Chapter 5

In vivo Xanthine Oxidase inhibition studies

1 Introduction

Few studies have been undertaken to collect data of the pharmacological effects of tea polyphenols in animal models. The majority of studies focused on the anticarcinogenic and antimutagenic activities of tea polyphenols. The anticarcinogenic activities of tea polyphenols are evaluated by their ability to a) prevent the biotransformation of a pro-carcinogen to a carcinogen as measured in urine (Dashwood, 1999), b) prevent DNA-adduct formation, c) reduce the amounts and sizes of tumors in lungs and the colon (Narisawa, 1993) and d) induce the activities of specific phase I and phase II detoxification enzyme (Sohn, 1994). The antimutagenic properties were tested in animal models where mutations were induced with UV radiation or oral administration of 12-O-tetradecanoylphorbol-13-acetate (TPA). The prevention of mutagenesis is evaluated by a) determining the amounts and sizes of the induced tumors (Conney, 1999), (Landau, 1998) and b) measuring mRNA levels of tumor-promotion marker molecules such as interleukin-1α (Katiyar, 1995).

Metabolic studies are normally conducted with rats as the animal model. No specific strain was preferred for the pro-carcinogen biotransformation studies. Previous studies have used several strains e.g. CDF (F344), Cl/Br, Sprague Dawley, BDVI and Wistar rats. In all the studies young adult (6-8 weeks) or adult (10 weeks) male rats were used. Female mice were used as animal model where the antimutagenic activities were evaluated. The SENCAR and A/J strains were used frequently.

XO inhibition studies were conducted in both rat (male Sprague-Dawley) and mice (male ICR) models (Osada, 1993), although rat model seemed to be used more predominantly. Again there was no preference towards the strains that were used. Studies have been conducted with Sprague-Dawley and Wistar rats. From a practical point of view, adult male rats are more suitable. Their large body weight makes it possible to collect serum and urine in larger volumes or at more frequent time intervals.

The dosage of tea, in the drinking water, used for treatment in animal models varied between 0.002% (w/v) and 6.25% (w/v). In all studies tea was available *ad libitum* to the groups that were treated with it. If it is assumed that a full grown rat of 275 gram drinks approximately 20 ml of fluid per day and the tea extracts contain 10% polyphenols (Wiseman, 1997), then the doses can be converted to 0.145 mg/kg – 453 mg/kg body weight. The dosage of XO inhibitors, such as allopurinol and TEI-6720 (Osada, 1993) are generally in the range of 5 – 50 mg/kg body weight. In all the studies the control groups had only water to drink.

2 Materials and Method

2.1 Experimental Strategy

Unlike humans, rats catabolize uric acid further to allantoin. This results in allantoin being the major excretion product of purines in the urine as opposed to uric acid in humans. Rats were treated with potassium oxonate to inhibit the enzyme uricase and prevent uric acid from being turned into allantoin. This effectively renders the rats hyperuricemic, simulating hyperuricemia in humans. For each hyperuricemic group of rats there was a control group.

The best *in vitro* inhibitor was selected for *in vivo* inhibition experiments in a Sprague Dawley rat model. Three different inhibitors were tested namely allopurinol, EGCg, and a tea polyphenol mix (TPM). The polyphenolic content of TPM is listed in Table 5.1. TPM was included in the study to examine the possibility of synergistic inhibition of XO by several catechins.

Table 1. The catechin contents in Polyphenon-70S (TPM in this study). Caffeine content is less than 0.5%

Compound	Weight %	Molecular weight
(-)-EC	18.3	290.28
(-)-EGC	8.6	306.27
(-)-EGCg	35.9	458.38
(-)-GCg	3.5	458.38
(-)-ECg	11.2	442.38
(-)-Cg	0.0	442.38
Total	77.5	

2.2 Animals

Inbred male Sprague-Dawley strain rats of 4-6 weeks of age were obtained from South African Vaccine Producers (Modderfontein, RSA). They were kept in single cages for one week to acclimatize to the environment. They were transferred to metabolic cages to enable urine collection after treatment commenced. All animals were kept in an air-conditioned room and given standard chow and water *ad libitum* for the duration of the study.

