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A Novel Capillary Zone Electrophoresis Technique for Pharmacokinetic Studies on Porphyrin-C_{60} Based Neuroprotectors. Preliminary Report

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Abstract

A novel, fast-n-reliable, Quartz capillary silica zone electrophoresis required to upgrade an arsenal of analytical tools for current pharmacokinetics studies on porphyrin-fullerene nanoparticles has been proposed. The *fullerene*(C_{60})-*tetra*(*p-hydroxyphenyl*)*porphyrin* structures, known for their capabilities to paramagnetic $^{25}Mg^{2+}$ delivery, were found quantitatively detectable in a total rat brain cytosol (S125) fraction, once these nanocationites administered in a single, either 1.0 mg/kg or 20.0 mg/kg, i.v. injection followed by 12 hrs long animal-drug exposition time. Driven by pressing needs in ongoing preclinical trial platform developments, the CZE track proposed might make an impact on both convenience and efficiency of pharmacokinetic estimate of medicinal nanocationites.

Keywords

Neuroprotectors, Porphyrin-Fullerenes, Ischaemic Brain Stroke, Preclinical Trial, Capillary Zone Electrophoresis (CZE)

1. Introduction

A controversial record of porphyrin-fullerenes, autonomous pharmacophores and the nanocarriers for some drug delivery cases, counts nearly 20 years [1]-[5].

Particularly, several water-soluble cyclohexyl(C_{60})-porphyrines like PMC16 nanoparticle family members [6] [7] were found efficient to provide an essential antihypoxia activity *in vivo* being engaged with the overproduction of nucleoside-

triphosphates induced by $^{25}Mg^{2+}$ paramagnetic ions [8] [9]. These *magnetic isotope effect* (MIE) promoting ions are capable to get bound, carried, and eventually released by porphyrin-fullerene PMC16 nanocationites in response to the hypoxia tissue, acidosis conditions [1] [4]-[6] [8] [9]. Concerning both mechanisms and an applied pharmacological validity of MIE *per se*, this relies upon its impact on the Mg^{2+}-kinases controlled correction of ATP disbalance in hypoxia suffering mammalian cells and tissues [10]-[13].

Most of pharmacokinetics, suitable analytical techniques like GC-MS, LC-MS and HPLC are time-consuming and rather expensive methods requiring the highest purity organic solvents, relatively large (10 - 100 μL) test sample volumes, and the sorbent "aging" related change (regeneration) of columns, while the separation efficiency mode usually does not exceed 50,000 - 60,000 theoretical plates [13]-[15].

On the contrary, a CZE approach is about to grant hundreds of thousands of theoretical plates (unreachable for HPLC) [14] being convenient for inexpensive express tests with 1.0 - 5.0 μL water soluble samples and with no need in either regeneratable columns or A-grade organics [14] [15]. So this peculiar approach could be a right choice to develop a fast, robust and reliable technique for the detection of negatively charged polar (amphiphilic) molecular NPs such as PMC16.

In the present study, we have developed a novel CZE technique to meet these expectations.

2. Materials and Methods

2.1. Nanoparticles

Water soluble *fullerene*(C_{60})-*tetra*(*p-hydroxyphenyl*)*porphyrine* molecular NP, indexed PMC16-RX [7], were kindly provided by a courtesy of Dr. N. Amirshahi, Amir Kabir University of Technology, Tehran, Iran.

2.2. Animals

Wistar Albino Glaxo male rats, 180 - 220 g, were kept under a standard vitaminized diet, starving for 24 hrs before the experiment. Three animals per each experimental point, 5 - 6 repetitions for every measurement were carried out.

2.3. NP Administration

1.0 mg/kg and/or 20.0 mg/kg of NP was administered to rats in a single i.v. injection. Solvent: 15 mM Tris-HCl (pH 7.80). Animals were decapitated 12 hrs after injection, brain tissue samples were removed and homogenized in 5 - 7 vols of 20 mM Tris-HCl (pH 8.0)/10 mM $MgCl_2$/1.5 mM NaCl/2.0 mM EDTA/25 mM sucrose/2.0% Triton X-100 (v/v). Potter glass-teflone homogenizer, 1800 r.p.m. (+4˚C), has been employed.

