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Short communication

High-performance liquid chromatographic determination of quercetin and isorhamnetin in rat tissues using β-glucuronidase and acid hydrolysis

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Abstract

Quercetin is a plant polyphenol which is present in the diet as an aglycone and as sugar conjugates. Despite potent vasodilatory and antioxidant effects in vitro, destruction by intestinal organisms has been assumed to limit its nutritional relevance in the rat. However, we have refined extraction techniques using β-glucuronidase followed by acid hydrolysis. Following this with HPLC methodology with post-column derivatisation, we have detected significant concentrations of quercetin and its metabolite, isorhamnetin, in tissues of rats maintained on quercetin-rich diets. Percentage recoveries are greater than 95% and intra-batch variation does not exceed 7% suggesting that the method may be useful in further studies of the biological role of this flavonoid. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Quercetin; Isorhamnetin; β-Glucuronidase

1. Introduction

Quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one; 3,3′,4′,5,7-pentahydroxy flavone) is a polyphenolic product of the phenylpropanoid biosynthetic pathway in plants and is therefore an integral part of the mammalian diet both as an aglycone and as β-glycosidic conjugates [1]. In vitro models indicate that this flavonoid has numerous biological roles, including antioxidant function, vasodilatory and platelet anti-aggregation effects and the maintenance of capillary integrity [2]. Early studies with rats indicated that quercetin had little nutritional relevance as the aglycone and the β-glycosides appeared to be destroyed by intestinal microorganisms in the small intestine and lower bowel [3,4]. Recently, however, quercetin and its methylated derivative, isorhamnetin, have been detected in plasma and urine of rats maintained on quercetin-rich diets [5]. This suggests that a significant proportion of the aglycone may be absorbed and further metabolised by tissues. Consequently, to further elaborate on the bioavailability of quercetin, we have developed methodology for extraction using β-glucuronidase incubation and acid hydrolysis with subsequent HPLC detection to quantify tissue con-

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centrations of quercetin and isorhamnetin in rats supplemented with the aglycone for 2 weeks.

2. Experimental

2.1. Animals and diets

Ten individually-housed weanling male rats of the Rowett Hooded Lister strain were offered, ad libitum, for 10 weeks a standard torula-yeast based semisynthetic diet [6]. Five rats were then offered the diet containing quercetin at a concentrations of 5 g/kg for a further 2 weeks and the remainder were maintained on the original ration. Diets were stored at −40°C before use.

Rats were anaesthetised with ether and blood removed by cardiac puncture into heparinised evacuated tubes (Becton Dickinson, Oxford, UK). Plasma was obtained by centrifugation (1500 *g*, 10 min, 4°C). The livers were perfused in situ with chilled 0.15 *M* KCl and removed along with the kidneys and heart. All tissues and plasma were immediately snap frozen in liquid nitrogen and stored at −80°C.

2.2. Chemicals

Sodium acetate, acetic acid, hydrochloric acid and butanol (all Analar grade) were obtained from Merck (Poole, UK). Aluminium nitrate nonahydrate, trifluoroacetic acid (TFA), diethyldithiocarbamic acid sodium salt and β-glucuronidase (grade H-2 from *Helix pomatia*) were purchased from Sigma (Poole, UK) whereas HPLC grade methanol and acetonitrile were supplied by Rathburn Chemicals (Walkerburn, UK). Isorhamnetin was obtained from Apin (Abingdon, Oxon, UK) and quercetin was from Fluka (Gillingham, UK). All solutions were prepared where appropriate with double distilled, deionised water.

2.3. Extraction procedures

Tissues (~1 g fresh weight) were homogenised on ice in 2 ml phosphate buffered saline (pH 7.4) and 0.6 ml of the homogenate (or plasma) was incubated with the same volume of β-glucuronidase (activity 5000 U/ml of 0.2 *M* acetate buffer, pH 5.0) for 30 min at 50°C. Following addition of 0.6 ml of methanol containing diethyldithiocarbamic acid (40 m*M*) and a similar volume of 4.8 *M* hydrochloric acid, the homogenate was incubated for a further 2 h at 90°C. Once cooled the sample was shaken vigorously for 2 min with 5 ml of distilled water and 2 ml of butanol and centrifuged (2000 *g*, 5 min). An aliquot (1 ml) of the butanolic supernatant was dried under vacuum (Savant Speed Vac 110A), reconstituted with 600 μl of a solution containing 50% methanol and 50% of a mixture consisting of 30% methanol, 15% HCl (4.8 *M*), 15% distilled water and 40% of the initial mobile phase (i.e. 85% trifluoroacetic acid: 15% acetonitrile) [7]. Inclusion of methanol in the reconstitution mixture was found to greatly facilitate the solubilisation of the sample and did not markedly affect the peak intensity. Samples were then filtered (0.2 μm Vectaspin Micro Anapore) prior to the injection of 100 μl onto the HPLC column. Quercetin and isorhamnetin standards and homogenates spiked with standards to assess recovery rate were subjected to identical procedures.

