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ON THE FATE OF TRICLOSAN IN HUMANS

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ABSTRACT

Triclosan is a chlorinated organic compound which, due to its antibacterial properties *in vitro*, has found widespread use in a variety of products such as textiles, plastics and healthcare products. Humans are directly and chronically exposed to triclosan via dermal and mucosal contact from soap and toothpaste, upon which triclosan is rapidly absorbed into the body. Owing to the hydroxyl group on triclosan and the quick phase II metabolism, the turnover of triclosan in the human body is relatively fast and the plasma half life is less than a day. Nevertheless, triclosan has been found in human blood plasma and milk. A cause for concern in this regard, is that *in vitro* and *in vivo* animal studies show that triclosan is able to exert adverse effects on hormone homeostasis and metabolic mechanisms, connected to a diverse array of possible toxicological endpoints.

Before this work began, the information about the exposure to triclosan in humans was scarce. Furthermore, the methods for analyzing triclosan in human body fluids lacked sensitivity. There was a need to identify the main sources of triclosan in humans and to investigate the exposure to triclosan in different subgroups of human populations. Also, the fact that triclosan had been found in human plasma warranted further study to examine the transfer rate of triclosan from plasma to milk, and to elucidate if breast milk is a significant source of triclosan to infants. In addition, there was a clear need to study if the *in vivo* triclosan exposure in humans had an impact on cytochrome P450 3A4 enzyme activity and/or thyroid hormone homeostasis. The scope of this thesis was to contribute to filling these knowledge gaps.

Study I describes a sensitive and precise method for the analysis of triclosan in human body fluids, enabling the study of background levels of triclosan. Study II showed that triclosan was omnipresent in plasma and milk from Swedish nursing mothers, and that the level of exposure was highly correlated to the use of triclosan containing personal care products. The transfer rate of triclosan from plasma to milk was low, and the risk of adverse effects from triclosan exposure of the breastfed infant via the milk was judged to be negligible. The results from Study III showed that, apart from some small gender and age variations, the exposure was quite homogenous among serum pools of different genders, ages, regional cohorts and sampling years from the Australian population. Study IV showed that the *in vivo* exposure to triclosan via toothpaste in humans is not likely to change cytochrome P450 3A4 enzyme activity and/or thyroid hormone homeostasis.

While this work shows that there is widespread human exposure to triclosan as a result of the use of triclosan containing personal care products, more research is needed to assess potential negative effects thereof.

LIST OF PUBLICATIONS

This thesis is based on the following papers. They are referred to by their roman numerals in the text.

I. Allmyr M, McLachlan MS, Sandborgh-Englund G, Adolfsson-Erici M
Determination of Triclosan as Its Pentafluorobenzoyl Ester in Human Plasma and Milk Using Electron Capture Negative Ionization Mass Spectrometry
Analytical Chemistry 2006: 78;6542-6546

II. Allmyr M, Adolfsson-Erici M, McLachlan MS, Sandborgh-Englund G
Triclosan in plasma and milk from Swedish nursing mothers and their exposure via personal care products
Science of the Total Environment 2006:372; 87–93

III. Allmyr M, Fiona Harden, Leisa-Maree L. Toms, Jochen F. Mueller, Michael S. McLachlan, Margaretha Adolfsson-Erici, Gunilla Sandborgh-Englund
The influence of age and gender on triclosan concentrations Australian human blood serum
Science of the Total Environment 2008: 393;162–167

IV. Allmyr M, Panagiotidis G, Sparve E, Diczfalusy U, Sandborgh-Englund G.
Human exposure to triclosan via toothpaste does not provoke CYP3A4 activity or disruption of thyroid hormones
Accepted for publication in: Basic & Clinical Pharmacology & Toxicology

CONTENTS

LIST OF ABBREVIATIONS

CV	Coefficient of variation
CYP3A4	Cytochrome P450 3A4
GC/ECNI/MS	Gas chromatography electron capture negative ionization mass spectrometry
hPXR	Human pregnane X receptor
LD_{50}	Lethal dose for 50% of the animals in toxicity testing
LOD	Limit of detection
LOQ	Limit of quantification
mRNA	Messenger ribonucleic acid
NOAEL	No observed adverse effect level
PBDE	Polybrominated diphenyl ether
PCB	Polychlorinated biphenyl
SSNC	Swedish society for nature conservation
T3	Triiodothyronine
T4	Thyroxine

1 INTRODUCTION

1.1 GENERAL PROPERTIES AND USES

Triclosan (2,4,4′-trichloro-2′-hydroxydiphenyl ether, CAS 3380-34-5) (Fig. 1) is a lipophilic and phenolic compound (log K_{OW}=4.76; pKa=7.9) (Syracuse Research Corporation, 2009; Merck Index 12:3, 2000). The manufacturer and patent holder of triclosan since 30 years is the Ciba Specialty Chemicals Corporation. The chemical structure of triclosan is similar to other organic environmental pollutants, such as polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs).