2.3 Materials

The (-)-EGCg and Polyphenon-70S (TPM) were gifts from Mutsui Norin (Tokyo, Japan). The allopurinol, oxypurinol, xanthine, hypoxanthine, uric acid, potassium oxonate and Gum Arabic were obtained from Sigma Chemical Company (St Louis, MO, USA). The internal standard 9-methylxanthine was obtained from ICN Pharmaceutical (CA). All buffer salts and solvents needed for running buffers were of analytical grade. All solutions were prepared with distilled water that was deionized with a Milli-Q system (Millipore Corp., Bedford, MA. USA).

2.4 Test drugs

Allopurinol was tested at three concentrations, namely 5, 20 and 50 mg/kg. The EGCg and TPM were tested at 50 and 100 mg/kg. The inhibitors were prepared as suspensions in water with 5% Gum Arabic at concentrations of 5, 20 and 50 mg/ml for allopurinol and 50 and 100 mg/ml for both EGCg and TPM. Control groups were also included for both the normal and hyperuricemic rats. Control animals were treated with carrier only.

2.5 Treatment protocol

The rats were randomly divided into groups of six. The groups of normal rats were given a single dose of the test compound orally at t=0h. Blood and urine samples of t=0h were collected the day before the actual experiment. Samples for t=2h-8h were collected every two hours up to eight hours after the treatment. Groups of hyperuricemic rats were injected with 250 mg/kg potassium oxonate at t=-2h and dosed with a single dose of test compound at t=0h. Blood and urine samples for t=-2h were also collected on the day before the actual treatment. The samples for t=0-8h

were collected every two hours afterwards up to eight hours. The control samples of t=0h for normal rats and t=-2h for hyperuricemic rats were collected the previous day to familiarize the animals to being handled, reduce stress on the animals and to be able to collect larger volumes of blood over all the intervals. Blood samples (0.5-1.0 ml) were centrifuged immediately at 11000g for five minutes before the serum supernatants were collected. The serum (100-400 µl) and urine samples were snap-freezed in liquid nitrogen and all samples were marked and stored away at –20°C until they were analyzed. The samples of each animal was analyzed individually.

2.6 Measurement of analytes in urine and serum samples

Samples were spiked and deproteinated. The levels of uric acid, xanthine, hypoxanthine, allopurinol and oxypurinol were quantified with the CZE method as described in Chapter 4.

2.7 Statistical analysis

The data from both *in vitro* and *in vivo* experiments are presented as means ± SEM. The *in vivo* results were statistically analyzed using the Student's t-test (unequal variance).

3 Results

3.1 Hypouricemic effects of inhibitors on hyperuricemic rats.

Upon treatment with potassium oxonate a significant increase in the uric acid levels could be detected in both serum and urine (Fig. 5.1). The uric acid levels were elevated between 3 and 5 fold in both serum and urine after 2h and it took longer than 8h for them to return to normal levels (Fig 5.2). With progression of time the uric acid

levels decreased since the inhibition effect of the potassium oxonate wore of due to excretion. The oxonate did not interfere with the analysis, for it could not be detected with the CZE method at 280 nm with the concentrations used.

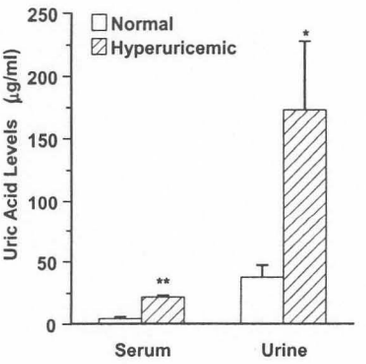

Figure 5.1 The hyperuricemic effect of 250 mg/kg potassium oxonate on serum and urine urate levels of normal rats. The data presents the mean with SEM of 6 rats. The serum and urine results for normal rats t=–2h (control) and t=0h (2 h after potassium oxonate treatment of normal rats) were compared. The results were statistically significant with * representing $P < 0.05$ and ** representing $P < 0.0001$.