2.4. Brain Homogenate Treatment

To isolate the cytosol fraction (S125), homogenates were subjected to ultracentrif-

ugation at 125,000 g, 4 hrs, +4˚C, Spinco L5-65B Ultracentifuge (Beckman, USA), rotor SW 27.1. Superatants (S125) were carefully collected, protein measurements were performed by a routine Bradford colorimetric method.

S125 samples were mixed with 10 vols of ice-cold acetone followed by an overnight incubation at +4˚C. The resulted pellets were precipitated at 20,000 r.p.m., 20 min, +4˚C and removed. Supernatants were collected for further use in UV-VIS spectrophotometry and CZE studies.

To elucidate the target product extractability degree, the acetone precipitated dry pellets were dissolved in 15 mM ammonium phosphate (pH 8.80)/0.1% SDS/2.5 mM EDTA/1.0% 2-mercaptoethanol (20:1, v/w) with a consequent sonication treatment at 60 KHz, 40˚C, 60 min, followed by the below specified CZE analysis of A_{440}-pool heterogeneity. In all tests conducted, no PMC16-RX traces found.

2.5. Spectrophotometry

5 mL Portions of the S125 acetone-soluble pool were lyophilized and then dissolved in the same volumes of 20 mM ammonium-phosphate (pH 9.0) buffer. UV-VIS of these solutions were conventionally registered, along with the PMC16-RX standards (same buffer) controls, in Lambda 1050 Analytical System (Perkin Elmer, USA).

2.6. CZE Procedure

Acetone-soluble S125 extracts were concentrated in a rotor evaporizer to the final volume of 0.2 mL followed by addition of 30 mM ammonium-phosphate (pH 8.80), 25:1 (v/v).

10 µL of a sample was inserted into the P/ACE MDQ Plus CZE Analytical System (ALGIMED, Belarus) coupled to the UV-VIS 770 KS detector, 440 nm monochromatic filter (Prince Technologies BV, Netherlands) with a following 10 min run at +6˚C: Quartz (50 µ diameter/7.5 cm effective length) capillaries packed with the UV-transparent silica saturated by SJX40 electrolyte pH 8.80 (SCIEX BV, Netherlands), 115 V/60 Hz/300 W per cap. Data acquisition unit: DAX DATE 220 LK (SCIEX BV, Netherlands).

3. Results and Discussion

As seen from the data presented in **Figure 1**, UV-VIS-detection would be no doubt an appropriate way to employ for CZE/PMC16-RX pharmacokinetic purposes. Thus, 439, 548 and 662 nm λ_{max} values revealed allow to be sure of an accuracy of the 440 nm monochromatic filter use in CZE analysis of certain PMC16 containing compositions (**Figure 2**).

A mere comparison of spectra 1, 2, 3 and 4 (**Figure 1**) makes it reasonable to choose the UV-VIS -detection mode for simple and reliable calibration of the CZE patterns required for quantification of NPs in biomaterial studied (**Table 1**).

Using this calibration chart (**Table 1**), the following numerical link was

achieved: 1.0 mg/kg PMC16-RX, i.v., 12 hrs exposition → 7.0 - 8.0 ng/mg S125 protein of the NP concentration. In similar control experiments, 20.0 mg/kg injection used to lead to as high as 120 - 125 ng/mg protein drug content in the brain tissue cytosol fraction.

