Over the past decade interest in the biochemical and biological properties of polyphenols has grown considerably, as epidemiological evidence for their beneficial effects on health continues to increase. Dietary polyphenol antioxidants are reported to have many ‘health promoting’ properties, including anti-inflammatory, vasoprotection, anti-cancer and anti-obesity effects. However, their absorption and metabolism are as yet not fully elucidated, particularly with regard to their interactions with other metabolized compounds, such as nicotine. Epidemiological studies have shown that coffee-drinking cigarette smokers are more likely to smoke less in comparison to non-coffee drinking cigarette smokers. Nicotine is primarily metabolized by the hepatic cytochrome P450 enzyme CYP2A6 and may be inhibited by the metal chelating properties of polyphenols via haem interactions with their hydroxyl groups. To model nicotine metabolism, bovine liver microsomes, shown to suitably represent human CYP2A6 metabolism, were isolated by calcium precipitation and differential centrifugation. Fluorometric analysis of 7-hydroxycoumarin, the CYP2A6 metabolite of coumarin and known probe for CYP2A6 activity, was used to model nicotine metabolism in vitro and to quantify the degree of CYP2A6 inhibition imposed by caffeic acid and quercetin. It was found that both caffeic acid and quercetin, major polyphenolic constituents of coffee containing beverages, significantly inhibited CYP2A6 activity in vitro by 37.9% \( (P < 0.05) \) and 48.2% \( (P < 0.05) \), respectively. Thus, this study demonstrates the first reported biochemical evidence in support of previous epidemiological observations, where it is suggested that polyphenol interaction with CYP2A6 prolongs the pharmacological effects of nicotine by decreasing its rate of elimination.

**Key words:** polyphenols, CYP2A6, caffeic acid, quercetin, nicotine.

**Introduction**

Polyphenols are the most abundant antioxidants in the diet, with a total dietary intake that exceeds that of vitamin C, vitamin E and carotenoids.\(^1, 2\) Their main dietary sources include fruits and plant-derived beverages such as fruit juices, tea, coffee and red wine. Vegetables, cereals, chocolate and dry legumes also contribute to the total polyphenol intake. These dietary nutraceuticals have been shown to contribute significantly to the prevention of disease,\(^2\) where a recent study by Mink *et al.*\(^3\) revealed that flavonoids found in fruits and vegetables are strongly associated with reduced cardiovascular disease mortality. These health promoting effects are thought to be due to bioactivities that include anti-inflammatory,\(^5\) vasoprotection and anti-obesity effects.\(^6\) In addition, phenolic antioxidants commonly consumed in our diets are being proposed as preventative treatments against chronic human diseases.\(^3, 7-11\)

However, if these food-based antioxidant approaches are to be employed as prevention or treatments for chronic diseases, clarification of their little known metabolism must be achieved, particularly given the negative findings from studies of antioxidant supplementation\(^12, 13\) and the controversy that exists within the field regarding the bioavailability and bioactivity of many phytochemicals. Furthermore, little research has been conducted regarding the interactions between dietary antioxidants and other environmental promoters of oxidation, such as cigarettes.
Cigarette smoking, the foremost form of nicotine addiction, continues to be one of the world’s most serious public health problems and is considered to be the major risk factor of ischaemic heart disease, lung cancer and chronic obstructive pulmonary disease. A key factor in reducing the pharmacological and addictive effects of nicotine would be to improve its removal by the drug metabolizing pathways of the liver. However, interactions with dietary components, particularly polyphenol antioxidants, may hinder its removal. Of the nicotine absorbed through smoking, 80% is metabolized in the liver by the cytochrome P450 enzyme CYP2A6 via C-oxidation to cotinine. CYP2A6, therefore, plays a pivotal role in the removal of nicotine in humans. The relationship between nicotine and polyphenol metabolism is indicated in a review by Swanson et al. who stated that 86.4% of cigarette smokers consume coffee compared with 77.2% of non-smokers, and also in a study by Kozlowski demonstrating that coffee-drinking cigarette smokers smoked more when they ingested almost no caffeine than when they ingested an amount of caffeine, in the form of coffee, ranging from 75 to 300 mg. Caffeic acid and quercetin (Figs 1 and 2) represent major antioxidant constituents within caffeine containing beverages and are potential CYP2A6 inhibitors. Thus, in the present study, the CYP2A6 inhibition potential of caffeic acid and quercetin was investigated, where it was hypothesized that CYP2A6 inhibition is imposed by caffeic acid and quercetin and it is this interaction responsible for the epidemiological observation that coffee-drinking cigarette smokers smoke less than non-coffee-drinking cigarette smokers.