2.4. Apparatus and chromatographic conditions

Samples were loaded (100-μl loop) from a Gilson (Anachem, Luton, Beds, UK) 321 autosampler fitted with an 832 temperature controller maintained at 4°C onto an Jones Genesis (Jones Chromatography, Henygoed, Mid Glamorgan, UK) C_{18} column (15 cm×4.6 mm, 4 μm) preceded by a Waters (Watford, UK) Symmetry guard cartridge. The C_{18} column and the derivatisation coil (see below) were contained in a column oven (Jones) at 40°C. The HPLC system consisted of a Gilson 305 and 306 pump with 811b dynamic mixer and 805 manometric module maintaining a flow-rate of 0.75 ml/min. To increase sensitivity, postcolumn derivatisation was carried out to convert quercetin into a fluorescent quercetin–aluminium complex [8]. However, to prolong column longevity, the concentration of aluminium nitrate was reduced to 0.1 *M* as preliminary experiments indicated that this did not markedly affect the chromatography (A. Crozier, personnel communication). Prior to passing through a fluorescence detector (Shimadzu RF 535; excitation 420 nm/emmission 480 nm), the eluent was mixed in a PEEK coil at

Table 1
Gradient conditions for optimal separation of quercetin and isorhamnetin in plasma and tissue homogenates

Time (min)	Solvent A[a] (%)	Solvent B[b] (%)
0	85	15
2	85	15
8	60	40
12	80	20
17	80	20
35	10	90
45	85	15

[a] Trifluoroacetic acid H_2O (pH 2.5).
[b] Acetonitrile.

40°C (Reeve 9802 low pressure pump, flow-rate 0.8 ml/min) with 0.1 M $Al(NO_3)_3$ in methanol containing 7.5% (v/v) acetic acid. The optimal chromatographic separations were obtained using an elution gradient (Table 1). The mobile phases were continuously sparged with helium and the system was managed and data integrated using a computer with Gilson UNIPOINT software (version 1.65).

2.5. Extraction efficiency and precision

Replicate ($n=5$) liver homogenates and plasma samples were spiked with quercetin, and isorhamnetin (final concentrations 500–1667 nM) and using the extraction procedures described above, the percentage extraction recovery (ER) was calculated. Intra-assay precision was estimated as the coefficient of variation calculated from the values obtained from five independent extractions from a plasma pool and liver homogenate. All tissue data was standardised to protein content and is given as mean±SEM.

3. Results and discussion

The chromatographic peaks of quercetin and its methylated derivative, isorhamnetin, were well separated (retention times 13.07±0.02 and 28.54±0.79 min, respectively) (Fig. 1). There was some indication of the coelution of endogenous components with quercetin, although the peaks were comparatively small. Moreover, a peak with a retention time of 24.82±0.20 min may be kaempferol which differs from quercetin only by the absence of an hydroxyl group on position 5 of the B-ring. Although the spiking of samples leads to the proportional augmentation of peak area, definitive identification of kaempferol is lacking and the breadth of the peak and possible small shoulder may indicate the presence of additional components.

