house with a Glassman High Power Supply model PS-MJ30P04400X88 (Glassman High Voltage Inc. Whitehouse Station, NJ, USA), a collinear fluorescence detector described elsewhere [44] equipped with a Hamamatsu Photo Multiplier Tube Model H-9306 (Hamamatsu Co, Middlesex, NJ, USA) and a Cobolt solid state, 488 nm, continuous wave laser model 0488-06-01-0060-100 (Cobolt MLD, Solna, Sweden). The signals were collected by a National Instruments data acquisition card, model PCI-6221 (National Instruments Co. Austin, TX, USA) in a computer programmed with custom-made software both for acquisition and processing.

2.6. Sample treatment

With previous consent from all participants, the samples were collected from 12 patients averaging 66 years old and ranging from 48 to 82 years old and 12 controls averaging 61 years old and ranging from 42 to 78 years old in the Neurology Unit of the University of the Andes Hospital. The tip of the finger was pricked with an automatic lancing device. The drop of blood was collected into hematocrit tubing and centrifuged for 5 min at 375*g* to separate plasma from cells. A volume of 10 μl of RBC was mixed with 10 μl of pure water to destroy the plasma membrane of the RBC, vortexed for 30 s and centrifuged for 5 min at 375*g*. Once centrifuged, 10 μl of the supernatant were mixed with 10 μl of acetonitrile to precipitate proteins. The sample was centrifuged again at 375*g* for 5 min and 10 μl of the supernatant was collected and reacted with 10 μl of the derivatizing solution. In the case of plasma, 10 μl was directly mixed with 10 μl of acetonitrile to deproteinize and these were then centrifuged at 375*g* at room temperature for 5 min. Ten microliters of the supernatant were collected and mixed with 10 μl of the derivatizing solution. After 16 h in darkness the samples and the standard were ready for CZE analysis. To identify the putrescine peak in the samples each of them was divided in three equal volumes. A solution of

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