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Determination of quercetin in human plasma using reversed-phase high-performance liquid chromatography

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Abstract

A method is reported for the measurement of quercetin in human plasma using reversed-phase high-performance liquid chromatography (HPLC). Quercetin and kaempferol (as internal standard) were spiked into plasma samples and extracted using C_{18} Sep-Pak Light cartridges (efficiency >85%). Flavonoids were eluted with aqueous acetone (50% v/v, pH 3.5), dried down and redissolved in aqueous acetone (45% v/v, pH 3.5). The increased osmolarity promoted a phase separation and the water-saturated acetone layer, containing the flavonoids, was analysed by HPLC with aqueous acetone mobile phase (45% v/v acetone in 250 m*M* sodium dihydrogen sulphate. The mixture was adjusted to pH 3.5 with phosphoric acid and used at a flow-rate of 1.0 ml/min) and μBondapak C_{18} column (150 × 3.9 mm I.D., 10 μm particle size). The detection limit ($A_{375\ nm}$) for quercetin in plasma was 0.1 μg/ml (300 n*M*). The method also detects metabolites of quercetin, although these are not yet identified.

1. Introduction

Flavonoid glycosides are present in many fruits and vegetables and consumed in a healthy diet at levels of 1–2 g per day [1]. Many flavones and flavonol glycosides have been newly-discovered in recent years; their structures, distribution, metabolism and chemistry have been reviewed extensively [2]. Quercitrin, a naturally occurring flavonoid, is the most common dietary component and is hydrolysed in the gut by intestinal bacteria, releasing free quercetin [3]. Quercetin has a wide range of biological activities, which include a strong and prolonged anti-inflammatory effect [4], inhibition of histamine release from mast cells [5] and platelet deposition on blood perfused collagen strips at 50 n*M* [6]. Quercetin has been used with therapeutic intent to treat inflammation, allergy, bee sting, ulcer [7] and varicosity [8]. Recent studies have reported that quercetin exerts powerful antiproliferative activity on human breast [9], leukaemia [10], gastro-intestinal [11] and ovarian [12] tumour cells at micromolar concentrations. It has also been demonstrated that quercetin exhibits a synergistic antiproliferative effect with cisplatin [7], and acts as an inhibitor of HIV-reverse transcriptase [13]. All these results suggest that quercetin could be a compound with potential clinical applications.

In 1973 it was reported that quercetin levels in blood and urine could be measured indirectly

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following derivatisation of the flavonoid to produce a fluorescent quercetin–tetraphenyldiboroxide complex [14]. This method is complicated and may lack specificity. In this paper we report the development of an HPLC method which shows sufficient specificity, sensitivity and simplicity for the measurement of quercetin in human plasma, and which is being applied routinely in a clinical trial to determine quercetin blood levels [15].

2. Experimental

2.1. Chemicals and solvents

Quercetin, fisetin, morin, myricetin and naringenin (Fig. 1) were obtained from Aldrich (Gillingham, UK) and kaempferol was purchased from Sigma (Poole, UK). All solvents were of analytical or HPLC grade and obtained from BDH (Poole, UK), except for acetone which was from Fluka (Poole, UK). Water was purified (18 MΩ) by a Milli-Q water purification system (Elga, High Wycombe, UK).

2.2. HPLC analysis

Chromatographic analysis of flavonoids was performed using a Kontron HPLC system (Watford, UK) equipped with a high-pressure mixing solvent delivery system (HPLC 422); an automated sample injector (HPLC465) and diode-array spectrophotometric detector set to the known absorbance peak at 375 nm (HPLC 440). System control, data collection and data evaluation were performed using an IBM PC with a 450 MT_2 software data package (Kontron, Watford, UK). The resolution of quercetin and other flavonoids was compared using μBondapak C_{18}, Spherisorb S5 C_8 and Hypersil ODS analytical columns with a range of mobile phases (vide infra). Quercetin has a molar extinction coefficient of 20 900 at 373 nm [16].

