Chapter 6

Concluding discussion

According to Chinese tradition, Emperor Shennong was the first to drink tea. He was one of the mythical emperors, the one the Chinese worshiped since ancient times as the deity of medicine and agriculture. It is since then, approximately 3000 BC that tea has been used as a medicinal drink by the Chinese. By the end of the sixth century AD they began to consume it as a beverage. The western nations were introduced to it at the beginning of the seventeenth century. Tea polyphenols contribute to both the pharmacological and quality properties of tea. A family of flavanols, known as catechins, has been identified as the bioactive compounds in tea. The catechins have been shown to have both anti-carcinogenic and anti-mutagenic activities. Tea polyphenols are antioxidants and scavenge oxygen radicals that can cause DNA mutations and result in disease such as atherosclerosis. They also modulate the activities of phase I and phase II detoxification enzymes. This reduces the amount of pro-carcinogens that are converted to DNA-mutating carcinogens. The catechins have also been shown to inhibit enzymes in the signal transduction pathways that are important in regulating transcription and cell-signaling.

Polyphenols and coumarins from other plant sources have been shown to inhibit XO *in vitro*. XO is a liver enzyme that converts hypoxanthine to xanthine and xanthine to uric acid. It has a low substrate specificity for aromatic heterocyclic compounds, making it possible that xenobiotics may also be hydroxylated by this enzyme. XO also produces reactive oxygen radicals (superoxides or peroxides) during the catalytic process. Oxygen

radicals have been linked to carcinogenesis. Since natural plant products inhibit the production of a potential cancer-causing oxygen radicals, it is a possible way by which tea polyphenols may prevent carcinogenesis. This has not been tested before.

Capillary electrophoresis is a very powerful technique and is becoming more and more popular in investigations regarding natural product chemistry and pharmacology. Several different modes of capillary electrophoresis exist which include micellar electrokinetic chromatography and capillary zone electrophoresis. This makes it a versatile analytical technique that can analyze ionized and neutral analytes simultaneously.

The first aim of this study was to develop a MEKC method that is suitable for the analysis of the five major tea catechins, as well as theanine, caffeine, gallic acid and ascorbic acid. This method would be used for studies related to quality determination. The second aim was to determine the *in vitro* inhibitory effects of tea polyphenols on XO and to present a model explaining the structure activity relationship for the observed results. Finally the *in vivo* inhibitory effects were tested in a rat model. CZE was used to detect the levels of XO substrate, product and inhibitor levels in rat urine and serum.

A univariate optimization approach was used to develop a MEKC method that could analyze some of the organoleptically important compounds in tea. The influence of pH, surfactant concentration, background electrolyte concentration, organic modifier concentration and applied voltage was determined and optimal conditions were selected for each parameter. The pH parameter was the most important factor influencing the separation.

From the literature a correlation could be drawn between the pH, the type of buffer that was used and the physiochemical properties that were exploited to achieve separation. In our study the pH was varied in the range of pH 6.8 to 8.0. A pH of 7.0 was selected as the optimum condition. At this pH the polyphenols are usually only slightly negatively charged. The separation is achieved mainly with the differential hydrophobic interaction that the polyphenols have with the surfactant in the buffer. The interaction with the surfactant was almost sufficient to resolve all the analytes. Organic modifiers were added to try and increase the selectivity of buffer and obtain better separations. Acetonitrile, 2-propanol and methanol were tested as organic modifiers. Organic modifiers alter the distribution of the polyphenols between the aqueous phase and the surfactant phase. Increased organic solvent concentrations increase the solubility of the polyphenols in the aqueous phase. The addition of organic modifiers has an additional benefit by reducing the migration time of hydrophobic analytes. Methanol was found to increase the selectivity of the buffer the most. A concentration of 6% (v/v) methanol in the running buffer was considered as optimal for analysis of green and black tea samples. A methanol concentration of 5% (v/v) in the running buffer was used when iced tea samples fortified with ascorbic acid were analyzed. The optimal conditions for the analysis of all tea samples were 100 mM SDS, 25 mM phosphate and 6% (v/v) methanol at pH 7.0. An uncoated open fused silica column with an effective length of 50 cm was used. An applied voltage of 14 kV was found to provide good resolution and speedy analysis without resulting in excess Joule heating.

