High performance liquid chromatography tandem mass spectrometry dual extraction method for identification of green tea catechin metabolites excreted in human urine

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Abstract

The simultaneous analysis of free-form and conjugate flavonoids in the same sample is difficult but necessary to properly estimate their bioavailability. A method was developed to optimise the extraction of both free and conjugated forms of catechins and metabolites in a biological sample following the consumption of green tea. A double-blind randomised controlled trial was performed in which 26 volunteers consumed daily green tea and vitamin C supplements and 24 consumed a placebo for 3 months and urine was collected for 24 hours at 4 separate time points (pre- and post-consumption) to confirm compliance to the supplementation and to distinguish between placebo and supplementation consumption. The urine was assessed for both free and conjugated metabolites of green tea using LC-MS^2 analysis, after a combination extraction method, which involved an ethyl acetate extraction followed by an acetonitrile protein precipitation. The combination method resulted in a good recovery of EC-O-sulphate (91±7 %), EGC-O-glucuronide (94±6 %), EC (95±6 %), EGC (111±5 %) and ethyl gallate (74±3 %). A potential total of 55 catechin metabolites were investigated, and of these, 26 conjugated (with methyl, glucuronide or sulphate groups) and 3 free-form (unconjugated) compounds were identified in urine following green tea consumption. The majority of EC and EGC conjugates significantly increased post-consumption of green tea in comparison to baseline (pre-supplementation) samples. The conjugated metabolites associated with the highest peak areas were O-methyl-EC-O-sulphate and the valerolactones M6/M6'-O-sulphate. In line with previous studies, EC and EGC were only identified as conjugated derivatives, and EGCG and ECG were not found as mono-conjugated or free-forms. In summary, the method reported here provides a good recovery of catechin compounds and is appropriate for use in the assessment of flavonoid bioavailability, particularly for biological tissues that may contain endogenous deconjugating enzymes.

1. Introduction

Green tea is the second most popular tea beverage, with the majority of consumption in Asian countries and North Africa, and with a recent increase in popularity in Western countries [1]. Green tea consists of catechins (or flavan-3-ols), which are a sub-group of the flavonoids. In comparison to other teas, green tea has the highest flavanol content (35-50 %) [2], including catechin (C), epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG) and epigallocatechin gallate (EGCG) (Fig. 1).

Daily consumption of green tea has been highly associated with beneficial health effects, studied both in vivo and in vitro [3-7]. Epidemiological studies have linked green tea consumption with reducing the incidence of cancer onset [8], factors associated with cardiovascular disease [9-11] and factors related to the onset of diabetes [1,12]. Research has become more focused on the presence of conjugated metabolites in biological fluids following green tea interventions [13-15]. However, analysis of flavonoids in biological samples presents several challenges. The free and aglycone forms of flavonoids are most soluble in lipophilic solutions, but are unstable during analysis [16-18]. Above pH 7 and 37 ºC, the stability of free-form catechins is reduced due to degradation and epimerisation, and therefore, most free-form analysis utilises a low pH and low temperature environment with the addition of an antioxidant, such as ascorbic acid, to improve the stability [19-21]. As most of the conjugated catechins are commercially unavailable, enzyme deconjugation of green tea catechin metabolites present within biological samples [22-25] allows for the assessment of the metabolites relative to the free-form standards. However, the employment of this method has been questioned due to inefficient and incomplete hydrolysis [26], and as a result the more stable conjugated catechin forms have been assessed by monitoring the fragmentation patterns of the compounds using liquid chromatography mass spectrometry (LC-MS^2) analysis [27-31]. Due to the hydrophilic nature of the conjugated catechins, the majority of the methods that omit deconjugation utilise a protein precipitation of urine with acetonitrile prior to LC-MS^2 analysis [28,30]. A direct injection of the filtered urine sample following the consumption of green tea can also be employed, however, this can lead to ion suppression [32,33].