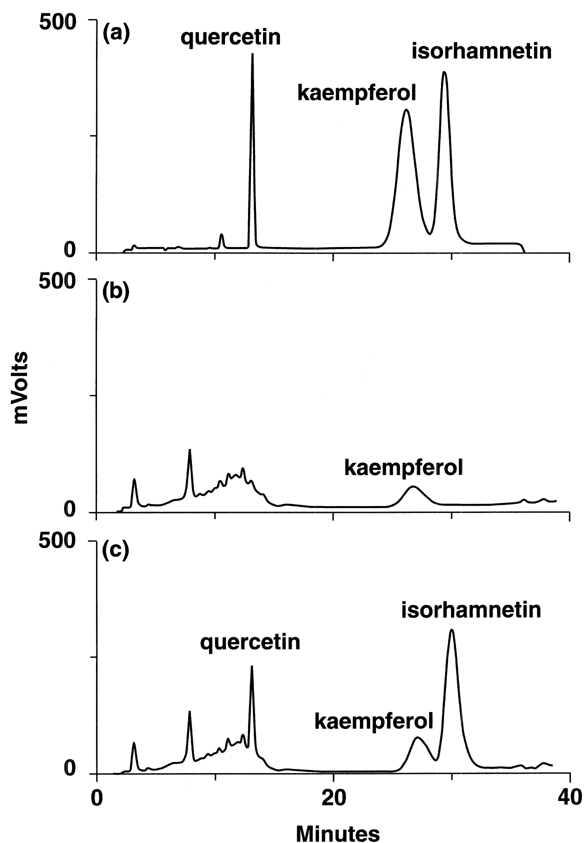

Fig. 1. Example of chromatogram showing the presence of quercetin and isorhamnetin in liver homogenates from rats maintained on the quercetin rich diet for 2 weeks (c) compared with animals maintained on the standard ration (b). A solution containing 2 μM quercetin, 1 μM kaempferol and 1 μM isorhamnetin is also shown for comparison (a).

Mean (±SEM) percentage recoveries from spiked samples were: plasma: quercetin 96.7±1.0; isorhamnetin 95.2±4.8; liver homogenate: quercetin 92.0±4.7, isorhamnetin 88.8±6.5. Estimates of intra-batch variation were calculated from five replicates of plasma and liver tissue using a quercetin standard curve with a maximum value of 2 μM and an isorhamnetin curve with an upper concentration of

Table 2
Concentrations of quercetin and isorhamnetin following enzymatic digestion and hydrolysis of tissues and plasma of rats on standard semisynthetic ration (S) or supplemented with 5 g quercetin/kg diet (+Q)[a]

Tissue	Quercetin		Isorhamnetin	
	S	+Q	S	+Q
Plasma (n*M*)	0.34±0.03	15.3±2.2	0.02±0.01	51.3±7.4
Liver (nmol/mg protein)	1.2±0.02	6.9±1.3	0.03±0.01	9.8±2.1
Heart (nmol/mg protein)	0.20±0.06	2.6±0.6	0.07±0.01	11.1±1.8
Kidney (nmol/mg protein)	0.96±0.15	15.4±1.4	0.04±0.01	25.1±2.2

[a] Data as mean±SEM, five rats/group.

1 μ*M*. Mean (±SEM) plasma quercetin concentrations in these plasma and liver samples were 1229±10 n*M* and 393±3 n*M*, respectively. For isorhamnetin, plasma values were 74±3 and liver values were 507±3 n*M*. The intra-batch coefficients of variation for estimations in plasma were quercetin 6.6%; isorhamnetin 7.2% and for liver homogenate were 3.2 and 1.7% for quercetin and isorhamnetin, respectively. The assay was highly linear over a range of standards (0–4 μ*M*) (r=0.995 and 0.999 for quercetin and isorhamnetin, respectively; $P<0.001$; degrees of freedom, 35) and limits of detection (LOD) were 5 n*M* for quercetin and 10 n*M* for isorhamnetin. The limited material available from a rat meant that it was not feasible to obtain a value for the interbatch coefficient of variation by measuring the same sample over numerous successive days. However, analysis of the same liver sample on two occasions 1 day apart gave quercetin concentrations of 2.12 and 2.42 μmol/g and isorhamnetin concentrations of 0.77 and 0.78 μmol/g, respectively.

Consumption of the quercetin-rich diet was associated with marked increases in concentrations of quercetin and isorhamnetin in plasma and tissues compared with animals maintained on the standard ration (Fig. 1 and Table 2). The lack of isorhamnetin in plasma and tissues of the rats maintained on the standard ration suggests that its presence in the quercetin-fed rats is due to 3′-O-methylation of quercetin in vivo. The plasma concentrations of quercetin and isorhamnetin (Table 2) are similar to that detected in quercetin-fed rats by Manach et al. [5]. To our knowledge, the present study is the first to determine quercetin in a range of tissues and the results indicate that significant accumulation of quercetin and its metabolite, isorhamnetin, occurs in liver, kidney and heart when rats consume quercetin-rich diets for 2 weeks. In the present study, only the aglycone of quercetin rather than quercetin glycosides were added to the diet. However, it should be noted that the current method would result in the conversion of any quercetin glycosides present to the aglycone and thus both species would be measured simultaneously. It will be necessary to develop extraction procedures which do not employ acid hydrolysis to differentiate the flavonoid glycosides in tissues. It is unclear at present how this may be achieved. However, once extraction procedures are resolved the chromatographic determination of selected glycosides may be feasible using HPLC with photodiode array detection for example [9]. Nevertheless, the method described suggests that the bioavailability and physiological relevance of quercetin in the rat may be greater than previously assumed [10].

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