XO inhibition studies were conducted *in vitro* and an attempt was made to explain the results with kinetic models and *in silico* models. Since polyphenols have previously been shown to inhibit XO, it was not surprising to find that catechins also inhibit the enzyme. All the catechins exerted an inhibitory effect. The most potent inhibitor was EGCg which had a K_i value of 0.76 μM comparable to that of allopurinol (0.30 μM). The most intriguing aspect was the vastly different modes of inhibition that was displayed by the structurally related catechins. Competitive, uncompetitive and mixed-type inhibitions were observed. Similar inhibition results have been observed before with other classes of polyphenols as well as coumarins.

A model is proposed based on the ping-pong bi-bi catalytic action of the XO. The enzyme cycles between an oxidized form (E_{ox}) and a reduced form (E_{red}). According to three-dimensional data obtained from the X-ray crystallography structures of AOR there are no significant structural differences in the spatial arrangement of the amino acids in the active center or anywhere else on the two enzyme forms. The only significant difference is in the Mo coordination complex where the bond length of the apical sulfur atom has increased upon conversion from the E_{ox} to E_{red} enzyme forms.

The catechins are structurally very similar and it is likely that they all bind to the same region on the enzyme. Since EGCg showed competitive inhibition it is possible that they may bind to the active center of the enzyme. In the XO ping-pong catalytic cycle the inhibitors can bind to the active centers of either the E_{ox} or E_{red} enzyme forms. The inhibitors simulate "alternative substrates" for the enzymes. When the steady-state

kinetics equations are derived it can be seen that an inhibitor binding only to the active center of the E_{ox} enzyme form results in competitive inhibition and an inhibitor binding only to the E_{red} enzyme form results in uncompetitive inhibition. An inhibitor that has the ability to bind to both forms of the enzyme with different affinities results in mixed type inhibition. K_I is defined as the inhibitory constant of an inhibitor for the E_{ox} enzyme form. K_i is the inhibitory constant of an inhibitor for the E_{red} enzyme form. A competitive inhibitor only has a K_I value and an uncompetitive inhibitor has only have a K_i value. Mixed type inhibitors have both K_I and K_i values. For mixed type inhibitors the ratio of K_i/K_I will be used to assess their structure-activity relationships.

When comparing the structures of different xanthine oxidase inhibitors, two different regions can be distinguished. The first is called the primary binding region and mimics the structure of xanthine oxidase substrates. It requires a heterocyclic ring and hydrogen bond forming groups. The secondary binding region is responsible for enhancing the affinity and to modulate the selectivity of the inhibitor for the different enzyme forms. Large hydrophobic moieties increase the overall affinity of the inhibitor for the enzyme. In polyphenols the C3 of the C-ring is important in modulating the K_i/K_I ratio. A hydroxyl present and orientated in the plane or behind the plane in the (-)-configuration allow the binding to the E_{ox} enzyme form. A lack of a hydroxyl or a hydroxyl oriented towards the front of the C-ring plane in the (+)-orientation only allows binding to the E_{red} enzyme form. The amount of hydroxylation of the B-ring also influences the K_i/K_I ratio. Binding to the E_{ox} enzyme form is favored with a decrease in hydroxylation on the B-ring.

Since the tea catechins showed promise as inhibitors of xanthine oxidase *in vitro*, the next logical step was to test them *in vivo* in an animal model. A rat model was selected, since rats are best suited for metabolic studies. A CZE analytical method was used to quantify the *in vivo* concentrations of xanthine, uric acid, oxypurinol and allopurinol. The principle of stacking was used to increase the sensitivity of the method. The method was sensitive enough to detect the basal uric acid levels in the rat serum. It was not sensitive enough to detect the basal levels of xanthine in all the rat serum samples. Upon inhibition of XO, xanthine accumulates in the serum. Even with weak inhibition, levels of 1 μg/ml xanthine is to be expected. The limit of detection for xanthine was 0.5 μg/ml, making the method sufficiently sensitive to detect the inhibition of xanthine oxidase in a rat model.