Treatment with allopurinol reduced the elevated uric acid levels (Fig. 5.3). The serum uric acid levels decreased faster in allopurinol treated rats than in the control rats (Fig 5.2). The levels of uric acid were decreased in a dose-dependant manner by treatment with allopurinol. Both the 20 and 50 mg/kg allopurinol reduced the uric acid levels to below the levels expected in normal rats. The 50 mg/kg EGCg treatment decreased the uric acid levels but no statistically significant change could be detected in the uric acid levels with the 100 mg/kg EGCg treatment. Both the TPM doses had no significant effect on the uric acid levels. The serum uric acid levels decreased in a similar manner the control animals and the animals treated with EGCg or TPM (Fig.

5.2). None of the polyphenol inhibitors inhibited XO since no increases in xanthine levels were detected. With allopurinol treatment, the levels of xanthine increased in a dose-dependent manner.

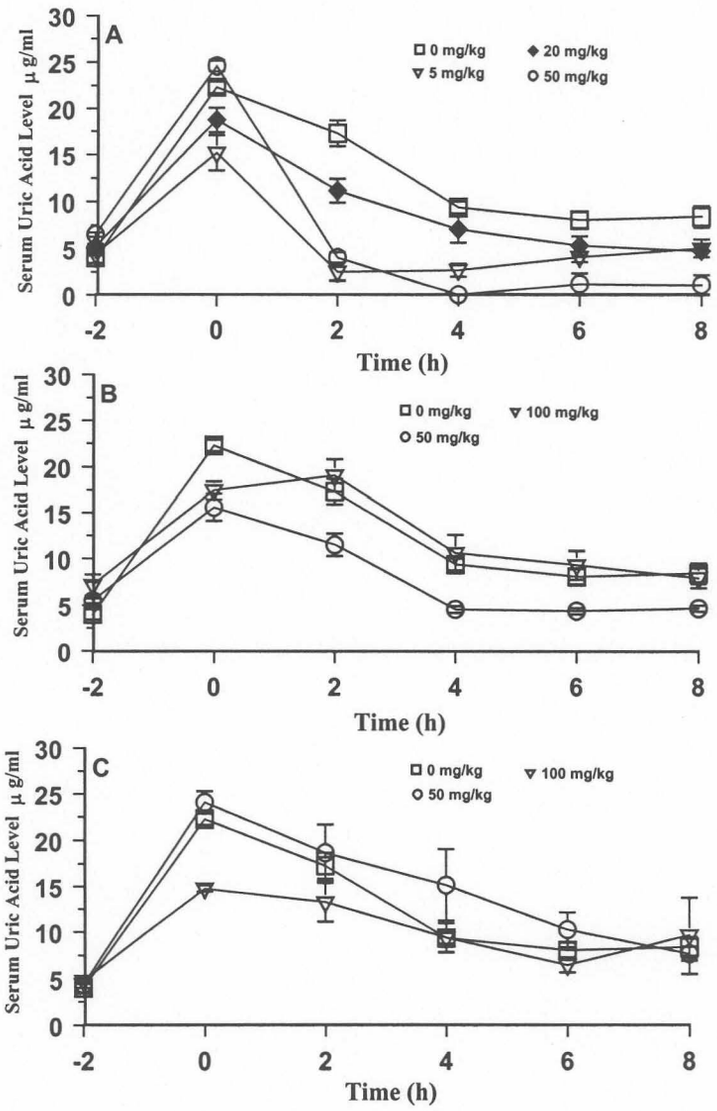

Figure 5.2 A Time course of the effect of allopurinol on serum uric acid levels of hyperuricemic rats. (□) Square, (∇) triangle, (◆) diamond and (○) circle represents doses of 0, 5, 20 and 50 mg/kg respectively. **B**: Time course of the effect of EGCg on serum uric acid levels. (□) Square, (○) circle and (∇) triangle represents doses of 0, 50 and 100 mg/kg respectively. **C**: Time course of the effect of TPM on serum uric acid levels. (□) Square, (○) circle and (∇) triangle represents doses of 0, 50 and 100 mg/kg respectively. The data represents the means and SEM for 6 rats.