Materials and methods

Materials and reagents

Potassium dihydrogen phosphate, di-potassium monohydrogen phosphate, sucrose, calcium chloride, sodium cholate, sodium cacodylate, glucose-6-phosphate, acid molybdate, sodium dodecyl sulphate, ascorbic acid, sodium hydroxide, copper sulphate, sodium potassium tartrate, sodium carbonate, Folin reagent, bovine serum albumin, coumarin, caffeic acid, quercetin, nicotinamide adenine dinucleotide phosphate (NADP), perchloric acid and 7-hydroxycoumarin were purchased from Sigma Aldrich Co. (UK). Bovine liver samples were supplied to Staffordshire University by a local abattoir.

Liver microsome isolation

Whole bovine livers were delivered within ~4 h of animal death and placed directly on ice. Liver microsome isolation was performed using modification to the methods stated by Ernster et al. and Hamilton et al. Liver tissue samples of 80–100 g were cut from the whole bovine liver, rinsed with 0.25 M sucrose in 25 mM potassium phosphate buffer (pH 7.4) and minced using a scalpel blade. Minced liver samples were transferred to a homogenization tube in 20–25 g portions, and homogenized for ~2–3 min with 10 ml 0.25 M sucrose in 25 mM potassium phosphate buffer (pH 7.4) per portion. The homogenate was centrifuged at 1000 g for 10 min at 4°C using a Sigma Howe 3K30 centrifuge with Sigma 12156-H rotor. Following careful aspiration of the thin floating lipid layer, the remaining supernatant was then transferred to another centrifuge tube and the pellet discarded. The supernatant was centrifuged at 12 000 g for 15 min at 4°C. Again, the thin floating lipid layer was carefully aspirated and the supernatant retained. This supernatant fraction was called the post-mitochondrial fraction (PMF). A volume of 8 mM calcium chloride, 7.5 times the volume of the PMF was added dropwise to the PMF with constant stirring and left to stir for 15 min at 4°C. This mixture was then centrifuged at 8000 g for 10 min at 4°C. The supernatant was removed and the pellet re-suspended in 0.25 M sucrose in 25 mM potassium phosphate buffer (pH 7.4) at a volume of 0.3 ml for each gram of original tissue. The suspension was vortexed and treated with 0.5% (w/v) sodium cholate, at a volume one-tenth the final suspension volume, for 20 min at 4°C. This suspension, called the microsome isolation, was stored at −80°C until necessary.
Protein determination

The protein assays were performed, in duplicate, using the method developed by Lowry et al. The microsome isolation was diluted 1:100 with 0.5 M sodium hydroxide and 0.2, 0.4, 0.6, 0.8 and 1 ml of the diluted microsome isolation was made up to a final volume of 1 ml, respectively, with 0.5 M sodium hydroxide. A blank was prepared using 1 ml 0.5 M sodium hydroxide. To all diluted microsome isolation samples was added 5 ml of copper reagent (containing 2% (w/v) sodium carbonate in 0.1 M sodium hydroxide, 1% (w/v) copper sulphate and 2% (w/v) sodium potassium tartrate, prepared at a ratio 100:1:1 by volume, respectively). These mixtures were then vortexed and allowed to stand for 30 min at room temperature. Finally, 0.5 ml 1 N Folin reagent was added to all the samples and left to stand for 30 min at room temperature subsequent to vortexing. Absorbance was read at 750 nm on a Cecil CE1010 spectrophotometer after zeroing on the blank. Protein concentration was estimated by direct interpolation from a standard curve.

A standard curve was constructed using a stock solution of bovine serum albumin (BSA) at a concentration of 100 µg/ml in 0.5 M sodium hydroxide. BSA stock solutions at volumes 0, 0.2, 0.4, 0.6, 0.8 and 1 ml were made up to a final volume of 1 ml with 0.5 M sodium hydroxide (equivalent to 0, 20, 40, 60, 80 and 100 µg protein/ml, respectively). The standard curve solutions were then processed as described earlier.