In humans uric acid is the excretion product of purine degradation. In rat however uric acid is converted to allantoin by the uricase enzyme. This enzyme is inhibited with oxonic acid treatment. The uric acid accumulates in the serum, rendering the treated rats hyperuricemic. Hyperuricemia is defined as uric acid levels of 2 S.D. above the population mean. In humans the mean uric acid levels are 40-50 μg/ml and hyperuricemia occurs at saturation levels of uric acid which is 60-70 μg/ml. The normal rats had a mean serum uric acid concentration of 5-8 μg/ml and the oxonic acid treated rats had a mean serum uric acid concentration of 25 μg/ml. The uric acid levels are not as high as in humans, but increased between 3 and 8 fold above the norm for rats. This ensures that the oxonic acid treated rats are hyperuricemic according to the definition.

Treatment with allopurinol served as positive control for the *in vivo* inhibition experiment. Treatment with allopurinol decreased the uric acid concentration in both the serum and urine of normal and hyperuricemic rats. An equimolar accumulation of xanthine was observed for hyperuricemic rats. The inhibition with allopurinol also occurred in a dose-dependant manner. These results indicated that the treatment protocol and the analytical procedures were successful.

Treatment of the hyperuricemic group with 50 mg/kg (-)-EGCg resulted in a low but statistical significant decrease in serum uric acid concentration. The 100 mg/kg (-)-EGCg dose and both TPM doses did not result in any statistically significant changes of the serum uric acid levels of hyperuricemic rats. No accumulation of xanthine was observed for any of the catechin treatments. The treatment of normal rats with either (-)-EGCg or TPM at both the 50 and 100 mg/kg doses resulted in a low but statistically significant decrease in serum uric acid concentration. No accumulation of xanthine was observed, indicating that XO was not inhibited. The decrease in uric acid levels did not seem to be dose-dependant. Since the change is small, the lack of dose-dependence may be due to variance in the animal model and analytical method. The tea polyphenols may have a weak uricosuric effect on the normal rat groups or an enzyme further up-stream of XO in the purine degradation pathway may be inhibited.

The *in vitro* studies showed that allopurinol and (-)-EGCg have similar K_i values. Yet in the i*n vivo* model only allopurinol inhibited the XO enzyme. The systemic absorption and bioavailability of (-)-EGCg in rats are very low. According to a pharmacological study

only 1% of the (-)-EGCg became bioavailable and the serum concentration was in the order of 1μM. Since the K_i of (-)-EGCg is 0.76 μM, one would still expect to see at least 50% inhibition with a competitive inhibitor. There are however other factors that influences the amount of free (-)-EGCg that are available. Polyphenols have been shown to bind to proteins. The aromatic hydroxyl groups form hydrogen bonds with the peptide carbonyl groups. This interaction is not very selective, resulting that polyphenols can easily bind to any serum proteins. Polyphenols bind more readily to globular proteins with a loose tertiary structure such as albumin. Albumin is present in plasma at concentrations of 35-45 g/l (600 μM). It is very likely that a large percentage of the absorbed EGCg can bind to serum albumin. This will reduce the effective amount of free EGCg significantly. The biotransformation of polyphenols occurs via UDP-glucuronosyl transferase and phenolsulfotransferase. Both these enzymes are liver enzymes and have some affinity for EGCg. If the affinity of EGCg is higher for the detoxification enzymes (cytochrome P 450) than for XO, it is unlikely that the 1 μM EGCg that reaches the liver will even bind to the XO (Yang, 1999). It is also unlikely the biotransformed EGCg will be a good inhibitor of the XO enzyme.

In the future a novel class of XO inhibitors with a polyphenol-like structure could be developed. Research studies can be conducted to investigate and improve the absorption and specificity. Studies will have to be conducted to elucidate the molecular mechanism of the inhibitory actions. Modeling software may be used to predict enzyme-inhibitor interactions. Several spectroscopic methods may be used to determine whether the inhibitor binds in close proximity of the Mo complex. Once the molecular basis of the

action is known, the inhibitor structure can be altered to improve the affinity and selectivity. When new inhibitors are designed it should be taken into consideration that the bioavailibility and overall bioavailability (affinity for proteins, affinity for detoxification enzymes and excretion) should also be improved. Software packages are available that can help predict the absorption and organ selectivity properties of drugs. This software can be used and *in vivo* studies preformed to determine the absorption and bioavailability of drugs. Since tea polyphenols have no adverse effects a slightly modified tea polyphenol inhibitor may be a better therapeutic drug for gout than allopurinol and colchicine.