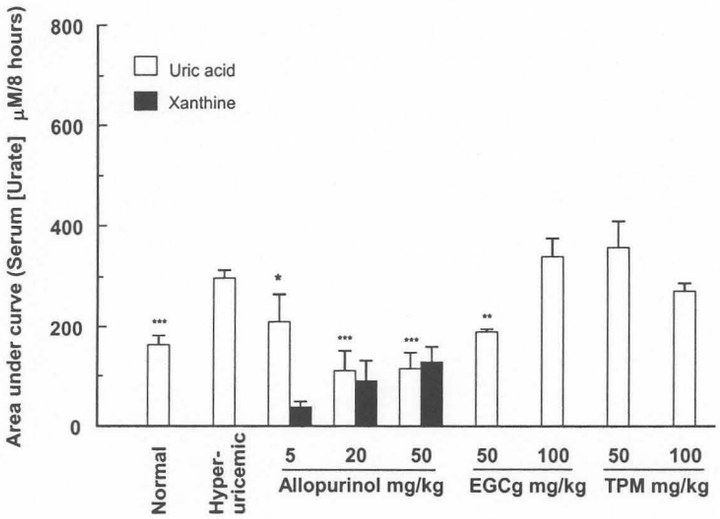

Figure 5.3 The effect of different doses allopurinol, (-)-EGCg and TPM on the serum urate and xanthine levels in hyperuricemic rats. The results were shown as the area under curve from 0-8h for both urate and xanthine concentrations (µM). The data represents the mean with the SEM for 6 rats. Statistical analysis shows significant difference between the treated groups and the hyperuricemic control with * $P < 0.02$, ** $P < 0.005$ and *** $P < 0.0002$.

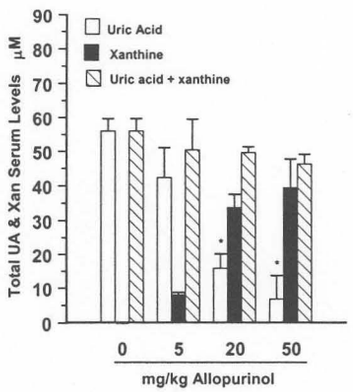

Figure 5.4 The total amount of xanthine and uric acid 4h after the treatment of hyperuricemic animals was conserved. The graph shows that with an increase in allopurinol dosage the concentration xanthine increase (solid bar) and the uric acid concentration decrease. The total amount of uric acid and xanthine (hashed bar) is nearly equal indicating that the 1 µM xanthine is detected for every 1 µM uric acid that has not been formed. The data represents the mean and SEM from 6 rats. The decrease in uric acid concentrations were statistically significant with * representing $P < 0.001$.

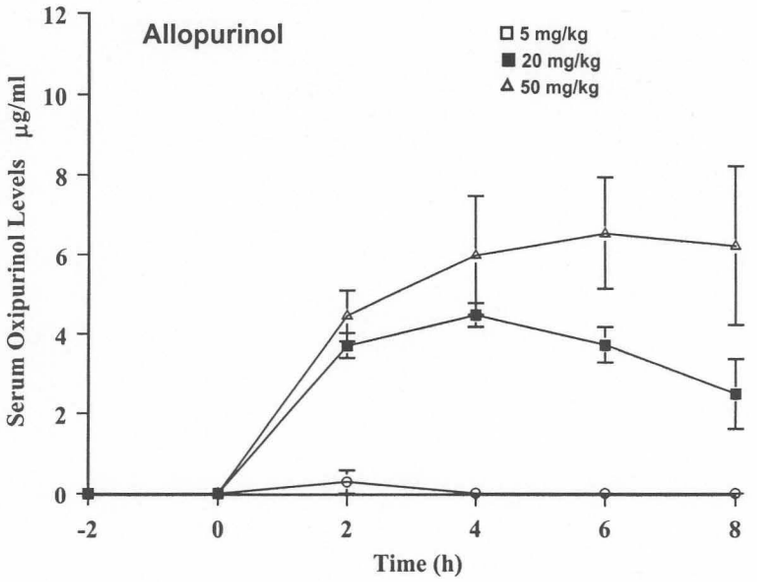

Figure 5.5 The serum levels of oxypurinol increased with an increase in the allopurinol dosage concentration. The (□), (■) and (▲) represent 5, 20 and 50 mg/kg respectively. For low levels of oxypurinol (5 mg/kg) the maximum serum level is reached 2h after dosage and for higher dosages (20 and 50 mg/kg) the maximum serum levels are reached 4-6h after treatment. The data represent the mean ± SEM for 6 rats.