Glucose-6-phosphatase assay

This assay was performed, in duplicate, using a modification to the methods stated by Taussky and Shorr and Nordlie and Arion. Initially, 50 µl 20 mM glucose-6-phosphate and 20 µl 160 mM sodium cacodylate were pre-incubated for 10 min at 30°C. The incubation reaction was initiated with the addition of 90 µl microsome isolation and incubated for 20 min at 30°C. The incubation reaction was stopped with the addition of 1.6 ml Burchell’s reagent consisting of 0.42% (w/v) acid molybdate, 5% (w/v) sodium dodecyl sulphate and 10% (w/v) ascorbic acid prepared at the ratio 6:2:1 by volume, respectively. The incubation mixture was then placed on ice for 15 min and re-incubated for 20 min at 47°C. The absorbance was measured on a Cecil CE1010 spectrophotometer at 820 nm only after the incubation mixtures had stabilized to room temperature. In control samples glucose-6-phosphate was added after the 20 min incubation period. Organic phosphate concentration was estimated by direct interpolation from a standard curve as described in what follows. For samples with an absorbance exceeding the range of the standard curve, a 1:1 dilution was performed and the absorbance re-measured.

A standard curve was constructed using 0.5 mM potassium di-hydrogen phosphate at volumes 0–150 µl (final phosphate concentrations 0, 20, 25, 40, 50, 75 mM, respectively), made up to a final volume of 160 µl with 160 mM sodium cacodylate. 1.6 ml Burchell’s reagent was added to the standard curve solutions and incubated as stated earlier.

CYP2A6 activity assay

This CYP2A6 activity assay was performed, in duplicate, using a modification to the methods stated by Creaven et al., Koenings et al. and Ngui et al. Initially, 25 µM coumarin in 25 mM potassium phosphate buffer (pH 7.4) was pre-incubated with 2 ml microsomal isolation for 3 min at 37°C. The reaction was initiated by the addition of an NADPH generation system consisting of 10 mM glucose-6-phosphate and 0.5 mM NADP (final incubation volume, 6 ml). After 10 min at 37°C, the incubation was quenched with the addition of 100 µl 6 N perchloric acid, set on ice for 2 min and centrifuged at 1000 g for 10 min using a Sigma Howe 3K30 centrifuge with Sigma 12156-H rotor. Controls (blanks) were prepared with the addition of the NADPH generation system after the incubation period. Fluorescence was recorded on a Kontron SFM 25 fluorometer at excitation and emission wavelengths of 351 and 454 nm, respectively, zeroed against the blank. An additional CYP2A6 activity assay was performed with the incubation time increased to 15 min (substrate control) to ensure the assay substrates were not depleted during incubation.

The amount of metabolite (7-hydroxycoumarin) formed by the CYP2A6 activity assays were quantified with a standard curve generated from known amounts (0–100 ng/ml) of 7-hydroxycoumarin in 25 mM potassium phosphate buffer (pH 7.4) and 100 µl 6 N perchloric acid, made up to a final volume of 6 ml with distilled water.

CYP2A6 inhibition assay

This assay was performed, in duplicate, following the method described earlier with the addition of 25 µM caffeic acid and quercetin, respectively, to the incubation mixture.

Statistical analysis

Repeated measures analysis of variance was applied as a three factor test to determine between-treatment effects followed by Turkey HSD and Bonferroni post-hoc tests using Windows SPSS (version 15) software.

Results and discussion

Many animal species have been shown to possess CYP2A6 like activity, where CYP2A6 has been shown to be the major coumarin 7-hydroxylase in bovine liver with activities similar to that of human liver. Thus, bovine liver samples were used to model human CYP2A6 coumarin 7-hydroxylation within this study. CYP2A6 is the major, if not the only, coumarin 7-hydroxylase in humans, where coumarin is almost exclusively metabolized into...
7-hydroxycoumarin (68–92% of the dose). As coumarin is almost exclusively metabolized by CYP2A6 to produce 7-hydroxycoumarin, a highly fluorescent compound, it was an ideal probe and marker for CYP2A6 activity and inhibition studies. Thus, within this study coumarin had been substituted for nicotine as an alternative CYP2A6 substrate to model in vitro nicotine hydroxylation.