The extraction of free-form catechin metabolites in acetonitrile is associated with a poor recovery [28], and therefore, these molecules cannot be efficiently monitored in biological samples using extraction techniques that are suitable for the hydrophilic conjugated forms. In the present study, a combination of using an in initial ethyl acetate extraction to isolate free-form metabolites followed by protein precipitation of the remaining urine sample with acetonitrile, which allows the identification of green tea conjugated metabolites, was utilised for the first time. This method is applicable for future human intervention studies in which both the free and conjugated forms can be monitored in biological samples following consumption of green tea, particularly for samples where β-glucuronidase and sulphatase are already present such as tissue extracts.

2. Experimental

2.1 Materials

Epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), epigallocatechin gallate (EGCG), catechin (C) and taxifolin were purchased from Extrasynthése (Genay, France). Ethyl gallate and 3-methyl gallic acid were obtained from Apin Chemicals Ltd (Oxfordshire, UK), hippuric acid, benzoic acid and 3-hydroxybenzoic acid were purchased from Fluka (Dorset, UK), ascorbic acid (AA) was from Sigma Aldrich (Dorset, UK) and syringic acid and gallic acid were purchased from Alfa Aesar (Lancashire, UK). All standards were of a HPLC purity (>90 %). A Millipore Q water purifying system (Millipore, Hertfordshire, UK) was used to provide ultrapure water (≥18.2 MΩ cm at 25 °C) for LC-MS^2 analysis. EC-O-sulphate and EGC-O-glucuronide were synthesised using the method by Wong et al (2010) [34].

2.2 Human intervention

In total, 50 volunteers enrolled on the 3-month double-blind randomised controlled trial conducted in the Photobiology Unit, Dermatology Centre, Salford Royal NHS Foundation Hospital (Manchester, UK). Ethical approval was received from the North Manchester Research Ethics Committee (reference 08/H1006/79) and the study conformed to the Declaration of Helsinki principles. Inclusion criteria for the study included white Caucasian adults (aged 18-65 years) with sun-reactive skin type I-II. Volunteers were excluded from the study if they had a history of skin cancer or photosensitivity, underwent sunbed use or sunbathing in the 3 months prior to the study, consumed photoactive medication or nutritional supplements, consumed >2 cups of tea per day or were pregnant or breastfeeding. The volunteers were allocated either placebo or green tea supplements that were the equivalent of 5 cups of green tea per day (5.25 mg C, 31.25 mg EC, 123.25 mg EGC, 65 mg ECG and 181.5 mg EGCG), along with 50 mg vitamin C to improve the gastrointestinal stability of the green tea supplements. The placebo and supplement were encapsulated and coded to maintain the blind aspect of the study. Urine was collected for 24 hours at four time points during the intervention: baseline (pre-supplementation), day one (post-supplementation), week 6 and week 12. Urine was excreted into HCl-washed flasks containing AA (approximately 1 g/L) and

was stored at -20ºC prior to analysis. Compliance to the study was tested through monitoring the presence of EC-O-sulphate and EGC-O-glucuronide excreted into urine during the trial at four time points, and by counting residual supplements.

2.3 Urine analysis

Urine was defrosted and 50 µL AA (6 mM in water, 1 mM final concentration in urine) and 50 µL ethyl gallate (internal standard; 4 µg/mL in water) were added to 200 µL urine (technical duplicates from one biological sample) in a 1.5 mL microcentrifuge tube (Eppendorf UK Ltd., Stevenage, UK) on ice. Samples were vortexed for 10 sec before the addition of 500 µL ice cold ethyl acetate. Samples were then vortexed for 30 sec and placed on ice for 2 min before centrifugation at 17,000 g (4 ºC) for 2 min. The extract (supernatant) was placed in a new microcentrifuge tube on ice. The process was repeated again and the extracts were pooled and dried down under nitrogen flow on ice. The dried down sample was then placed at -80 ºC.