The total concentrations of uric acid and xanthine were conserved as shown in Fig. 5.4. High levels of oxypurinol could be detected in body fluids 2 hours after allopurinol treatment. Very little or no allopurinol was detected after 2 hours. The oxypurinol levels stayed elevated throughout the 8 hours of monitoring (Fig. 5.5). In agreement with literature, allopurinol is converted to oxypurinol very rapidly and the the *in vivo* inhibition is the result of oxypurinol and not allopurinol (Massey, 1970).

3.2 Hypouricemic effects of inhibitors in normal rats

From Fig. 5.6A it can be seen that allopurinol reduced the uric acid levels in normal rats in a dose-dependent manner. The biggest decrease in uric acid levels occurred at

2 hours after treatment, but maximal inhibition only occurred at 4 hours for the two higher doses. With the 5mg/kg dose the uric acid levels returned to normal after 4 hours. With the 20 mg/kg dose the uric acid levels have not returned to normal even after 8 hours, and with the 50 mg/kg dose uric acid levels were still undetectable after 8 hours. As in the hyperuricemic group, the xanthine levels increased with higher doses of allopurinol, indicating that XO was inhibited. From Fig. 5.6B and C it can be seen that EGCg and TPM caused a slight but statistical significant reduction in the uric acid levels. In the *in vivo* system, inhibition is observed as an increase in the concentration of xanthine in urine and serum. Although EGCg is a competitive inhibitor of XO with a relative high affinity for the enzyme *in vitro*, it failed to effect an increase in xanthine concentration in the serum or urine of treated rats. It seems that concentrations of up to 100 mg/kg EGCg had no XO inhibitory effect *in vivo* in a rat model.

3.3 Bioavailability of catechins

In pharmacokinetic studies it has been found that less than 1μM (0.67 mg/l) EGCg occurred in the serum of rats, after an intragastrical (i.g.) dose of 500 mg/kg body weight (Yang, 1999), (Nakagawa, 1997(b)). Less than 1% of the total administered EGCg became bioavailable. A large amount of the EGCg was found in the feces, suggesting a lack of absorption. After repeated doses, the highest catechin concentrations were found in the tissue of the esophagus, large intestines, kidneys, bladder, lungs and prostate. Some of these organs are in direct contact with the catechins as they are consumed and move through the digestive tract to be excreted. This may explain the catechin accumulation in the tissue of these organs. The fact that catechins are found in the lungs, kidneys and prostate is an indication that they are

absorbed and do have some organ specificity to some extent. Very little catechins