Each microsomal isolation procedure yielded a 30 ml microsomal isolation mixture. Using the Lowry protein determination method it was shown that microsome isolations yielded a protein concentration of 10.89 mg.ml\(^{-1}\) (SD ± 3.11, \(n = 7\)). This protein concentration provided only an estimate for the quantity of microsomes isolated and did not quantify CYP2A6 content. However, for the purpose of this study it was not necessary to quantify CYP2A6 content as protein concentration provided sufficient means by which subsequent assays could be standardized and compared with previously reported data. Microsome isolations showed glucose-6-phosphatase activities of 0.44 pmole.mg protein\(^{-1}\).min\(^{-1}\) (SD ± 0.1, \(n = 6\)). Although this activity was lower than previously reported glucose-6-phosphatase activities in rats,\(^{36–38}\) it was still significantly higher than control samples \((P < 0.05)\). Thus, the liver microsome isolations demonstrated evidence for the presence of rough endoplasmic reticulum (RER) membrane enzymes, such as CYP2A6, as glucose-6-phosphatase is an RER membrane-bound protein.

The bovine liver samples showed a mean rate of coumarin 7-hydroxylation of 48.66 pmole.mg protein\(^{-1}\).min\(^{-1}\) (SD ± 28.38, \(n = 6\)) under uninhibited conditions in comparisons to a mean rate of 30.33 (SD ± 11.36, \(n = 6\)) pmole.mg protein\(^{-1}\).min\(^{-1}\) in the presence of caffeic acid and quercetin, respectively (Fig. 3), with caffeic acid and quercetin demonstrating significant inhibition of coumarin 7-hydroxylation \((P < 0.05)\). This equated to a CYP2A6 activity inhibition of 37.9 and 48.2% by caffeic acid and quercetin, respectively, at a concentration of 25 \(\mu\)M. While the maximum plasma concentration of caffeic acid is reported to be 3.8 \(\mu\)M\(^2\) following 200 ml of oral coffee consumption, excessive consumption may significantly increase this plasma concentration. Studies by Olothof et al.\(^{21}\) and Rechner et al.\(^{39}\) have shown that between 0.3 and 5% of ingested chlorogenic and caffeic acid may be excreted in the urine. Thus, with coffee beverages containing up to 478 \(\mu\)g/ml chlorogenic acid, it is possible that hepatic concentrations of caffeic acid may be considerably increased with excessive coffee consumption, particularly since the polar phenolics may be subject to enterohepatic circulation and accumulation within the gallbladder.\(^{40}\) Therefore, while the concentrations of phenolic acid within this study exceed reported physiological levels, a higher concentration was selected as an initial trial to determine inhibition potential and in response to previously reported data, in which concentrations of chlorogenic acid exceeded 30 \(\mu\)M.\(^{41}\) Further studies should be conducted to assess inhibition at physiological concentration. However, this study provides a positive indication for the inhibitory potential of these polyphenols on nicotine metabolism.

Little previously reported data was found regarding the inhibitory effects of caffeic acid on phase I metabolizing enzymes and no data regarding CYP2A6 inhibition, although a study by Teel and Huynh\(^{42}\) showed that caffeic acid did significantly inhibit the activities of cytochrome P450 enzymes benzyloxyresorufin O-dealkylase (BOD) and methoxyresorufin O-demethylase (MROD) by 46 and 40%, respectively. A study by Obach\(^{41}\) evaluated the inhibitory effects of chlorogenic acid on five CYP enzymes (CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4) and found no CYP inhibition by chlorogenic acid at concentrations \(\geq 30 \mu\)M. The average plasma concentration of quercetin achieved by dietary means is reported to be 1.46 \(\mu\)M,\(^{43}\) however, an equal concentration as caffeic acid was selected in this study to allow comparison to caffeic acid and previously reported data.\(^{41}\) Thus, CYP2A6 inhibition by quercetin showed similar results to that of caffeic acid. This compares favourably with previously reported data,\(^{41}\) showing that quercetin inhibited CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 by 50% at concentrations of 7.5, 47, >100, 24 and 22 \(\mu\)M, respectively. No studies were found regarding quercetin inhibition of CYP2A6.

