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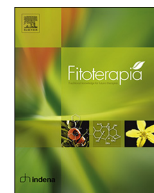

Trans-crocin 4 is not hydrolyzed to crocetin following i.p. administration in mice, while it shows penetration through the blood brain barrier

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ABSTRACT

A novel, fit-for-purpose, highly sensitive, analytical UPLC-PDA methodology was developed and fully validated, according to ICH, FDA and EMA guidelines, for the rapid and accurate quantification of *trans*-crocin 4 (TC4) and crocetin (CRC) in mice plasma and brain after *i.p.* administration. A PDA based methodology shows a wider applicability as it is cost effective and can be easily and seamlessly adopted by the pharma industry. The separation of the analytes was performed on a C18 Hypersil Gold column with 2.5 min run time, employing the internal standard (ISTD) methodology. The two methods were successfully applied for the determination of CRC and TC4 in mouse plasma and brain after *i.p.* administration of TC4 (50 mg/kg) in a time range of 0–240 min. Due to the selection of *i.p.* administration route, the first-pass metabolism and/or gastric hydrolysis were bypassed, a fact that enhanced the bioavailability of TC4. Furthermore, TC4 was found to be capable of crossing the Blood Brain Barrier (BBB) and build up levels in the mouse brain, regardless of its highly hydrophilic character. CRC was not detected in any plasma or brain sample, although it has been reported that TC4 quickly hydrolyzes to CRC after *p.o.* administration. Therefore *i.p.* administration could be used in the case of TC4 for the accurate determination of its biological role. Overall, the developed methodology offers important information about the bioavailability of TC4 in mouse plasma and for the first time, demonstrates the ability of TC4 to penetrate the BBB and localize inside the brain.

1. Introduction

Saffron, the dried stigmas of the flower *Crocus sativus* L. is considered to be among the most expensive spices in the world. *Crocus sativus* L., a stemless perennial herb of the Iridaceae family which is native to Greece and Southwest Asia, was first cultivated in Greece for its' red stigmas (style branches). Saffron is mainly used as a traditional herbal medicine but also as a flavoring and food coloring agent in everyday life. Chemical analysis of *Crocus sativus* L. *stigmas* has shown the presence of a wide variety of different constituents including carotenoids (e.g., a- and b-carotene), mono- and bis-esters of crocetin, picrocrocin and safranal [1]. Picrocrocin, a colorless glycoside, is the $\beta-$d − glucoside of hydroxysafranal (4 − hydroxy − 2,6,6 − trimethyl − 1 − cyclohexenhe − 1 − carboxaldehyde) and its mainly responsible for saffron's well − known bitterness. Safranal on the other hand, is the principal substance of saffron's aroma [2]. The main bioactive saffron constituents are the crocins, which are mono − and bis − esters of crocetin (apocarotenoid) with glucose, gentiobiose and/or gentiotriose [3]. Depending on the type and amount of sugar(s) conjugated with crocetin as well as the space-configuration, there are many different crocins produced i.e. *cis/trans* − crocin − 2 (CC2/TC2), *cis/trans* − crocin − 3 (CC3/TC3), *cis/trans* − crocin − 4 (CC4/TC4) while TC4 is found to be the most abundant crocin in all saffron species studied so far [3].

Several studies indicate that saffron's constituents have been proven to be effective against a wide range of common disorders including coronary artery disease, [4-6] stomach disorders, hypertension [7], learning and memory impairment [8], dysmenorrhea and premenstrual syndrome (PMS) [9]. Furthermore, saffron or its main constituents have shown remarkable activity against some neurodegenerative diseases that nowadays affect significant percentages of the general population such as Alzheimer's Disease (AD) [10–12], Parkinson's Disease (PD) [13], depression [14] and schizophrenia [15]. Finally, saffron exhibits dose − dependent inhibitory response on breast cancer cells [16]. Due to

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its plethora of pharmacological properties saffron is not only regarded to be a precious spice but also a highly valuable and health-promoting herb [17].

TC4 is of raising interest due to its' promising pharmacological properties as well as its' nontoxic character [18, 19]. Interestingly *i.p.* administration of crocin up to 3 g/kg in mice did not show any mortality after 24 and 48 h. Therefore TC4 has been considered to be a practically low-toxic substance [20]. Anti-oxidant and anti-inflammatory activity of TC4 suggest its therapeutic potential against various nervous system disorders [8]. It has been shown that TC4 enhances the sexual activity of male rats [21], exhibits antidepressant effects in rodents [22], prevents oxidative stress in the hippocampus contributing to the prevention of deficits in spatial learning and memory [23] and also enhances the learning ability and improves memory [24]. However, recent studies have demonstrated that TC4 also possesses anti-AD and anti-PD activity as it has shown significant inhibitory effect on the fibrillation of apo-alpha-lactalbumin (a-alpha-LA), under amyloidogenic conditions [25] and also on beta-amyloid (Aβ) fibrillogenesis [12]. CRC, the main metabolite of TC4, has been shown to inhibit Aβ fibrillization and attributed to the stabilization of Aβ oligomers [26], enhanced the angiogenesis in rats [27] and it is considered to be a potent antitumor agent as it has been shown to act as scavenger of free radicals [28]. Furthermore, CRC has shown neuroprotective effects against brain injuries as it inhibited apoptosis at early stages of the injury as well as promoted the angiogenesis step [27].