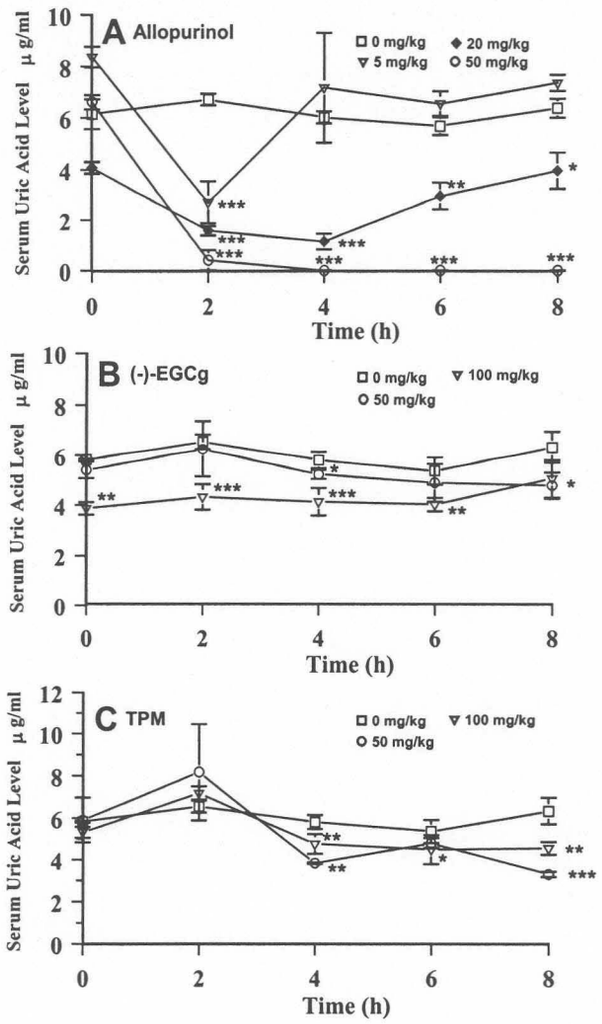

Figure 5.6 A Time course of the effect of allopurinol on serum uric acid levels of normal rats. (□) Square, (▽) triangle, (♦) diamond and (○) circle represents doses of 0, 5, 20 and 50 mg/kg respectively. **B**: Time course of the effect of EGCg on serum uric acid levels. (□) Square, (○) circle and (▽) triangle represents doses of 0, 50 and 100 mg/kg respectively. **C**: Time course of the effect of TPM on serum uric acid levels. (□) Square, (○) circle and (▽) triangle represents doses of 0, 50 and 100 mg/kg respectively. The data represents the means and SEM for 6 rats. Statistical significant differences are indicated with * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

accumulated in the spleen, liver and thyroid. The distribution studies in Sprague-Dawley rats showed that only 48 ng/ml EGCg occurred in the liver 60 min after a single i.g. dose of 500 mg/kg body weight. The absorption of the different catechins also varied. After a single i.g. dose, only 14% of the EGC and 31% of the EC became bioavailable, as compared to only 1% for the EGCg. This is an important clue in the designing of catechin-like drugs with an increased systemic absorption in rats.

Bioavailability of catechins

In a pharmacokinetic study with radiolabeled EGCg in CD1 mice, it was found that EGCg was absorbed into almost all the organs. The blood and liver contained the most radioactivity after 1h. The amount of radioactivity in the serum converted to approximately 1.2 mg/l EGCg, although it is not certain how much of the EGCg has been biotransformed (Suganuma, 1998).

Few catechin bioavailability determination studies have been conducted with humans. From the available results, it is clear that the bioavailability of the catechins depends heavily on the doses that the subjects receive. In one study human subjects ingested 1.2 g of decaffeinated tea in warm water. Blood EGCg levels reached 0.046-0.27 mg/l (0.1-0.6 μM) after 1h. The majority of these catechins were already conjugated with glucuronic acid or sulfate (Lee, 1995). In another study human subjects drank a tea infusion with approximately 400 mg of total catechins. Both EGCg and ECg were detected in plasma at a maximum concentration of 2 μM after 2h (Pietta, 1998). In the most recent study humans were dosed i.g. with different amounts of EGCg, the highest being 525 mg. The plasma EGCg reached a maximum of 4.4 μM after 90 min (Nakagawa, 1997 (c)).

As far as the bioavailability is concerned, a mouse or human model may be more appropriate than a rat model. Humans may be able to absorb up to 4 times more and mice up to two times more EGCg than rats. The catechin levels in mice and humans are still in the low micromolar range.

Although the EGCg serum level is in the order of 1μM, this should be sufficient to inhibit XO, since EGCg has an inhibitory binding constant (K_i) of 0.76 μM. Despite of this, inhibition was not seen. Several reasons exist why this is not the case. Polyphenols are known to interact with proteins. Proanthocyanidins are phenolic polymers that have the ability to bind to proteins and even precipitate proteins (Hagerman, 1981). Proteins are precipitated the easiest by proanthocyanidins at pH's near their iso-electric points (pI). In accordance with this, proteins with acidic pI's such as bovine serum albumin have a higher affinity for polyphenols at pH 4.9 than at pH 7.8 and basic proteins such as lysozyme have higher affinities at the higher pH. The relative affinities of proteins and polypeptides for polyphenols are influenced by the size of the polymer. Proteins with a molecular mass of less than 20 kDa have a rather low affinity for polyphenols. Proline-rich proteins, such as some salivary proteins, have a particularly high affinity for polyphenols (Murray, 1994). Proteins with compact globular structures such as cytochrome c and myoglobin have lower affinities for proanthocyanidins and polyphenols than loosely structured globular proteins such as bovine serum albumin and histone F. Hydrogen bonding between phenolic hydroxyl and peptide carbonyl is a major force stabilizing proanthocyanidin-protein complexes. With loosely structured proteins there is an increased accessibility to the peptide backbone, hence a higher affinity of proanthocyanidin for such proteins. The main binding proteins in plasma are albumin and α_1-glycoprotein. Albumin

consists of a single polypeptide chain and is present in plasma at a concentration of 35-45 g/l in normal healthy individuals. Despite its large molecular weight (68.5 kDa), albumin is not retained exclusively in the plasma compartment, but is distributed extravascularly. Albumin binds both acidic and basic drugs, while α_1-glycoprotein binds only basic drugs (D'Arcy, 1996). No catechin-albumin binding studies have been conducted, but it is very likely that a large part of the serum EGCg will bind to the albumin, influencing its availability, distribution and elimination.