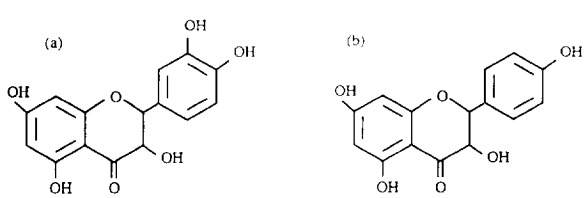

Fig. 1. Chemical structures of (a) quercetin and (b) kaempferol.

2.3. Standards and control samples

Quercetin (2.5 mg/ml) and kaempferol, fisetin and morin (each 1.0 mg/ml) were dissolved in methanol and all were stored at −20°C as stock solutions. Plasma standards were prepared by addition of quercetin stock solution to drug-free pooled human plasma giving final concentrations of 0.1, 1.0, 5, 20, 50, 100, 200 and 300 μg/ml. The standards were incubated in capped tubes for 1 h at 37°C, then divided into 1-ml aliquots and stored at −20°C prior to use. Standard curves were generated following the extraction and HPLC analysis of spiked plasma samples, as described below. Quality control samples were prepared in the same way, from different stock solutions, for determination of the within-day and between-day variability of measurements.

2.4. Solid-phase extraction procedure

Samples of human plasma (up to 1 ml, depending on anticipated quercetin content) were diluted and acidified with sodium phosphate buffer (pH 2.0, 0.33 *M*, 5 ml). Kaempferol (100 μl, 50 μg/ml) or fisetin (60 μl, 50 μg/ml) was added as an internal standard. Sep-Pak Light cartridges (Waters plc, Watford, UK) were pre-equilibrated with methanol (2 ml) and conditioned using sodium phosphate buffer (as above; 1 × 6 ml wash). The diluted plasma samples were loaded slowly onto the cartridges. Following a wash with sodium phosphate buffer (as above; 2 ml), flavonoids were eluted with aqueous acetone (4 ml, 50% v/v acetone, containing 250 m*M* NaH_2PO_4 and 0.1% v/v diethylamine, adjusted to pH 3.5 with orthophosphoric acid), dried down in a Gyrovap and redissolved in aqueous acetone (600 μl of 45% v/v acetone in 250 m*M* NaH_2PO_4; the mixture was adjusted to pH 3.5 with orthophosphoric acid). The

increased osmolarity from salts in the pellet promoted a phase separation of the aqueous acetone, with flavonoids remaining in the water-saturated acetone layer. Following centrifugation (13 000 *g*, 10 min) the acetone layer was used directly for HPLC analysis (sample size 5–80 μl, depending on flavonoid concentration). The mobile phase adopted (vide infra) was a mixture of acetone (45%) with 250 m*M* sodium dihydrogen phosphate solution (55%) adjusted to pH 3.5 with orthophosphoric acid. The flow-rate was 1 ml/min.

In order to estimate extraction efficiency, samples of plasma (1 ml) containing known amounts of quercetin were subject to the extraction procedure and redissolved in mobile phase containing kaempferol (3 μg/sample) as internal standard. The extraction efficiency was calculated by comparing the ratios of quercetin to fisetin peak areas in the HPLC traces of extracted samples with those of standards of identical concentrations prepared in mobile phase containing internal standard. All analyses were performed in triplicate.

2.5. Stability of quercetin added to plasma samples

Stability of quercetin added to plasma was evaluated at 37°C and −20°C, respectively. Plasma samples containing quercetin (5 μg/ml) were prepared and aliquots (1 ml) placed into capped tubes. Three samples were placed at −20°C and others were maintained (in triplicate) at 37°C for 2.5, 5, 10, 24, 34 h, and then stored at −20°C. Analyses were performed on the day following freezing. In separate experiments, storage stability at −20°C was determined by analysis of quercetin in plasma after several storage times up to 60 days.