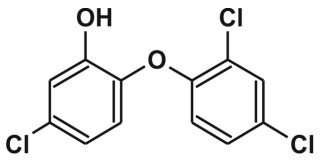

Figure 1. Chemical structure of triclosan

Due to its antibacterial properties, triclosan is incorporated into textiles, plastics and personal care products intended for everyday use, including toothpastes and soaps (Adolfsson-Erici et al., 2002; Bhargava and Leonard, 1996; Perencevich et al., 2001). Ciba Specialty Chemicals Corporation is not willing to release any figures about production and regional and global triclosan demand (Personal communication, CIBA). However, it has been estimated that the annual usage of triclosan is >300 tons in the US and 350 tons in Europe (Halden and Paull, 2005; Singer et al., 2002). The European Commission (2002) stated that more than one third of the triclosan used within the EU in 2002 appeared to reach consumers in oral care products, and a similar amount in skin care products. In 2001, 75% of liquid soaps in the US contained triclosan (Perencevich et al., 2001). A recent study reported that triclosan was common in detergents used for cleaning household foods (e.g. fruits and vegetables) in Thailand (Tsai et al., 2008).

Figure 2 displays the annual gross and net import of triclosan in chemical products to Sweden during the last 15 years. The information on the specific use of the imported triclosan is concealed due to secrecy. Both the gross and net import of triclosan to Sweden increased markedly until 1999. However, since the year 2000 there has been a decrease in imports.

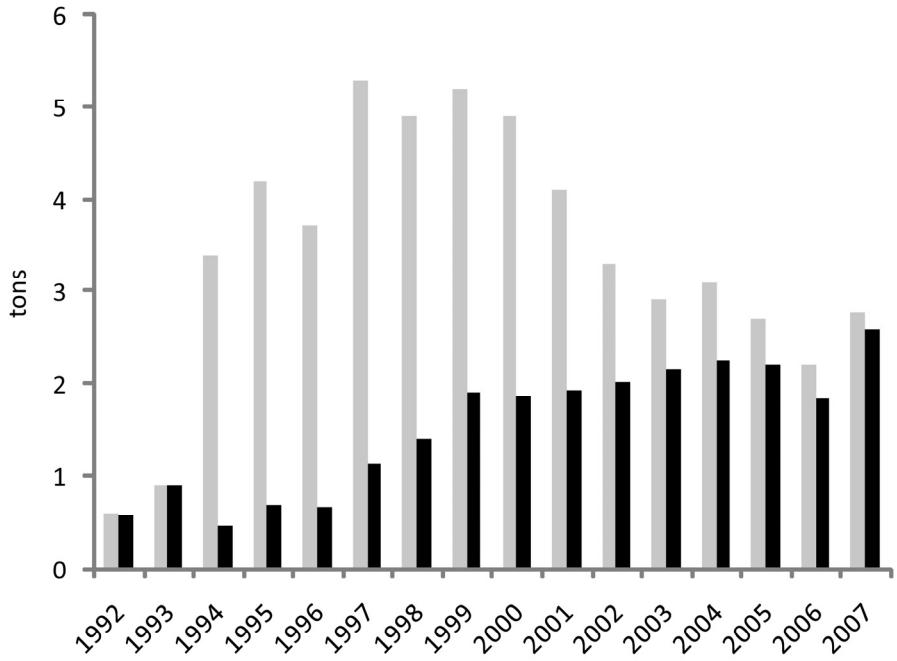

Figure 2. The annual gross (grey) and net (black) import of triclosan (tons) to Sweden as either technical triclosan or in chemical products (hygienic products not included) (KemI-Stat, 2009).

It is plausible that this slowdown is related to the dialogue that preceded and followed the joint recommendation of Swedish government agencies in the year 2000, which promoted a restricted use of triclosan and related antibacterial compounds (Swedish Chemicals Agency, 2001).The amounts presented in the graph do not include the annual import of 2-2.5 tons of triclosan in toothpastes and ~0.3 tons in cosmetics (Holmer O., personal communication). According to the Swedish Cosmetics, Toiletry and Detergent Association, approximately 20-25% of the toothpastes sold in Sweden contains triclosan (Holmer O., personal communication). However, this figure has probably decreased dramatically since the year 2007 due to the work by the non-governmental organisation the Swedish Society for Nature Conservation and voluntary actions from retailers (SSNC, 2007).