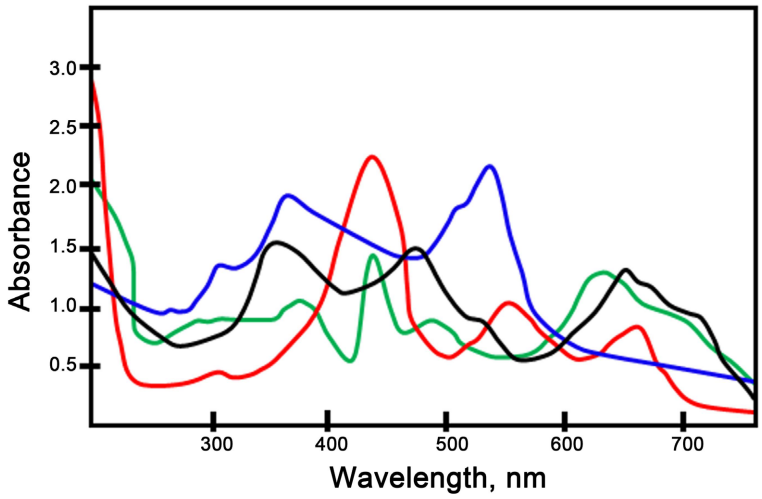

Figure 1. UV-VIS Absorbance spectra of the pmc16-rx containing/lacking solutions and biomaterials. 1-, PMC16/20 mM ammonium phosphate (pH 9.0); 2-, PMC16-RX/S125 acetone soluble pool obtained in a course of the *in vivo* drug administration experiment (pH 9.0); 3-, PMC16-RX/mixed with the S125 acetone soluble pool isolated from the intact rat brain homogenate, no *in vivo* drug injection administered (pH 9.0), 200 ng/mg protein; 4-, Pure S125 acetone soluble pool, no PMC16-RX involved.

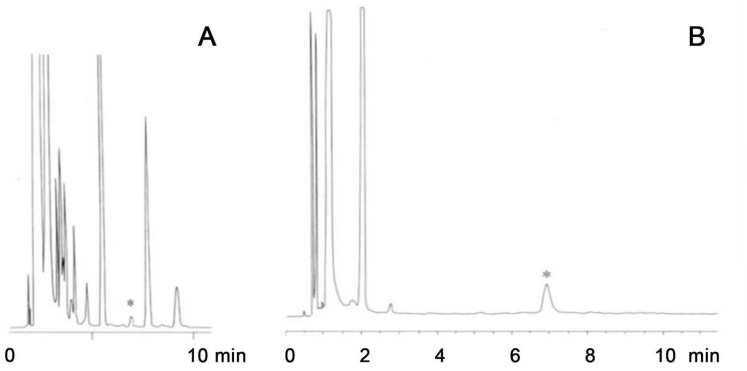

Figure 2. CZE fractionation of the pmc16-rx containing samples. (A) *Sample*: S125 acetone soluble pool isolated from the rat brain homogenate obtained 12 hrs after a single i.v. injection of PMC16-RX, 1.0 mg/kg. (B) *Sample*: PMC16-RX (internal standard) mixed with the acetone soluble fraction of cytosol (S125) isolated from the intact rat brain tissue, 200.0 ng/mg S125 protein. No *in vivo* drug administration beyond. ***Note.*** Pure PMC16-RX marker/20 mM ammonium phosphate (pH 9.0), CZE 10.0 min run: a single and clear peak revealed, R_t = 7.0 min. Star sign (*) indicates to PMC16-RX peak.

A zero PMC16-RX contamination of the acetone-insoluble, *i.e.* high molecular weight S125 compounds containing fraction, was found, showing a complete extractability of the target product from biomaterial studied.

Table 1. The internal standard contents as correlated to absorbance of CZE revealed target compound (PMC16-RX, R_t = 7.0 min).

PMC16-RX, ng/mg S125 protein	A_{440}/mL (M ± SEM)
1.0	0.09 ± 0.02
5.0	0.33 ± 0.08
10.0	0.61 ± 0.09
25.0	1.84 ± 0.08
50.0	3.87 ± 0.11
100.0	5.32 ± 0.50
200.0	8.55 ± 0.72
500.0	18.38 ± 0.88
1000.0	36.72 ± 1.06

***Note**: *CZE sample analysed*: S125 acetone-soluble pool mixed with the certain amounts (1.0 - 200.0 ng/mg S125 protein) of a target compound, PMC16-RX.

Last but not least, electrophoretic patterns for both the S125 extract added NP internal standard and the inside biomaterial traced NP detectable in *in vivo* experiments, as well as the pure NP retention time test (R_t = 7 min), are all in a favor to applied validity of the CZE technique presented. This shows its potential as a tool for PMC16 related pharmacokinetic research (**Figure 2**) which makes it promising for the brain hypoxia prevention/correction research, in particular.

On several occasions, porphyrin(C_{60})-fullerenes were successfully used for a targeted delivery of stable magnesium isotope $^{25}Mg^{2+}$ to the damaged heart muscle in rat models of myocardial hypoxia [1] [5], while it is hardly possible to exclude a similar result in other compartments of the whole organism [4] [8], including the brain. Hence, the MIE related ($^{25}Mg^{2+}$ engaging) anti-hypoxic activity of certain porphyrin-fullerenes is no doubt deserves to be tested in *in vivo* brain research as well. To conduct these tests, a simple and reliable analytical method should be proposed as the NP-pharmacokinetics specific tool. Our results are about to respond to this need (**Figure 1**, **Figure 2**, **Table 1**).