However despite the broad bioactivity of crocins, there are only few studies focused on the pharmacokinetic properties of TC4 and CRC while most of them include plasma and/or urine [29–34]. Many of the methods reported in previous studies did not exhibit adequate sensitivity for the quantitation of crocin in biological samples, and only its metabolite, CRC, could be detected and assessed. Despite the fact that LC-MS/MS methodologies developed are slightly more sensitive [33], the incorporation of the PDA detector offers a wider linear range as well as wider laboratory applicability because of its lower cost. Therefore, there is a need to develop a low-cost, fast and sensitive methodology in order to assess the bioavailability of crocin and its metabolites in brain and plasma.

Moreover, several factors contribute to a compound's bioavailability such as the administration route, its lipophilicity, sex, age, genotype, hormonal status etc. of the dosed organism as well as the individuality of each subject etc. [35–37]. So far there has been no other study describing the bioavailability of TC4 and CRC after *i.p.* administration in mice plasma, as well as the bioavailability of these compounds in mice brain. Given that saffron extracts have shown neuroprotective effects, it is of a great importance to discover the correlation between plasma and brain circulation levels following TC4 administration. In this study, we describe the development of two fully validated UPLC analytical methodologies for the simultaneous quantification of TC4 and CRC in both mice plasma and brain. This is a rapid, robust and fully automated procedure, with a simple sample pretreatment and a high sample turnover with only 2.5 min run time.

Although there is a more sensitive methodology appearing in the literature [33], the currently developed methodology shows comparably low detection limits (2 vs 10 ng/mL of plasma). Nevertheless, the UPLC-PDA methodology for plasma is fit-for-purpose, as it exhibits a broader linear range (10–6000 ng/mL), whereas the levels determined by the bioavailability study are well higher than the LLOQ. Preliminary pilot studies concerning the concentration levels of TC4 in plasma showed the circulation of relatively high levels (> 400 ng/mL in plasma) indicating that a method's sensitivity in the low ng/mL area is not actually required. Furthermore, a PDA based methodology receives wider applicability as it is cost effective and can be easily and seamlessly adopted by the pharma industry. Considering the administration route, an *i.p.* administration methodology has been adopted in order to bypass the first-pass metabolism and/or gastric hydrolysis and gain a more holistic bioavailability profile of the substance (elimination of the liver-induced metabolism as well as exposure of TC4 in the low pH of the stomach) [38]. Hence, this study demonstrates for the first time the absence of hydrolysis of TC4 to CRC in plasma after *i.p.* administration, and provides preliminary evidence on the ability of TC4 to penetrate the Blood Brain Barrier (BBB) and localize inside the brain.

2. Materials and methods

2.1. Study design

The mice study was conducted under medical supervision at National and Kapodistrian University of Athens Medical School. Twelve (12) male mice were treated with 50 mg/kg pure TC4 (dissolved in 0.9% aqueous NaCl) through *i.p.* administration. Plasma samples and the whole brain of the mice were collected at predefined time points (0, 15, 30, 60, 120 and 240 min after administration). The protocol was approved by the ethical committee of the National and Kapodistrian University of Athens and was conducted according to the ICH-GCP guidelines (ICH GCP, 1996). The study received a permit from the Veterinary Directorate of the Prefecture of Athens (Approval #: 478/2014) according to the Greek legislation conforming to the 2010/53/EU Council Directive.

2.2. Chemicals and analytical reagents

The analytical reference standard of TC4 was isolated from *Crocus sativus* stigmas following a procedure previously developed and described in our laboratory [39]. Plant material *C. sativus* dried stigmas (saffron) was kindly provided by the Cooperative De Safran (Krokos Kozanis, West Macedonia, Greece). The purity of TC4 was found to be > 95% by HPLC-PDA using the continuous peak purity approach and its structure was verified by ^{1}H Nuclear Magnetic Resonance (NMR) spectroscopy. Crocetin (CRC) was produced after saponification of TC4 with aq. sodium hydroxide (10% w/v) at 60 °C for 4 h. The solution was then acidified with phosphoric acid and the yielded precipitate was washed with water. CRC was recrystallized from dimethylformamide [17] and its purity was better than 95% (HPLC). The internal standard (ISTD) 4-nitro-aniline (Fig. 1) was purchased from Sigma-Aldrich ® (Darmstad, Germany). All solvents were of LC-MS grade. Acetonitrile, methanol and water, were purchased from Avantor ® (Gliwice, Poland) whereas trifluoroacetic acid (TFA) was purchased from Sigma-Aldrich ®. Blank mice serum and brain for the validation of the PK studies were obtained from healthy animals (wild type mice) under written consent.

2.3. Stock solutions, calibrators and QCs preparation

Stock solutions of TC4, CRC and ISTD were prepared at a concentration of 1 mg/mL in methanol and stored at dark place at −20 °C. Working solutions were prepared in a daily basis by diluting appropriate volumes of the stock solutions in methanol in order to achieve the following concentration levels: 100, 10, 1 μg/mL and 100 ng/mL for both TC4 and CRC in the same initial solution and 100 μg/mL for the ISTD. The TC4 and CRC calibration curve in mouse plasma was constructed in the dynamic range of 10–6000 ng/mL (10, 25, 50, 100, 250, 500, 1000, 2000, 4000, 6000 ng/mL) while the concentration of the ISTD was kept at 100 μg/mL. In order to proceed to the validation of the described methodology, a different set of solutions were used as Quality Control (QC) samples in concentrations 30, 80, 3000, 4600 ng/mL (LQC1, LQC2, MQC and HQC respectively). For the construction of the calibration curve in mice brain the dynamic range was set from 0.05–5 ng/mg (0.05, 0.125, 0.25, 0.5, 1.25, 2.5, 4, 5 ng/mg) while the concentration of the ISTD was also kept at 100 μg/mL. The concentrations of the QC samples were 0.2, 0.35, 3, 3.5 ng/mg (LQC1, LQC2, MQC and HQC respectively).

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