1.2 HUMAN EXPOSURE

When the present project was initiated, very little scientific literature on triclosan in human biological fluids was available. Triclosan had been qualitatively determined in human blood plasma from Swedish male adults, and furthermore found at <20-300 ng/g (lipid weight) in human milk samples (Adolfsson-Erici et al., 2002; Hovander et al., 2002). It had furthermore been shown that triclosan was present in human plasma

samples at levels in the low ng/g range, even though the individuals studied had no known sources of exposure to triclosan (Sandborgh-Englund et al., 2006).

The most obvious route of exposure to triclosan is direct contact with triclosan containing personal care products such as deodorants soaps and toothpaste. The triclosan content in toothpaste is 0.3%, resulting in a maximum exposure dose of the order of 6-12 mg/day. Furthermore, since triclosan is present in plasma from people without exposure to these products, it has also been suggested that food could be a contributing background source. One study showed triclosan levels of <1.4-8.4 ng/g in different types of fatty food (Remberger et al., 2002). This result could not be confirmed by our investigations, which showed that sample pools of dairy products, meat, fish and egg, composed on the basis of Swedish per capita consumption data, contained <0.02-0.15 ng/g triclosan (Adolfsson-Erici et al, 2007). This would account for an average triclosan intake of 16 ng/day via food for humans in Sweden.

Another source of background exposure to triclosan was suggested by the finding of triclosan in indoor dust samples from households in Spain, which contained triclosan at an average level of 1.1 μg/g (Canosa et al., 2007). Toddlers and adults may be estimated to have an average dust intake of 0.05 and 0.02 g/day, respectively (Jones-Otazo et al., 2005). Assuming 100% bioavailability, the dust would hence account for a relatively low intake of 55 and 22 ng/day for the two groups.

A simplified estimation of the theoretical steady state concentration of triclosan in plasma resulting from a chronic exposure to triclosan via food and dust can be made by applying the equation: $C_{ss} = R_0 / CL$, where C_{ss} = steady state concentration in plasma, ng/mL, R_0 = rate of infusion, ng/day, and CL = clearance of drug from plasma, mL/day (Rowland and Tozier, 1989). For the calculation, CL was derived from the clearance per fraction of dose absorbed per kg bodyweight, 0.041 L/h/kg, adopted from the pharmacokinetic study of triclosan by Sandborgh-Englund et al. (2006). (Conversion: CL = 0.041 L/h/kg •1000 (mL) • 24 (h) • mean body weight: 73.7 kg = 72520.8 mL/day). The mean weight, i.e. 73.7 kg, of men and women in the ages 17-79 years that participated in the Swedish consumption study Riksmaten (Becker and Pearson, 2002), was adopted for the calculations of exposure on a body weight basis. It is here assumed that all of the ingested triclosan is transferred to the blood, independent of the vehicle of administration, and that the pharmacokinetics at low and high doses are

comparable. Hence, the resulting calculated plasma net concentration of triclosan from a combined intake via food and dust in an adult would be 0.0005 ng/ml, which is four orders of magnitude lower than the background plasma triclosan concentrations measured in the Swedish population (Sandborgh-Englund et al., 2006).

1.3 PHARMACOKINETICS

Triclosan is rapidly absorbed and distributed in the human body (Sandborgh-Englund et al., 2006). Maximum concentrations are reached within three hours after oral intake. However, the metabolism and excretion of the compound is fast. The terminal plasma half life of triclosan is 21 h (Sandborgh-Englund et al., 2006). The fast turnover rate is due to an ortho positioned hydroxyl group on one of the aromatic rings, which makes it prone to phase II metabolism via sulfotransferase and glucuronosyltransferase enzymes (Wang et al., 2004). In humans the resulting conjugates are excreted primarily in urine (Sandborgh-Englund et al., 2006).

During chronic exposure to triclosan via toothpaste usage two times a day, the triclosan concentrations reach a steady state at a level dependent on the intake dose and clearance rate (Bagley and Lin, 2000). For normal use of triclosan containing toothpaste, the total triclosan concentrations in plasma, including both conjugated and unconjugated triclosan, are in the ng/ml range. Sandborgh-Englund et al. (2006) showed that the fraction of unconjugated triclosan in plasma was ~30% of the total following oral exposure. This figure has been contradicted by a recent study showing no unconjugated triclosan in human plasma (Ye et al., 2008a). However, in the latter study the origin of the samples was unknown. It is thus uncertain if these divergent findings are due to differences in routes of exposure to triclosan. For example, it has been shown that triclosan is conjugated to a large extent when it passes through human skin (Moss et al., 2000). It is possible that the passage of triclosan through buccal and intestinal mucosal epidermis is faster and results in a lower degree of conjugation.