The urine sample (still on ice) remaining after the ethyl acetate extraction was then mixed with 800 µL ice cold acetonitrile and vortexed for 2 min before centrifuging at 17,000 g (4 ºC) for 10 min. The supernatant was placed into a pre-weighed microcentrifuge tube and the organic solvent was removed by centrifugal evaporation (settings: HPLC fraction, lamp off; Genevac, EZ-2 plus model; Genevac, Suffolk, UK). The microcentrifuge tube was re-weighed to calculate the remaining aqueous portion of the sample, using 1 g = 1 mL. Samples were placed at -80 ºC until required. For LC-MS^2 analysis, 90 µL of the aqueous sample was added to the dried down ethyl acetate fraction and 10 µL taxifolin (to monitor any changes to samples during HPLC-MS^2 analysis); 20 µg/mL in 50 % acetonitrile and 1 % AA, final concentration of 5 % and 0.1 %, respectively) was also added prior to vortexing for 1 min and 5 min of sonication. The sample then underwent centrifugation for 5 min at 17,000 g (4 ºC) and was placed into an amber HPLC vial.

A 5 µL sample aliquot was injected onto a Kinetex C18 column (2.6 µm, 150 x 2.1 mm; Phenomenex, Cheshire, UK) connected to an Agilent 1200 SL system (column oven 35 ºC; Agilent Technologies UK Ltd., Berkshire, UK) and an Agilent 6410a triple quadrupole LC-MS^2 (negative

mode, multiple reaction monitoring (transitions monitored are shown in Table 1), gas temperature 350 ºC, a 30 psi nebulizer pressure, nitrogen flowing at 11L/min and tuned using ESI tuning mix).

A binary gradient of 95 % LC-MS grade water (solvent A: 5 % acetonitrile, 0.1% formic acid) vs. 95 % acetonitrile (solvent B: 5 % water, 0.1% formic acid) was run at 0.3 ml/min. The gradient began at 0 % solvent B and was held at 0 % for 6 min (5 column volumes), increasing to 100 % over 15 column volumes (by 24 min), remaining at 100 % for 3.6 min (3 column volumes) before returning to 0 % over 1.2 min (1 column volumes) and finally re-equilibrating over 4.8 min (4 column volumes). The total method length was 33.6 min.

Results

3.1 Identification of green tea catechin metabolites

LC-MS^2 was used to identify the green tea catechin metabolites present within the urine samples throughout the intervention study. Examples of the fragmentation patterns monitored are displayed in Fig. 2. The conjugated metabolites were measured as peak area relative to the peak area of the internal standard, ethyl gallate. Identification of EC-O-sulphate and EGC-O-glucuronide in the urine at significantly higher concentrations than baseline urine (approximately 9x higher for EC-O-sulphate, with EGC-O-glucuronide only found in a few baseline samples of volunteers) confirmed compliance with the daily supplementation and also identified volunteers consuming the placebo. For the placebo volunteers, EC-O-sulphate was also identified, however, there was no significant increase between pre- and post-supplement urine samples and therefore the urine samples for the placebo volunteers were not assessed further for other green tea catechin metabolites. In total, 24 volunteers consumed the placebo and 26 consumed the green tea and vitamin C supplements daily for 3 months, with 21 volunteers compliant with the green tea supplementation. Following the confirmation of compliance, the urine of the 21 volunteers was analysed for the total green tea catechin metabolite content.

Without reference standards the identification of green tea catechin metabolites can be achieved by monitoring the loss of conjugated moieties upon fragmentation. The loss of 176 atomic mass units (amu) represents cleavage of a glucuronyl unit, 80 amu is the removal of a sulphate moiety and 14 amu is the cleavage of a methyl group. Other changes in transitions and feasible fragmentation patterns of green tea catechin metabolites are displayed in Fig. 2 and Table 1. The collision energies were optimised by repeat analysis of each green tea catechin metabolite by using week 12 urine sample from one of the volunteers. The chromatograms recorded, corresponding to each green tea catechin metabolite within urine samples, are shown in Fig. 3. Without reference standards, the chiral form of particular catechins and their corresponding metabolites cannot be determined between e.g. conjugated forms of (-)-epicatechin and (+)-catechin, and M6 and M6'.