2.6. Clinical drug administration and blood sampling

Quercetin was administered to patients at doses up to 2000 mg/m^2 in a DMSO vehicle (100 mg/ml) by intravenous bolus injection into the side-arm of a rapid infusion of DMSO (30 ml, 50% v/v) as part of a phase I clinical trial [15]. Blood samples (10 ml) were collected intermittently into heparinised tubes via an indwelling intravenous catheter placed in the opposite arm. The blood was centrifuged at 2000 *g* for 5 min, and the plasma separated and stored at −20°C or −70°C until analysed.

3. Results and discussion

3.1. High-performance liquid chromatography

The aim of this present study was to develop a rapid HPLC assay capable of the measurement of quercetin in human plasma following intravenous administration.

Following comparison of various stationary phases a μBondapak C_{18} column was adopted for routine use since it afforded the greatest efficiency of flavonoid recovery and more symmetrical peaks than could be achieved using Spherisorb S5 C_8 or Hypersil ODS columns.

Several potential mobile phases were examined in conjunction with the μBondapak C_{18} column, including acetonitrile, methanol, propan-2-ol, acetone and various combinations used as organic modifiers. However aqueous methanol produced very broad peaks, acetonitrile gave poor resolution between quercetin, its metabolites and the internal standards, while the viscosity of propan-2-ol solutions resulted in unacceptably high column pressures. A mixture of acetone–sodium dihydrogen phosphate (250 m*M*) (45:55, v/v, adjusted to pH 3.5 with phosphoric acid) produced symmetrical peaks with retention times long enough to give an adequate separation between quercetin and the internal standards (Fig. 2). This mobile phase was therefore adopted for assay of quercetin, using kaempferol or fisetin as internal standards. Subsequently, addition of a small amount of diethylamine (0.1%, v/v) was found to improve peak symmetry further without affecting other parameters.

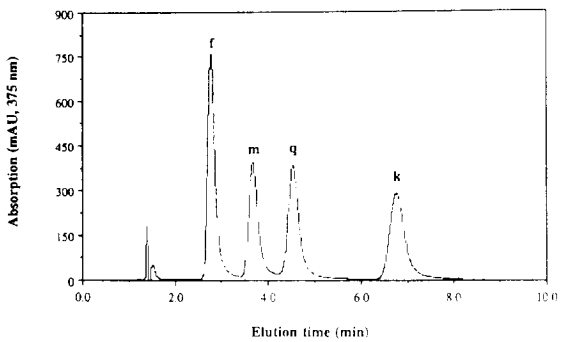

Fig. 2. HPLC resolution of flavonoids using a μBondapak C_{18} analytical column with aqueous acetone mobile phase as described in the text. Flavonoids were injected directly onto the column in aqueous solution (5 μg/ml). Peaks: f = fisetin, m = morin, q = quercetin, k = kaempferol.

3.2. Extraction of flavonoids from human plasma

Solid-phase extraction methods were investigated using C_2, C_8, C_{18}, CN, NH_2, Silica Diol and Alum B Sep-Pak extraction cartridges. The most efficient extraction of quercetin was obtained using CN and C_{18} cartridges, and the C_{18} cartridge was adopted for routine use. Thorough acidification of the extraction cartridge and plasma sample was found to be essential for optimal performance. The extraction efficiency for kaempferol (10 μ*M*) from spiked human plasma was found to be 86.2 ± 3.0%, while quercetin routinely showed over 90% extraction (Table 1). Calibration of the overall analytical procedure gave a linear signal ($r = 0.999$) over the quercetin concentration range 300 n*M*–3 m*M*.

Because of its poor solubility in water, quercetin was administered to patients in a DMSO vehicle which caused a brief but noticeable haemolysis. DMSO is known to cause mild haemolysis upon intravenous injection [17]. The extraction system described above was developed to minimise contamination of the HPLC traces by the products of haemolysis; notably the elution of flavonoids from the C_{18} column with aqueous acetone, drying down and subsequent phase partitioning produced much cleaner HPLC traces than could be obtained using alcoholic eluents. This was particularly important for accurate quantitation of trace amounts of quercetin.