1.4 TOXICOLOGICAL ASPECTS

In acute toxicity tests in mammals, triclosan displays low toxicity, with an LD_{50} value of > 5000 mg/kg body weight in the rat. Furthermore, triclosan is not carcinogenic, genotoxic, mutagenic or teratogenic. However, mouse and rat studies have revealed hepatic changes and increased incidence of retardation of ossification sites with a NOEL of 25 mg/kg/day (Bhargava and Leonard, 1996).

An increasing number of studies show that triclosan is able to affect specific biological functions. Triclosan is structurally similar to non-coplanar ortho-substituted PCBs and has similarly been shown to weakly increase aryl hydrocarbon receptor activity and to potently interact with ryanodine receptors and stimulate cellular Ca^{2+} mobilization (Ahn et al., 2008). The disruption of Ca^{2+} homeostasis in the brain by compounds altering ryanodine receptors may contribute to alteration of neurodevelopment (Gafni et al., 2004; Wong et al., 2001). Another interesting result from *in vitro* studies is that triclosan showed dose dependent antagonism at low uM levels towards estradiol and testosterone mediated activation of estrogen and androgen receptors (Ahn et al., 2008; Chen et al., 2007). In addition, recent research has shown that the oral administration of triclosan at doses of 10 and 20 mg/kg/day causes inhibition of testicular androgen production in male rats (Kumar et al., 2008; Kumar et al., 2009).

Mustafa et al., performed a study in which fibroblast cells were incubated with ^{14}C-triclosan (Mustafa et al., 2003). After equilibrating for 24h, 84% of the radioactivity was found in the cytoplasm of the cell, while 16% was found in the nuclear fraction. However, after repeatedly washing the fibroblast cells the radioactivity in the cytoplasmic fraction decreased by 77% while the level in the nuclear fraction remained unchanged. Whether or not triclosan has a similar strong association with the nucleus of other cell types in the human body during systemic exposure is not known, but this could be relevant for the ability of triclosan to exert biological effects.

In the work presented in this thesis, thyroid disruption and Cytochrome P450 3A4 (CYP3A4) enzyme induction are two toxicological aspects of triclosan that have been considered in order to investigate their potential relevance to humans as a result of *in vivo* exposure to triclosan via triclosan containing toothpaste.

Thyroid disruption

Organohalogen pollutants, such as PBDEs and PCBs, with a structural resemblance to thyroxine, have been recognized as being thyroid disruptors from *in vitro* and *in vivo* studies of rats and mice (Crofton, 2008; Darnerud, 2008). In addition, even though not entirely consistent, human epidemiological studies have indicated negative correlations between organohalogen pollutants and thyroid hormone status (Boas et al., 2006). The toxicological endpoint of thyroid deficiencies due to exposure to xenobiotics in adults could in theory result in reversible symptoms such as fatigue and muscle tension

(Ladenson et al., 2000). Thyroid imbalance during early development of the brain is, however, known to cause irreversible damage, affecting cognitive and learning capacity later in life. The fetus' supply of thyroxine is dependent on the thyroidal status of the mother. Hence, special attention should be paid to pregnant women when assessing thyroid disturbances from environmental pollutants (de Escobar et al., 2004).

Triclosan has also been identified as causing thyroid disturbances in animals in *in vivo* studies. Crofton et al. (2007) demonstrated that triclosan dose dependently decreased the circulating concentrations of thyroxin in the serum of rats during a 4-day *in vivo* oral exposure experiment. In that study, weaning rats were fed triclosan at doses ranging from 0 – 1000 mg/kg/day (body weight). The lowest dose that gave a significant effect on the T4 levels was 100 mg/kg/day and the benchmark dose was calculated to 70 mg/kg/day.

Zorilla et al. (2009) later presented a more thorough investigation, confirming the thyroid disruptive properties of triclosan. Rats were fed triclosan for 30 days from postnatal day 23 to 53. After the exposure, liver weight was measured and circulating serum levels of thyroid hormone T4, tri-iodothyronine T3 and stimulating hormone were measured. At doses of 30 mg/kg/day and above there was a significant and dose dependent decrease of T4 concentrations. The no observed effect level was 3 mg/kg/day. The T3 levels were affected only at triclosan doses of 200 mg/kg/day. A plausible explanation for the observed reduction of thyroid hormones could be the induction of uridinediphosphate glucuronosyltransferase, as has been shown for other chemicals (Vansell and Klaassen, 2002). Support for that hypothesis was provided by the observation that liver weight increased in highly exposed rats, which is indicative of increased hepatic enzyme activity. In addition, although non-significant, the analysis of rat liver samples showed a doubling of glucuronosyltransferase enzyme activity.

