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Enantioseparation of the constituents involved in the phenylalanine-tyrosine metabolic pathway by capillary electrophoresis tandem mass spectrometry

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ABSTRACT

Catecholamines dopamine, norepinephrine, and epinephrine are well-known neurotransmitters playing different roles in the nervous and endocrine system. These compounds are biologically synthesized in the phenylalanine-tyrosine pathway which consists on the successive conversion of L-phenylalanine into L-tyrosine, L-3,4-dihydroxyphenylalanine (L-DOPA), dopamine, norepinephrine, and epinephrine. This work describes the development of an enantioselective CE-ESI-MS^2 methodology enabling, for the first time, the simultaneous enantioseparation of all the constituents involved in the Phe-Tyr metabolic pathway, since all these compounds except dopamine are chiral. The developed method was based on the use of a dual CDs system formed by 180 mM of methyl-β-CD and 40 mM of 2-hydroxypropyl-β-CD dissolved in 2 M formic acid (pH 1.2) and presented the advantage of avoiding the use of any time-consuming labelling procedure. LODs ranged from 40 to 150 nM and the unequivocal identification of the compounds investigated was achieved through their MS^2 spectra. The applicability of this methodology to the analysis of biological samples (rat plasma) was also demonstrated.

1. Introduction

The determination of neurotransmitters is an important tool to increase the knowledge of neurological processes. The catecholamines dopamine (DA), norepinephrine (NE), and epinephrine (EP) are well-known neurotransmitters playing important roles in many biological processes and which can also act as hormones in stressful situations [1]. Quantification of catecholamines in biological fluids is an interesting tool in the diagnosis of several disorders such as schizophrenia, Parkinson's disease or pheochromocytoma [2,3]. These catecholamines are biologically synthesized in the Phe-Tyr metabolic pathway (Fig. 1). A known pathology related to this metabolic pathway is phenylketonuria, an inborn error of metabolism which results in large excess of Phe in blood and urine [4], what can derive in mental retardation as a consequence of low level of Tyr and catecholamines [5]. Previous works also demonstrated the relationship between some mental disorders and the availability of precursors of catecholamines in cerebrospinal fluid of monkeys [6]. Likewise, the reduction of 3,4-dihydroxyphenylalanine (DOPA) synthesis by using the alpha-methyl-*p*-tyrosine inhibitor also results in a transient decrease in catecholamine synthesis [7].

All the compounds involved in the Phe-Tyr metabolic pathway (see Fig. 1) except DA are chiral, therefore it is of high interest to study their stereochemistry, especially since only the L-enantiomers of the involved amino acids can lead to these important neuroactive molecules. Thus, D-Phe and D-Tyr were found to be present in different physiological fluids such as plasma, urine, cerebrospinal fluid, and amniotic fluid [8]. Moreover, it has been shown that D-Tyr has deleterious effects inhibiting the growth of mice [9]. On the other hand, Parkinson's disease therapy includes

Abbreviations: CMT, counter migration technique; DA, dopamine; DOPA, 3,4-dihydroxyphenylalanine; EMO, enantiomer migration order; EP, epinephrine; HP-β-CD, (2-hydroxypropyl)-β-cyclodextrin; LVSS, large volume sample stacking; M-β-CD, methyl-β-cyclodextrin; NE, norepinephrine; PFT, partial filling technique.

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administration of the L-form of DOPA in order to replenish DA, whereas that D-DOPA not only cannot be converted to DA but also presents side effects [10,11].

The study of the chirality of biological molecules acquires great importance since, although in the past it was assumed that only L-amino acids were the natural existing form [12], later studies demonstrated that the D-forms were also present in some micro-organisms [13], in amphibians and invertebrates and even in mammals, including humans [14] either as a result of spontaneous racemization of L-forms in the structure of polypeptides when ageing [15,16] or as endogenous substances [17–19]. The amino acid D-Serine is an example of this, as it is an important neuroactive molecule whose levels in mammals can even exceed the concentration of many L-amino acids [20]. Thereby, it is of high importance to increase the knowledge of the activity of enantiomers, whose determination still remains as a promising topic in the analytical sciences field.

Due to the fact that a pair of enantiomers presents the same physicochemical properties with the exception of opposite optical rotation of plane-polarized light and differential strength of the interactions with chiral compounds, their separation is a challenging task that could require the MS in order to achieve the necessary sensitivity and the unequivocal detection of the investigated compounds when these are in complex samples. Regarding the Phe-Tyr metabolic pathway, GC, LC, and CE have been the separation techniques employed to achieve the enantiomeric separations of its individual components or partial mixtures of them using different detection systems. Thus, the enantiomeric determination of Phe and Tyr, generally in mixtures with more proteinogenic amino acids, has been reported in numerous articles but their determination in biological fluids has been less frequent. Over the last 20 years, some of these works include the use of GC–MS employing Chirasil-L-Val as stationary phase [21,22], LC–MS employing either cinchona alkaloid based chiral stationary phases [23,24] or a chiral derivatization reagent [25–27], CE-UV with a chiral labelling agent [28], CE-LIF with a chiral derivatization reagent [29] or cyclodextrins [30,31] and CE-MS employing a chiral crown ether [32]. Note that all these methodologies required time-consuming derivatization steps except the last one [32]. Regarding the other chiral constituents of the Phe-Tyr metabolic pathway, DOPA, NE and EP, a considerable minor number of articles reporting their enantiomeric separation have been published. In the last 20 years, enantioseparation of DOPA, NE, or EP has not been reported by GC. In the case of LC, none described the enantioseparation of DOPA, while the individual or simultaneous enantioseparation of NE and EP was reported only in two works [27,33]. Concerning CE, some articles have been published dealing with the enantiomeric separation of DOPA [34–40], as well as mixtures of DOPA with Phe and Tyr using MS detection [41], or even the enantiomeric separation although not simultaneously of DOPA, and other components of the Phe-Tyr metabolic pathway using UV detection [42]. Regarding the simultaneous enantioseparation of NE and EP by CE, some studies have been described using UV [1,43–47] or electrochemical detection [48], but these compounds have never been enantioseparated using a CE-MS system.