3.2 Quantification of green tea catechin metabolites

Standard curves were created by spiking known amounts of free-form catechin reference standards into baseline urine samples (range, 0.2-0.0002 mg/mL; R^2 range, 0.90-0.99), and the limit of detection (LOD) and limit of quantification (LOQ) were identified for each free-form compounds (average LOD, 110 ± 50; range, 4-650 pmol; and average LOQ 170 ± 60; range 4-820 pmol, respectively). The precision of the method was assessed by repeat injection of EC and EGC standards over the standard curve range, and the relative standard deviation was 3% for EGC and 14% for EC. A total amount (µmol) excreted in urine was determined for each conjugated metabolite as an equivalent to its associated free-form, relative to the internal standard (ethyl gallate). This can result in over-quantification of metabolites due to differences in response factors for each compound [35].

Recoveries (performed by analysing three concentrations in duplicate) were determined for EC-O-sulphate (91±7 %), EGC-O-glucuronide (94±6 %), EC (95±6 %), EGC (111±5 %) and ethyl gallate (74±3 %) by spiking known amounts of the compounds into blank urine and relating the peak area from the spiked urine sample to the peak area from the direct analysis of an equivalent volume by LC-MS. When analysing the recoveries of the free-form catechins using acetonitrile only, the recovery is clearly reduced to 3±2% for EC and 13±6% for EGC, which is consistent with another study [28], and highlights the importance of using a method that is suitable for extracting the free-form catechins as well as the conjugated forms. The most common method for assessing free-form catechins following consumption of green tea is using enzyme deconjugation of the biological sample followed by an ethyl acetate extraction. This results in a good recovery of EC by 91±5% and EGC by 88±9%. Therefore, using a combination of ethyl acetate and acetonitrile allows for a good recovery and assessment of both free-form and conjugated catechins. The green tea catechin conjugated metabolites were quantified as equivalents to their corresponding commercially available free-form standards, relative to the internal standard.

Of the 55 conjugated and free-form metabolites investigated, 29 were detected in the urine samples collected for 24 h at 4 separate time points (Table 2). The majority of compounds were present in the conjugated form, with only three compounds identified in the free-form; 3-O-me-gallic acid, 3-hydroxybenzoic acid, and hippuric acid. Of the catechins, EGCG and ECG were not identified in any form as they were either not present within the samples, or because they were below the limit

of detection. Hippuric acid had the largest corresponding peak area, followed by O-me-EC-O-sulphate and M6/M6'-O-sulphate. For the first time, glucuronide and sulphate derivatives of 3-hydroxybenzoic acid, benzoic acid, hippuric acid and syringic acid were identified in urine following the consumption of green tea. Through using quantification by free-form equivalents due to the lack of validated metabolite standards, it is unknown whether large quantities of the metabolites were excreted or whether the result is due to more efficient ionisation. Although there is a large inter-individual variability for the green tea catechin metabolites, there are statistically significant differences between the pre and post-supplementation samples (Table 2).

4. Discussion

The optimisation of an analytical method that allows the determination of the bioavailability and biotransformation of the green tea catechins is important as this will allow for future identification of compounds that may be related to the putative health benefits associated with green tea consumption. This may also lead to the tailoring of studies to understand the mechanisms of the health benefits at a molecular level through use of the identified metabolites.

The present study confirmed that a combination method utilising both an ethyl acetate extraction for the more non-polar compounds and acetonitrile to precipitate the protein content of the urine, results in a good recovery of the compounds assessed (93±13 %). As sulphate and glucuronide conjugates of green tea catechins are not commercially available, previous studies have reported the identification of urinary green tea catechin metabolites as either deconjugated free-forms (using sulphatase and β-glucuronidase) [22,36] or through monitoring the loss of the conjugate through fragmentation during LC-MS^2 analysis [27-31]. More recently, studies have focused on the latter as the information obtained is more realistic of the compounds actually present post-consumption of green tea. Problems with using sulphatase have also been highlighted recently as it has been shown that the use of the enzyme results in underestimation of the bioavailability of green tea catechins due to incomplete hydrolysis and also because of the instability of the resulting free-forms during analysis [26].

