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Synthesis of the flavonoid 3′,4′,5′-trimethoxyflavonol and its determination in plasma and tissues of mice by HPLC with fluorescence detection☆

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ABSTRACT

3′,4′,5′-Trimethoxyflavonol (TMFol) was synthesized as a potential colorectal cancer chemopreventive agent. An HPLC method for determination for TMFol in murine plasma and tissues was developed and validated using human plasma. Analyte was separated (C_{18} column; fluorescence detection 330 nm excitation, 440 nm emission) using 69% methanol and 0.1 M ammonium acetate buffer (pH 5.1) as mobile phase. The method was linear for 50–2500 ng/ml plasma and 0.05–10 μg/g tissue ($r > 0.99$). TMFol was recovered from plasma or tissues using solid phase columns or organic solvent protein precipitation, respectively. Recovery at low, medium and high concentrations was 97.6–107.3%, with inter- and intra-day coefficients of variation of <10%. The lower limit of quantitation for plasma was 50 ng/ml. The method was applied to measure steady-state TMFol plasma and tissue levels in mice which received dietary TMFol (0.2%).

1. Introduction

Flavonoids occurring in the diet, typified by the flavonol quercetin (Fig. 1), are associated with beneficial health effects. Prominent among these effects is the ability to prevent malignancies or delay their onset [1]. However, flavonoids can also exert detrimental effects. Quercetin for example has been shown to be mutagenic [2,3] and to compromise kidney function when administered at high doses in a phase I clinical trial in cancer patients [4]. The mutagenicity of quercetin has been linked to the presence of hydroxyl functionalities in the A ring (for structure see Fig. 1) [3]. Only a handful of flavonoids including quercetin have thus far been the subject of pharmacological investigation, and it is completely unknown whether other flavonoid analogues exist with pharmacological properties superior to those of the molecules studied to date. Consequently, information on how structural features affect the pharmacological efficacy of flavonoids is extremely scarce. In the case of flavones, exemplified by tricin from rice and other grass species, the presence of methoxy moieties in the molecular scaffold has been suggested to impart cancer chemopreventive efficacy on the molecule [5,6]. In contrast, the influence of methoxy groups on the activity of flavonols is unknown. As part of a programme designed to generate flavonoids with optimal cancer chemopreventive properties we synthesized 3′,4′,5′-trimethoxyflavonol (TMFol, for structure see Fig. 1). The synthesis of TMFol was guided by the desire to omit the mutagenic toxicophores of quercetin from the molecule, ie hydroxy groups in ring A, and to include methoxy functions in ring B, probable pharmacophoric features in cogeneric flavones [5,6]. In preliminary, as yet unpublished, studies TMFol inhibited murine adenoma cell growth *in vitro* with an IC_{50} of 1.3 μM, exhibiting much higher antiproliferative potency than quercetin or apigenin (unpublished). Currently TMFol is lined up to undergo exploration of putative cancer chemopreventive activity in rodent models of colorectal carcinogenesis. Pharmacokinetic studies are necessary components of the development of such agents. Therefore we report here the development and validation of a specific, precise and sensitive HPLC method which will allow detection and quantitation of TMFol in mammalian biomatrices *in vivo*.

2. Experimental

2.1. Chemicals and reagents

TMFol was synthesized using a procedure adapted from Sobottka et al. [7]: sodium (13.52 g, 0.59 mol) was dissolved in ethanol (500 ml) and 2-hydroxyacetophenone (20 g, 0.15 mol) was added. To this suspension 3,4,5-trimethoxybenzaldehyde (28.8 g, 0.15 mol) was added. The resulting solution was stirred at 30 °C for 18 h, after which H_2O (1 L) was added, and the solution was acidified

Abbreviations: TMFol, 3′,4′,5′-trimethoxyflavonol; HPLC, high performance liquid chromatography; PMF, 3′,4′,5′,5,7-pentamethoxyflavone; DMSO, dimethyl sulfoxide; LLOQ, lower limit of quantification; LoQC, low quality control sample; MeQC, medium quality control sample; HiQC, high quality control sample; AIN93G, American Institution of Nutrition optimally formulated rodent diet.