In summary, to the best of our knowledge, the constituents of the Phe-Tyr metabolic pathway have never been simultaneously enantioseparated. Only an old method published in 1996 [42] described the separation within 60 min of these compounds using sulfated-β-CD as chiral selector in CE-UV but, the simultaneous separation was not possible due to the overlapping of DA, and NE and EP enantiomers. In addition, this methodology is not compatible with MS detection under the conditions assayed. Thus, it is of special interest to develop a methodology enabling the simultaneous enantioseparation and unequivocal detection of the compounds involved in the Phe-Tyr metabolic pathway in a single run, without the need of using time-consuming derivatization steps.

Since CE offers numerous advantages for chiral analysis such as high separation efficiency, excellent enantioselectivity and low reagents and solvents consumption (including chiral selectors), what makes it an environmental-friendly technique [49–52], this technique was employed in this work with the aim of developing an enantioselective CE-ESI–MS^2 methodology to carry out, for the first time, the simultaneous enantioseparation and unequivocal detection of all the Phe-Tyr metabolic pathway constituents avoiding the use of any derivatization procedure. Firstly, investigation on the enantiodiscrimination of several chiral selectors towards these compounds is presented in CE-UV. Secondly, the methodology is implemented in a MS system and, after testing its analytical characteristics, it is applied to the analysis of rat plasma. We consider that testing our enantioselective CE-ESI–MS^2 methodology to separate and detect all the Phe-Tyr metabolic pathway constituents in biological samples such as rat plasma could be particularly relevant due to the enormous potential of this biological sample in translational research.

2. Materials and methods

2.1. Reagents

All reagents were of analytical grade. MS-grade acetonitrile and methanol were obtained from Scharlau Chemie (Barcelona, Spain) while formic acid, perchloric acid, ammonium carbonate, ammonium hydroxide, and EDTA were from Sigma (St. Louis, MO, USA). Ammonium acetate was from Merck (Darmstadt, Germany), ascorbic acid was obtained from VWR (Radnor, PA, USA) and isoflurane from Abbott (Madrid, Spain). The employed water was MilliQ quality (Millipore, Bedford, MA, USA). β-CD, sulfated-β-CD (degree of substitution (DS): 12–14), methyl-β-CD (M-β-CD) (DS 1.7–1.9), 2-hydroxypropyl-β-CD (HP-β-CD) (DS ~4.2), 2,6-di-*O*-methyl-β-CD (DM-β-CD), and carboxymethyl-β-CD (CM-β-CD) (DS ~3) were purchased from Fluka (Switzerland). Acetyl-β-CD (A-β-CD)(DS 7), γ-CD, methyl-γ-CD (M-γ-CD) (DS ~12), 2-hydroxypropyl-γ-CD (HP-γ-CD) (DS ~4.5), HP-γ-CD (DS ~3.2), carboxyethylated-β-CD (CE-β-CD) (DS ~3), carboxymethylated-γ-CD (CM-γ-CD) (DS ~3), and carboxyethylated-γ-CD (CE-γ-CD) (DS ~3) were purchased from Cyclolab (Hungary). DL-Phe and DL-EP were from Aldrich (St. Louis, MO, USA), L-Phe, D-Tyr, L-Tyr, and DL-NE in Fluka (Buchs, Switzerland) and DL-DOPA, L-DOPA, L-NE, L-EP, DA, and trigonelline hydrochloride in Sigma (St. Louis, MO, USA).

2.2. CE-UV conditions

Electrophoretic experiments were carried out on a HP^{3D}CE system from Agilent Technologies (Palo Alto, USA) with a diode array detector. The electrophoretic system, and the data collection were controlled by HP^{3D}CE ChemStation software. The BGE employed consisted of a dual CDs system based on 100 mM M-β-CD and 60 mM HP-β-CD dissolved in 2 M formic acid (pH 1.2). Separations were performed in an uncoated fused-silica capillary of 50 μm I.D. and a total length of 88.5 cm, purchased from Polymicro Technologies (Phoenix, USA) at +30 kV and 15 °C. Injections were carried out applying 5000 Pa (50 mbar) for 5 s. Detector parameters were a wavelength of 200 ± 5 nm. At the beginning of the day the capillary was flushed with buffer solution for 10 min and at the end of the day with MilliQ-water for 5 min. In order to ensure the repeatability between injections, the capillary was flushed with buffer solution for 2 min and BGE for 5 min.