The majority of these studies assess biological samples obtained following green tea consumption with a protocol that utilises acetonitrile to precipitate the protein from the biological sample [28,30], but other studies have used direct injection following filtration onto the LC-MS [27,32]. The free-form catechins have been reported previously to have a poor recovery following isolation using acetonitrile [28], which is also supported within this study. Direct injection allows for identification of both free-form and conjugated green tea catechins without further processing. However, direct injection without sample preparation can lead to filtration problems throughout the LC-MS system and also suppressed ionisation due to matrix effects [33].

Throughout the 3-month human intervention study, which was a double-blind randomised controlled trial of green tea supplements or placebo simultaneously with ascorbic acid to improve the gastrointestinal stability of the catechins, 29 green tea catechin metabolites were identified out of the 55 investigated, with only 3 present in the free-form. EC and EGC were largely conjugated with methyl ester, sulphate ester or glucuronic acid, and the free-forms were not detected. EGCC and ECG were also not identified in any form, which is comparable to other studies. It is possible that they undergo biliary excretion [37] and are therefore not present within the urine, are conjugated in forms that were not targeted or were below the detection limit. The data reported are all from the supplemented volunteers as confirmed following the compliance testing, and as no difference was evident between the excretion of EC-O-sulphate and EGC-O-glucuronide in the urine samples from the placebo volunteer, the other metabolites were not assessed further.

In previous studies the conjugated forms have also been reported, with only minimal identification of the free-forms. EGCG and ECG are not present in any form in the present study, which is in line with previous reports [24,25,28,38]. The method developed in the present study may be utilised for the assessment of other biological samples (e.g. organ tissues) that have sulphatase and β-glucuronidase present within cells, and therefore may possibly have free-form compounds present.

The large inter-individual variability for the green tea catechin metabolites has been reported previously [39]. This is due to individual differences in gut microbiota leading to a variation in metabolism [40]. Hippuric acid had the largest corresponding peak area relative to the peak area of ethyl gallate, followed by O-methyl-EC-O-sulphate and M6/M6'-O-sulphate. Without the validated

metabolite standards, it is unknown whether large quantities of the metabolites were excreted or whether the result is due to more efficient ionisation, which is the case for sulphated metabolites. Hippuric acid is an endogenous metabolite and although it is also detected post-consumption of tea, the only significant increase in excretion in post-consumption urine samples compared with baseline was at day one. The majority of EC, EGC and valerolactone (M4 and M6/M6') conjugates excreted were significantly increased when post-consumption urine samples were compared to baseline samples, and after day 1, there was no significant change in the metabolite elution across the 12-week supplementation period except for EC-O-glucuronide (day 1 vs. week 6; $p=0.03$) and 3-hydroxybenzoic acid-O-sulphate (day 1 vs. week 12; $p=0.04$).

Catechin conjugates in urine have previously been quantified as aglycone equivalents [28]. The volunteers consumed a lower catechin dose compared to the present study (141 and 28 mg EGC and EC for Stalmach et al, 2009 [28] compared with 370 and 94 mg EGC and EC, respectively), however, the excretion of EGC metabolites was relatively similar (EGC-O-glucuronide, 6.5 vs. 8 µmol; EGC-O-sulphate, 2.6 vs. 1.9 µmol for Stalmach et al [28] and the present study, respectively), whereas the difference in quantification of EC conjugates was substantial (EC-O-glucuronide, 1.5 vs. 10 µmol; EC sulphate, 6.7 vs. 35 µmol, O-methyl-EC-O-sulphate 10.9 and 500 µmol for Stalmach et al (2009) and the present study, respectively). This difference may be partly explained by the tuning of the LC-MS with a solution of EC in the study by Stalmach et al (2009). This maximises the response of EC and related compounds, which may also result in smaller differences between the response factors from EC and conjugated derivatives. The comparison of the two studies highlights the importance of using validated reference standards, to enable an accurate measure of dose-response factors. This problem has also been reported previously for hydroxycinnamic acid conjugates which were over-estimated when expressed relative to corresponding free-form (unconjugated) derivatives [35], and requires exploring further in future studies.

In conclusion, to the best of our knowledge, this is the first time that a combination method has been utilised that allows the identification of both free-form and conjugated derivatives of green tea metabolites. This method is applicable for future studies involving biological samples in which endogenous deconjugation enzymes are present.

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