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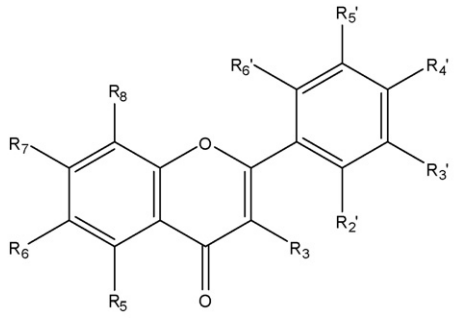

	R_3	R_5	R_6	R_7	R_8	$R_{2'}$	$R_{3'}$	$R_{4'}$	$R_{5'}$	$R_{6'}$
Quercetin	-OH	-OH	-H	-OH	-H	-H	-H	-OH	-OH	-H
Tricin	-H	-OH	-H	-OH	-H	-H	-OMe	-OH	-OMe	-H
TMFol	-OH	-H	-H	-H	-H	-H	-OMe	-OMe	-OMe	-H
Internal Standard	-H	-OMe	-OMe	-OMe	-OMe	-OMe	-H	-H	-OMe	-H

Fig. 1. Chemical structure of tricin, quercetin, TMFol and 2′,5′,5,6,7,8-hexamethoxyflavone, the internal standard.

(addition of 10% $HCl_{aq.}$ to pH 1). The product was extracted with $CHCl_3$ (3× 1 L), dried (Na_2SO_4) and evaporated. This chalcone intermediate was recrystallised from hot ethanol and then suspended in methanol (800 ml). 3 M $NaOH_{aq.}$ (150 ml) was added to produce a red solution. $H_2O_{2aq.}$ (30%; 166 ml, 1.47 mol) was added and the mixture was stirred (room temperature) for 2 h. The resulting suspension was poured into 10% $HCl_{aq.}$ (1 L) and the product was extracted into $CHCl_3$ (3 × 1 L), dried (Na_2SO_4) and evaporated to produce a yellow solid, which was recrystallised from hot ethanol to yield pale yellow crystals (18.24 g, 38%). M.pt. 181–183 °C; λ_{max}(MeOH)/nm 239 (ε/dm^3 mol^{-1} cm^{-1} 11334), 317sh, 355 (9978); δ_H(400 MHz; $CDCl_3$) 3.92 (3 H, s, C(4′)-OMe), 3.93 (6 H, s, C(3′,5′)-OMe), 7.37 (1 H, ddd, *J* 8.0, 7.1 and 0.7, C(6)H), 7.50 (2 H, s, C(2′,6′)H), 7.54 (1 H, dd, *J* 8.6 and 0.7, C(8)H), 7.66 (1 H, ddd, *J* 8.6, 7.1 and 1.6, C(7)H), 8.20 (1 H, dd, *J* 8.0 and 1.6, C(5)H); δ_c(75 MHz; $CDCl_3$) 56.13 (C(3′,5′)-OMe), 60.84 (C(4′)-OMe), 105.26 (C(2′,6′), 118.03 (C(8)), 120.45, 124.39 (C(6)), 125.26 (C(5)), 126.07, 133.41 (C(7)), 138.08, 139.80, 144.61, 153.00 (C(3′,5′)), 155.03, 173.10 (C(4)); HRMS (FAB) 329.10252 (M+H$^+$, $C_{18}H_{27}O_6$ requires 329.10202).

2′,5′,5,6,7,8-Hexamethoxyflavone supplied by the NCI Developmental Therapeutic Program's Open Compound Repository (NCI, Bethesda, USA) was used as internal standard (IS; for structure see Fig. 1). The IS and TMFol were >99% pure as determined by HPLC analysis. Ammonium acetate, ammonium hydroxide, acetic acid, formic acid, acetone and dimethyl sulfoxide (DMSO), all AnalaR grade, were obtained from Sigma (Poole, UK). HPLC fluorescence grade methanol was purchased from Fisher Chemicals (Loughborough, UK), and water for analysis was generated in a Nano-Pure water purification system (Barnstead, UK).

