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Validation of UHPLC–MS/MS methods for the determination of kaempferol and its metabolite 4-hydroxyphenyl acetic acid, and application to *in vitro* blood-brain barrier and intestinal drug permeability studies

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ABSTRACT

Sedative and anxiolytic-like properties of flavonoids such as kaempferol and quercetin, and of some of their intestinal metabolites, have been demonstrated in pharmacological studies. However, routes of administration were shown to be critical for observing *in vivo* activity. Therefore, the ability to cross intestinal and blood-brain barriers was assessed in cell-based models for kaempferol (KMF), and for the major intestinal metabolite of KMF, 4-hydroxyphenylacetic acid (4-HPAA). Intestinal transport studies were performed with Caco-2 cells, and blood-brain barrier transport studies with an immortalized mono-culture human model and a primary triple-co-culture rat model. UHPLC–MS/MS methods for KMF and 4-HPAA in Ringer-HEPES buffer and in Hank's balanced salt solution were validated according to industry guidelines. For all methods, calibration curves were fitted by least-squares quadratic regression with $1/X^2$ as weighing factor, and mean coefficients of determination (R^2) were >0.99. Data obtained with all barrier models showed high intestinal and blood-brain barrier permeation of KMF, and no permeability of 4-HPAA, when compared to barrier integrity markers.

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Abbreviations: 4-HPAA, 4-hydroxyphenyl acetic acid; ACN, acetonitrile; BSA, bovine serum albumin; Cal, calibrator; C_{Cl}, cell layer capacitance; CNS, central nervous system; CV%, coefficient of variation; CPT-cAMP, 8-(4-chlorophenylthio)-adenosine-3′,5′-cyclic monophosphate sodium salt; DMEM, dulbecco's modified eagle's medium; EMA, european medicines agency; ER, efflux ratio; ESI, electro-spray ionization; FBS, fetal bovine serum; FDA, food and drug administration; hBMEC, immortalized human brain microvascular endothelial cell line; HC, hydrocortison; HEPES, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); HPLC, high-performance liquid chromatography; IS, internal standard; KMF, kaempferol; LLOQ, lower limit of quantification; LogP, logarithm of partitioning coefficient; LY, lucifer yellow vs dilithium salt; min, minute; MRM, multiple reaction monitoring; MW, molecular weight; Na-F, sodium fluorescein; Papp, apparent permeability coefficient; PSA, polar surface area; QC, quality control; QCH, quality control high; QCL, quality control low; QCM, quality control medium; RBEC, primary rat brain capillary endothelial cell; RE%, relative error; RHB, Ringer HEPES buffer; Rpm, revolutions per minute; RT, room temperature; SD, standard deviation; SEM, standard error of the mean; SS, stock solution; TEER, transendothelial electrical resistance; ULOQ, upper limit of quantification; UHPLC–MS/MS, ultra-high performance liquid chromatography with tandem mass spectrometric detection; VA, vanillic acid; WS, working solution.

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1. Introduction

Flavonoids are a large and widely occurring class of natural products which are found in fruits, vegetables, wine, beer [1], and in medicinal plants [2,3]. Flavonoids reportedly possess a broad spectrum of pharmacological activities, such as anti-inflammatory, antiallergic, neuroprotective, cardioprotective, and cancer chemopreventive properties [1,4–9]. Sedative and anxiolytic-like activities of flavonoid-containing medicinal plants [10–14] and of selected purified flavonoids [15–19] have been substantiated in pharmacological studies. However, the route of administration appeared to be decisive, given that widely occurring flavonols such as kaempferol (KMF) and quercetin induced anxiolytic activities in mice only after oral administration (p.o.), but not upon intraperitoneal (i.p.) application [10].

Flavonols are metabolized by the intestinal microflora to phenolic acids, such as *p*-hydroxyphenylacetic, 3-phenylpropionic, *m*-hydroxyphenylacetic, *p*-hydroxyphenylpropionic and 3,4-dihydroxyphenylacetic acids [20–23]. Interestingly, phenolic acid metabolites of flavonoids, such as *p*-hydroxyphenylacetic acid (4-HPAA) and 3,4-dihydroxyphenylacetic acid showed anxiolytic activities after i.p. application [24]. Moreover, in gut sterilized mice no antidepressant-like effects were observed upon oral administration of KMF and quercetin [24]. Taken together, these findings suggested that flavonoids by themselves might not be the pharmacologically active compounds, but rather their metabolites produced by the intestinal microflora. At the same time, some of the intestinal metabolites of flavonoids occur in plasma as endogenous trace acids generated by oxidative deamination of amines such as dopamine and tyramine [25]. Plasma levels of the trace acids may vary significantly in several psychological disorders. It has been shown that plasma levels of 4-HPAA were significantly decreased in patients suffering from schizophrenia [26] and agoraphobia [27], as well as in violent offender prisoners [27], while they were increased in isolated aggressive mice [28]. Also, increased 4-HPAA and 3-HPAA levels in the brain were found upon treatment with anxiolytic and antipsychotic drugs such as chlorpromazine and sulpiride [29].