Within this study it was shown that both quercetin and caffeic acid inhibited CYP2A6 hydroxylation activity, although the nature of this interaction was not assessed. A study by Obach\(^{41}\) showed that quercetin demonstrates non-competitive inhibition of CYP1A2 and a study by Yang et al.\(^{43}\) suggests that phenolic compounds with free hydroxyl groups on the benzene ring are capable of inhibiting hydroxylation reactions. This is a possible validation
for the characteristics observed by both caffeic acid and quercetin within this and other studies. Additionally, as caffeic acid and quercetin showed no significant difference in their inhibitory effects ($P > 0.05$), it may be suggested that their inhibitory effect occurs via similar or generic mechanisms. Thus it may be hypothesized that caffeic acid and quercetin are intermediates of CYP2A6 that form a complex via interactions between the adjacent hydroxyl groups of the polyphenolic structures and the ferric portion of CYP2A6’s active site, effectively chelating the ferric portion of CYP2A6. Absorbance changes may thus be utilized in further studies to quantitatively describe caffeic acid and quercetin complexing (chelation) to the ferric protoporphyrin IX prosthetic group of CYP2A6 and other cytochrome P450s. By determination of the apparent spectral dissociation constant and the maximal spectral change elicited by the inhibitor, formally similar to the $K_m$ and $V_{\text{max}}$ values described by Michaelis–Menton kinetics, a measure of inhibitor affinity to cytochrome P450 may be determined. In addition, it is suggested that further experimentation to establish Lineweaver–Burk kinetics of this inhibitory reaction be conducted to assess competitive or non-competitive inhibition.

Although only a model system for nicotine metabolism, this study provides some evidence in support of the observations by Kozlowski$^{20}$ and Swanson et al.$^{19}$ linking coffee drinking with cigarette smoking. Furthermore, it is probable that drinking caffeine containing beverages increases the nicotine ‘hit’ received by coffee-drinking cigarette smokers by increasing the body’s exposure to nicotine. However, evidence suggests that most phenolic compounds are largely metabolized by colonic microflora,$^{44}$ where their metabolites are absorbed and further biotransformed in the liver and target cells, contradicting earlier studies stating that caffeic acid is metabolized largely by the liver.$^{21}$ It has been shown by Olthof et al.$^{45}$ that chlorogenic acid is hydrolysed by colonic microflora to form caffeic acid and quinic acid, which are subsequently absorbed more readily. However, during colonic metabolism and absorption of chlorogenic acid, caffeic acid is liberated, but dehydroxylated by microorganisms before absorption. Consequently, it is possible that during in vivo metabolism, caffeic acid may not exert its inhibitory effect even if it were present during phase I metabolism. Thus, although caffeic acid may inhibit CYP2A6 hydroxylation in vitro, CYP2A6 inhibition is not known in vivo. In order to evaluate the implications of this study, further in vitro and in vivo studies should be conducted to evaluate the metabolism and absorption of caffeic acid and quercetin.

**Conclusion**

It has been shown that caffeic acid and quercetin significantly inhibit the hydroxylation capacity of CYP2A6 in vitro and that these findings are concurrent with previously reported data. Consequently it is suggested that caffeic acid and quercetin are plausible inhibitors of nicotine metabolism. This study provides the first biochemical evidence in support of epidemiological observations of smoking behaviour and suggests that the caffeic acid, quercetin and similar polyphenolic structures present in caffeine containing beverages are responsible for coffee-drinking cigarette smokers smoking less than non-coffee drinking cigarette smokers. However, as nicotine metabolism was modelled by CYP2A6 coumarin hydroxylation activity in vitro, these results should be viewed as preliminary findings in justification of further study.

**Acknowledgements**

The author acknowledges the following scientists and staff of the Faculty of Sciences, Staffordshire University, for the use of their expertise and facilities during this investigation: Dr Robert Manning and Dr Sue Bird for their continued support and advice, Dr Stephen Merry for his guidance and input and Audra Jones for obtaining the bovine liver samples.

**Funding**

The present study was supported by the Faculty of Sciences, Staffordshire University, UK.

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