As seen from these data, the resolution and sensitivity of our CZE procedure are good enough to find out low but detectable level of PMC16-RX rat brain uptake estimated as 7.0 - 8.0 ng per 1.0 mg of total S125 protein. This amount of NP is detectable 12 hrs after a single i.v. injection of the agent, 1.0 mg/kg (see Methods), which is about 2.0% - 4.0% of the rat myocardium PMC16 uptake [1] [5]. A CZE/PMC16-RX calibration data beyond (**Table 1**). So the blood-brain barrier penetration for xenobiotic tested has been clearly shown.

The key *in vivo* test (1.0 mg/kg PMC16-RX), as compared to the results specified control one (20.0 mg/kg PMC16-RX), seems an argument not only for high sensitivity of the method and its remarkable separation capabilities but for a good enough PMC16-RX/BBB permeability as well.

Noteworthy, a relatively low «mass amount level» of the NP intralization in rat

brain cells (**Figure 2**) might have nothing to do with the agent's anticipated pharmacological impact since the latter would be determined by an extraordinary ATP overproduction, *i.e.* by the direct result of $^{25}Mg^{2+}$ MIE phenomenon [7] [8] [12]. Needless to outline that the drug intralization itself is a true priority in advanced pharmacokinetics studies.

A retention time value of the PMC16-RX CZE revealed peak, 7.0 min, was found a well-repeatable identification parameter. A separation of this key meaning peak is perfectly clear (**Figure 2**). Concerning the rest of peaks revealed, these 440 nm absorbing acetone soluble, e.g. low molecular weight, cytosol compounds are, most likely, presented by the variable and the brain tissue abundant polymorphic metabolites such as folate, ribitol, cyancobalamine and cyclopentaneperhydrofenantrene derivatives. Being totally focused on PMC16 (target compound) detection, we were not interested in detail specification of all CZE signals seen in **Figure 2**.

Another attention catching point deals with a comparison of the data presented in "A" and "B" parts of **Figure 2**. Even though an obvious detectability of a target compound, *i.e.* PMC16-RX peak (R_t = 7.0 min), was firmly proven, the whole CZ electrophoregrams were found different in "A" (*in vivo* experiment) and "B" (S125/PMC16-RX internal standard mixture) cases. This difference in (A_{440})-heterogeneity of samples "A" and "B" is, probably, caused by an impact of the NP tested on certain BBB functional peculiarities like its "filtration tolerance" to a variable protein, xenobiotic interfaces which results in increase of BBB permeability. In other words, in PMC16-RX *in vivo* administration tests, the protein pharmacophore complexes may play a role of the Trojan Horse for their activity in binding and carrying of some additional low molecular weight compounds from blood to the brain which, in turn, leads to increase of complexity of the above mentioned CZE profile [13]-[15].

A remarkable sensitivity of our method (**Figure 2(A)**) is nothing but a sign of its sharp-and-clear separation power: as a matter of fact, it is hardly possible to detect less than 35.0 - 40.0 ng PMC16 per 1.0 mg protein, once the conventional HPLC techniques employed in mammalian tissue extract analysis.

As seen from **Figure 3**, the porphyrin fullerene nanoparticles are the CZE-detectable family of similar C_{60}-derivatives.

Turning back to the target compound of a present study, PMC16-RX, we have to emphasize that this belongs to the unique group of water soluble porphyrin-(C_{60})fullerenes which holds great expectations and attracts intense interest, so there is a little doubt that future research will result in some more new pharmacological applications, as long as the pharmacokinetics dealing technological *tasks* would be properly solved. To the best of our knowledge, this is the first report ever on CZE application for this and related *tasks.*