Table 1
Efficiency of solid-phase extraction of quercetin from spiked human plasma

Concentration of quercetin (μg/ml)	Recovery ± (mean ± S.D.) (%)	Coefficient of variation (%)	*n*
300	91.8 ± 2.4	2.7	12
50	96.6 ± 3.4	3.4	12
5	107.8 ± 5.2	4.8	12
1	98.9 ± 9.8	9.9	11

Analysis of plasma samples taken at various times from patients administered quercetin showed a quercetin peak after 4.3 min. There were also a number of smaller peaks, thought to represent unidentified quercetin metabolites, rising transiently as the quercetin signal decreased.

3.3. Selection of internal standard

Several related flavonoids (fisetin, morin, myricetin, naringenin and kaempferol; see Fig. 1) were examined as possible internal standards. Myricetin, naringenin and morin showed poor recovery during C_{18} Sep-Pak Light solid-phase extraction, and were considered unsuitable for use as internal standards. When fisetin and kaempferol were applied as internal standards to samples obtained from quercetin-treated patients it was found that some sample-related peaks overlapped significantly with the fisetin peak, but not with the kaempferol peak (Fig. 3a). Kaempferol, therefore, was chosen as the internal standard for assay of clinical trial samples. The ratio of quercetin absorption to kaempferol absorption (both measured at 10 μ*M*, 375 nm) was found to be 0.83. Analysis of plasma samples from untreated volunteers showed no measurable signals (Fig. 3b).

Fig. 4 shows a typical HPLC trace generated by analysis of kaempferol-spiked plasma obtained from a patient 15 min following administration of quercetin (945 mg/m^2). The peaks are clearly resolved, permitting accurate quantification.

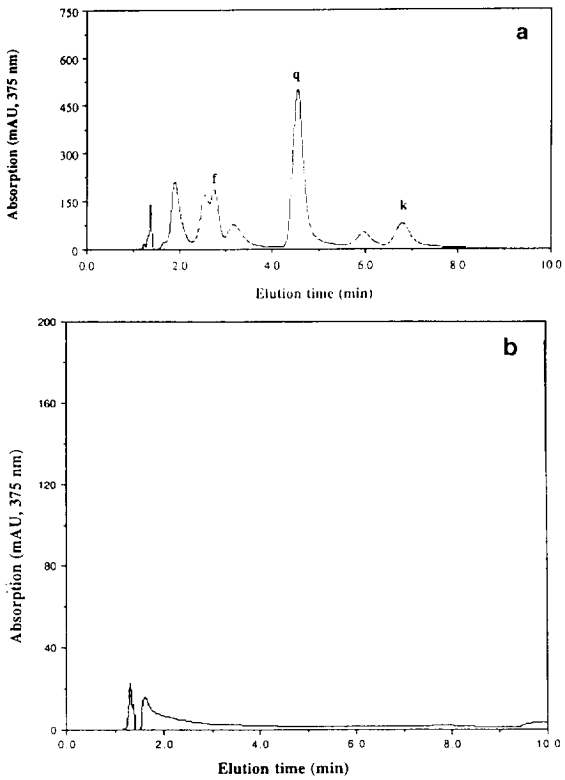

Fig. 3. (a) HPLC analysis of plasma sample isolated from a patient administered quercetin (630 mg/m^2) in DMSO vehicle and spiked with fisetin (50 μg/ml) and kaempferol (50 μg/ml) as potential internal standards. Labelled peaks represent: f = fisetin, q = quercetin, k = kaempferol. Unlabelled peaks are thought to represent quercetin metabolites. (b) HPLC analysis of blank human plasma sample extracted and processed in the same way as experimental samples, described fully in the text.

3.4. Linearity, precision and limit of quantitation for the analytical procedures

Direct HPLC analysis of quercetin gave a linear response over the concentration range of 300 nM to 3 mM ($r = 0.999$). The precision and reproducibility of the procedure for measurement of quercetin in plasma was examined by extracting and analysing replicate plasma samples containing 1, 5, 50 and 300 μg/ml of quercetin. The results are shown in Table 2, including the within-day and between-day coefficients of variation.