The biotransformation of catechins reduces the bioavailability even further. One pharmacokinetic study showed that the biotransformation of EC administered to male Wistar rats involves glucuronidation, sulfation and methylation (Piskula, 1998). UDP-glucuronosyl transferase activity was found in the liver and the intestinal mucosa. The highest glucuronosyl transferase activity was found in the intestinal mucosa, suggesting that the EGCg is biotransformed as absorption takes place. The highest phenolsulfo-transferase activity occurred in the liver. Catechol-O-methyl transferase activity was found in the liver and kidneys. Polyphenols and phenol-like compounds have been shown to be biotransformed by phenolsulfotransferases and glucoronosyl transferases in rats (*in vivo*) and by rat liver subcellular fractions (i*n vitro*) (Mulder, 1974). The majority of the conjugates that are formed in the liver and involve polyphenols are sulfo-conjugates. These conjugated polyphenols are eliminated predominantly via the biliary excretion path. The low levels of EGCg in the liver of rats could be a result of efficient biotransformation and excretion with the bile. The majority of the catechins in the plasma are in the conjugated forms only one hour after administration. The rapid conjugation of the polyphenols may be an indication that they have a high affinity for phase II enzymes. The polyphenols may have a higher

affinity for the active centers of the phase II detoxification enzymes than for the active center of XO. Since the catechins undergo extensive conjugation, it seems unlikely that their *in vitro* XO inhibition activity is retained *in vivo* in the rat model.

Intestinal bacteria also influence the bioavailability of catechins. In an *in vitro* study it was shown that human intestinal bacteria degrade both simple and gallated catechins. Simple catechins are degraded almost twice as efficiently as gallated catechins. Rat intestinal bacteria could degrade simple catechins, but not gallated catechins (Meselhy, 1997). Sulfated catechins are also formed in the intestines by intestinal bacteria arylsulfotransferase enzymes (Koizumi, 1991). It is not clear whether sulfated polyphenols are more readily absorbed from the intestines than the unmodified catechins.

The uric acid levels in normal rats treated with EGCg (Fig. 5.5B) and TPM (Fig. 5.5C) were slightly lower, but no xanthine substrate accumulation has been detected. EGCg may be a weak uricosuric agent, but this can only be confirmed when glomerular filtration studies are conducted.

4 Conclusion

Both hyperuricemia and hypouricemia could be induced with oxonic acid and allopurinol respectively. These controls indicated that the dosing procedure of the animals was successful and the analysis procedure could determine changes in the serum and urine xanthine and uric aid concentrations. The hypouricemic effect of allopurinol was dose-dependant as found with previous studies from other authors (Osada, 1993).

Treatment with EGCg and TPM did not result in xanthine accumulation in the urine or serum, therefore no inhibition of XO occurred. The tea catechins did cause a slight decrease in the serum uric acid levels of normal rats. Only the 50 mg/kg EGCg treatment showed a statistically significant decrease in the serum uric acid levels of hyperuricemic rats. No statistically significant changes in the serum uric acid levels of hyperuricemic rats could be observed with the 100 mg/kg EGCg and both TPM doses. It is possible that the tea catechins may have a weak uricosuric action. The total amount of uric acid excreted could not be determined to verify this, since the bladders of the rats were not cleared completely at each time interval.

Several factors may be responsible for the lack of inhibition. The low systemic uptake of EGCg, protein binding of polyphenols and the biotransformation of polyphenols must be overcome in order to produce a successful *in vivo* polyphenol inhibitor of XO. A better understanding of the absorption and distribution of the polyphenols may enable the sensible designing of novel polyphenol like inhibitors of XO.