4. Conclusion

The drug brain uptake, BBB permeability and a subsequent pharmacokinetic

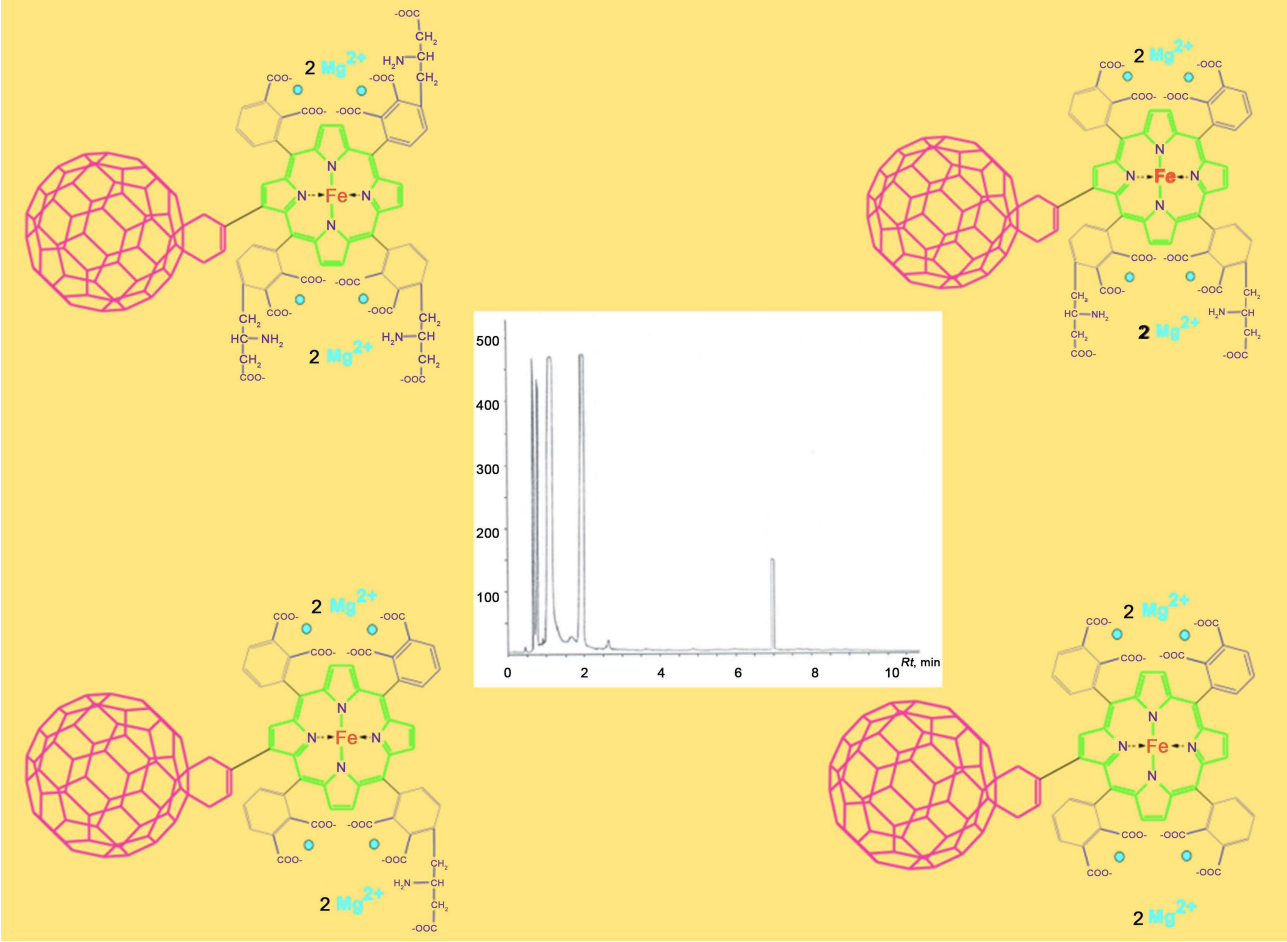

Figure 3. The *in vivo* detectable porphyrin-fullerene nanoparticles: CZE.

paths of the water-soluble porphyrin-(C_{60})fullerene nanoparticles are expected to be studied using an advanced, fast and reliable, Quartz capillary silica zone electrophoresis technique proposed in a present work. Separation quality as well as the high sensitivity of this method is in favor of this statement.

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Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest

This paper was not published or considered for publication/report elsewhere yet. There is no any conflict of interests which deals with this work whatsoever.

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Abbreviations

CZE:	Capillary zone electrophoresis,
MIE:	Magnetic isotope effects,
NP:	Nanoparticles,
BBB:	Blood-brain barrier.