The limit for determination of quercetin in human plasma using this protocol (defined as a signal-to-noise ratio of 3) was 0.1 μg/ml, equivalent to 300 nM.

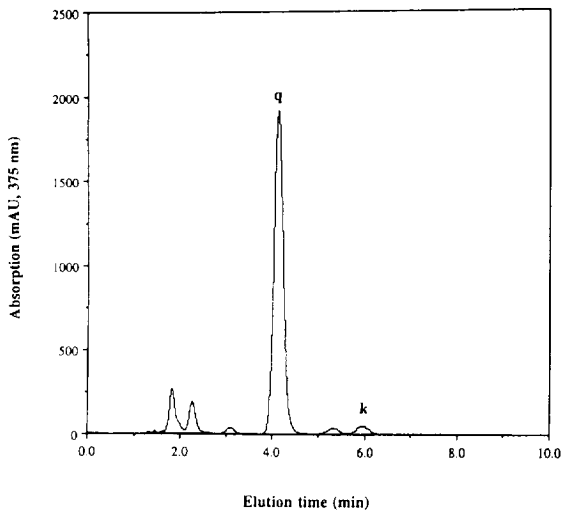

Fig. 4. HPLC trace obtained using plasma from a patient 15 min following administration of quercetin (945 mg/m^2) in DMSO vehicle, analysed as described in the text. The labelled peaks represent: q = quercetin, k = kaempferol. Unlabelled peaks are thought to represent quercetin metabolites.

3.5. Stability of quercetin in plasma and analytical samples

Quercetin added to plasma samples (5 μg/ml, approx. 17 μM) was found to be stable for 5 h at 37°C (stability defined as $\geq 90\%$ of initial amount remaining) although after 10 h the concentration determined fell to about 60%. When stored at −20°C, quercetin added to

Table 2
Precision of quercetin analysis in spiked human plasma

Concentration of quercetin (μg/ml)	Coefficient of variation (%)	
	Between-day (n)	Within-day (n)
300	2.7 (12)	2.2 (6)
50	3.4 (12)	1.7 (6)
5	4.8 (12)	2.0 (6)
1	9.9 (11)	6.5 (5)

plasma was found to be stable for at least 2 months.

As explained above plasma samples prepared from the blood of patients administered quercetin contained products of haemolysis. Such samples were reanalysed for quercetin following 3 months storage at −20°C or −70°C. Although some samples showed no measurable deterioration over this time, the level of quercetin determined in others fell as much as 36% compared with the starting level. Samples which deteriorated behaved similarly at both storage temperatures and consequently chromatographic analyses of clinical samples are now routinely performed within 2 weeks after sampling.

The possibility of flavonoid deterioration during the HPLC analysis was discounted since the signals generated by the internal standards showed no variation throughout the procedure. In addition, processing of standards relating to the calibration was always performed half way through the analysis and invariably produced straight lines with correlation coefficient ≥ 0.999, indicating no significant quercetin deterioration.

4. Conclusions

The method described in this paper represents a specific and sensitive HPLC assay for the determination of quercetin in human plasma following its administration in a DMSO vehicle. The method described previously [14] required a complex extraction procedure, subsequent coupling to a fluorophore and was unable to distinguish between quercetin and its putative metabolites. The method reported here is both simple and highly reproducible and is being used to study the pharmacokinetics of quercetin in patients with advanced cancer [15]. The levels of quercetin achieved 15 min after administration of an intravenous dose of 945 mg/m^2 are in the range 20–40 μM and are higher than those required in vitro to demonstrate antiproliferative effects against ovarian [12] or breast [9] cancer cell lines, to act synergistically with cisplatin [18] or to potentiate the action of cytosine arabinoside to kill human acute myeloid leukaemic cells ex vivo [10]. The investigation of quercetin as an anticancer agent [15] and sensitiser to conventional cytotoxics will be facilitated by the assay described in this paper.

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