Bioactive compounds need to cross several biological barriers in the human body to reach the target tissues. After oral administration they have to be absorbed from the gut via the intestinal epithelium into systemic circulation. Compounds acting as modulators of brain function subsequently need to cross the blood-brain barrier (BBB) to reach the central nervous system (CNS) [30]. However, the systemic distribution and brain penetration of flavonoids and their metabolites is not well understood. To date, only a limited number of studies on intestinal [31–35] and brain permeability [36–39] of flavonoids and flavonoid metabolites have been published. Barrington et al. [40] showed that in Caco-2 cells KMF undergoes extensive phase 2 metabolism to sulfate and glucuronide conjugates, and that only a small fraction of non-conjugated KMF penetrated Caco-2 cell monolayers. Very recently, Yang et al. [37] reported the permeation of KMF in Caco-2 cells, and in a rat cell-based BBB model. However, none of these studies were performed with quantitative methods validated according to international bioanalytical guidelines [41,42]. To date, no transport studies with 4-HPAA have been reported. Therefore, we here investigated the permeation of KMF and its main metabolite, 4-HPAA, in human and rat *in vitro* barrier models. Determination of KMF and 4-HPAA in corresponding transport media was carried out with UHPLC–MS/MS assays. Quantification methods were developed and validated according to international guidelines [41,42].

2. Materials and methods

2.1. Ethics statement

Animal studies for the establishment of primary cultures were done according to the 1998. XXVIII. Hungarian law about animal protection and welfare. Formal approvals for animal studies have been obtained from the local Hungarian animal health authorities (Permit number: XVI./834/2012).

2.2. Reagents and chemicals

KMF, 4-HPAA, vanillic acid (VA), Hank's balanced salt solution (HBSS, pH 7.4), sodium fluorescein (Na-F), hydrocortisone (HC), chlorophenylthio-adenosine-3′,5′-cyclic monophosphate (CPT-cAMP), bovine serum albumin (BSA) and Dulbecco's modified Eagle medium (DMEM) were obtained from Sigma-Aldrich (Germany). RO 201724 was from Roche (Switzerland). Fetal bovine serum (FBS) "Gold" was from PAA Laboratories (Austria). $^{13}C_{15}$-labeled KMF was purchased from IsoLife (Netherlands), and Lucifer Yellow VS dilithium salt (LY) was from Santa Cruz (Germany). Formic acid (99%), and acetic acid glacial were obtained from Biosolve (Netherlands). Ringer HEPES buffer (RHB) was prepared by dissolving the ingredients (all from Sigma-Aldrich) in water as follows: NaCl (150 mM), $CaCl_2$ (2.2 mM), $MgCl_2$ (0.2 mM), KCl (5.2 mM), $NaHCO_3$ (6.0 mM), 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid HEPES (5 mM) and glucose (2.8 mM), adjusted to pH 7.4, filtered through 0.2 μm filter and stored at 4 °C. HPLC grade acetonitrile was from Scharlau (Spain), and UHPLC grade methanol was from Lab-Scan (Poland). High quality water was prepared using EASYpure II purification system (Barnstead, USA). Tissue culture inserts for the BBB models were from Greiner Bio-one® (Germany). The inserts used in the Caco-2 model were from Corning Costar® (USA).

2.3. UHPLC–MS/MS instrument and chromatographic conditions

UHPLC–MS/MS analyses were performed on an Agilent 6430 Triple Quadrupole MS system connected to a 1290 Infinity LC system consisting of binary capillary pump G4220A, column oven G1316C, cooling system G1330B, and autosampler G4226A. Quantitative analysis by MS was performed with electrospray ionization (ESI) in MRM mode. Desolvation and nebulization gas was nitrogen. MS/MS data were analyzed with Agilent MassHunter Workstation software version B.06.00. The temperature of the autosampler was set at 10 °C.

2.3.1. Quantification of KMF

A ZORBAX Eclipse Plus C18 column (2.1 × 50 mm, 1.8 μm, Agilent) was used. The mobile phase consisted of high purity water (solvent A) and acetonitrile (ACN) (solvent B) both containing 0.1% formic acid. The following gradient (A–B, v/v) was used: 75:25 (0–1 min), 75:25 to 72:28(1–2.6 min), 72:28 to 0:100 (2.6–2.7 min), 0:100 (2.7–3.7 min), 0:100 to 75:25 (3.7–3.8 min), hold for one min (flow rate: 0.4 ml/min). Column temperature was set at 55 °C. Injection solvent was water-ACN (65:35, v/v) both containing 1 mM acetic acid. The needle wash solvent consisted of a water-methanol mixture (1:1, v/v). Mass spectrometry detection was performed in positive ESI mode. MRM transitions of KMF and the IS, and the corresponding collision energies and fragmentor voltages are provided in Table 1. Capillary voltage was set at 5000 V. The desolvation gas temperature was 350 °C, the flow rate for desolvation gas was 13 l/min, and the nebulizer was set at 60 psi. The dwell time was automatically set at 250 msec.

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