2.2. Preparation of standards and quality control samples

A stock solution of TMFol (1 mg/ml) prepared in DMSO was diluted to give working concentrations of 2.5–250 μg/ml. The purity of both IS and TMFol were analysed by the HPLC method described here, when injected on their own each gave one peak. Stock solutions of the IS in DMSO (2 mM) were prepared for spiking plasma and tissues. A fixed volume (1 μl) of the IS was added to aliquots of control plasma prior to extraction to generate quality control samples, its final concentration being 8.04 ng/ml. Concentrations of DMSO in samples did not exceed 2%.

2.3. Intervention

C57BL/6J mice were bred in the University of Leicester Biomedical Services facility using animals originally obtained from Charles River (Margate, UK). Experiments were performed under animal project licence PPL 80/2167, granted to the University of Leicester by the UK Home Office. The experimental design was assessed by the University of Leicester Local Ethical Committee for Animal Experimentation and met the required standards set by the UKCCCR guidelines [8]. Mice were randomised into control or treatment groups that received AIN93G diet or diet containing 0.2% TMFol, respectively, for a period of 1 week. At the end of the experiment animals were placed under terminal anaesthesia (halothane), and blood was collected (cardiac puncture) into a lithium–heparin tube. Plasma and tissues were collected and snap frozen in liquid nitrogen then samples were stored at −80 °C until analysis. Human plasma was obtained from the National Blood Transfusion Centre (Sheffield, UK).

2.4. Sample preparation

Frozen samples of plasma or tissues were thawed to room temperature. Aliquots of plasma (100 μl) were mixed with 2% formic acid (200 μl) and PBS (700 μl). Samples were loaded onto a Waters Oasis HLB Extraction Cartridge (Waters Corporation, Milford, USA), which had been pre-conditioned and equilibrated with methanol and water (1 ml each). The cartridge was subsequently washed with 1 ml each of 5% MeOH in 2% CH_3COOH, 5% MeOH in 2% NH_4OH and 5% MeOH in 0.1 M $NH_4^+CH_3COO^-$ buffer (pH 5.1). The analyte was eluted with 1 ml of acetone containing 0.1 M CH_3COOH. The eluate was evaporated to dryness (under nitrogen) and reconstituted in 100 μl of mobile phase. An aliquot (5 μl) of the supernatant was injected onto the HPLC column. In the case of the tissue samples, 1 part tissue was placed into 2 volumes PBS (w:v) and homogenized (Ystral × 10/20 homogenizer, Ballrechten-Dottingen, Germany). An aliquot of the homogenate (100 μl) was mixed with two volumes of acetone containing 0.1 M CH_3COOH, to precipitate protein and extract the analyte. After vortexing (2× 60 s) and centrifugation (13,000 rpm, 20 min) the sample was evaporated to dryness (under nitrogen) and reconstituted in 100 μl of mobile phase. An aliquot (5 μl) of the supernatant was injected onto the HPLC column. TMFol concentrations in the calibration standards and quality control samples ranged from 50 to 2,500 ng/ml and 0.05 to 40 μg/ml for plasma and tissue samples, respectively. In the case of murine plasma samples TMFol levels detected were at or below the limit of quantification, therefore 50 μl of sample rather than 5 μl was injected onto the column.

2.5. Chromatographic conditions

HPLC analysis was carried out using a Varian Prostar HPLC system (Varian, UK) which consisted of a Varian ProStar 230 solvent delivery system, a Pro-Star 363 fluorescence detector, a 410 Varian autosampler. HPLC separation was performed on a Gemini C_{18} column (4.6 mm × 150 mm, 3 μm, Phenomenex, UK) at a flow rate of 0.75 mL/min with 69% methanol in 0.1 M $NH_4^+CH_3COO^-$ buffer (pH 5.1) as an isocratic mobile phase. The content of TMFol in all samples was quantified using the internal standard method

3. Results and discussion

3.1. Method development

TMFol could be detected with adequate sensitivity by fluorescence detection with 330 and 440 nm as excitation and emission wavelengths, respectively. TMFol was well separated from the IS