Not all observations support the hypothesis of an increased phase II metabolism of thyroid hormones by triclosan. Schuur et al. (1998) demonstrated that triclosan weakly inhibited the sulfotransferase activity towards the di-iodothyronine hormone in rat liver cytosol *in vitro*. Additionally, at the same time as being a substrate for sulfotransferase and glucuronosyltransferase, triclosan acts as an inhibitor of these enzymes and has been shown to hinder the phase II metabolism of the environmental pollutant metabolite 3-OH-benzo[a]pyrene *in vitro* (Wang et al., 2004).

CYP3A4 enzyme induction

Triclosan has shown some potential to influence the activity of different enzymes in rats and mice (Hanioka et al., 1997; Hanioka et al., 1996; Jinno et al., 1997). However, inter species differences as to which and to what extent an enzyme is expressed as a result of xenobiotic exposure make direct extrapolation from animals to humans inappropriate (Jones et al., 2000; Pelkonen et al., 2008; Tabb et al., 2004). An important indication of triclosan-enzyme interactions relevant to humans was presented by Jacobs et al. (2005), who showed that triclosan is a medium affinity ligand to the human pregnane X receptor (hPXR) *in vitro*. hPXR mediates the transcriptional activation of, among other things, the CYP3A4 enzyme. CYP3A4 is one of the most abundant phase I metabolizing enzymes in the human body. The binding pocket of CYP3A4 is exceptionally smooth and large. This feature enables it to recognize a wide variety of endogenous and exogenous compounds. One problematic result thereof is that CYP3A4 is responsible for catalyzing the biotransformation of an estimated 50% of the commercially prescribed drugs (Lehmann et al., 1998; Luo et al., 2002). Therefore, the exposure to drugs that induce CYP3A4 poses a risk of increased metabolism of pharmaceuticals that are substrates to the enzyme. A well known example of drug-drug interaction is the conflicting co-administration of contraceptive agents that are substrates to CYP3A4, and the mild antidepressant St John's wort, a known hPXR ligand and CYP3A4 inducer (Moore et al., 2000; Murphy et al., 2005).

In the hPXR binding gene reporter assay study by Jacobs et al. (Jacobs et al., 2005), triclosan was tested at 10 μM, and the effect was ~50% relative to that of 10 μM rifampicin, which is a strong CYP3A4 inducer in humans (Kanebratt et al., 2008). Studies have shown good correlations between *in vitro* PXR activation in cell reporter assays and induction of CYP3A4 mRNA in human hepatocytes (Luo et al., 2002). Nevertheless, it is difficult to interpret receptor assay data and its relevance to *in vivo* exposure conditions. One recent study showed a correlation between CYP3A4 activity and concentrations of ΣPCBs in plasma from Faroese men, indicating that this enzyme might be induced *in vivo* in humans by a group of environmental contaminants which are structurally related to triclosan (Petersen et al., 2007). Interestingly, PCB 118 and PCB 153, two of the most abundant PCB congeners in the Faroese serum, were found to be weak activators of hPXR in the study by Jacobs et al., (Jacobs et al., 2005).

2 AIMS

The overall aim of this thesis was to increase the understanding of triclosan exposure, and the consequences thereof, in humans.

Study I

The aim of study I was to improve the analytical methods for analyzing triclosan in human body fluids such as blood and milk in order to be able to detect triclosan at lower background levels. The objective was to broaden the understanding of triclosan exposure in the general population.

Study II

The aims of study II were to screen for and to identify the main sources of triclosan in the general Swedish population, represented by a group of 36 nursing mothers. The transfer ratio from plasma to milk was analyzed, and the extent to which the infant is exposed to triclosan via mother's milk was estimated. An additional objective was to examine if the use of triclosan is based on informed choice.

Study III

The aim of study III was to investigate the internal exposure to triclosan of humans of different ages, genders and geographical regions in Australia by analyzing pooled serum samples. The data from a group of Australian females were compared to triclosan concentrations in human plasma from Swedish females of the corresponding age in study II.

Study IV

The aim of study IV was to elucidate if *in vivo* exposure to triclosan via toothpaste change the CYP3A4 enzyme activity and/or thyroid hormone homeostasis in humans.

3 MATERIAL AND METHODS

3.1 STUDY I

Chemical analysis

The method that was developed and then used for triclosan analysis in this work is briefly described in the following. After weighing 3 g milk or 3-5 g plasma, ^{13}C labeled triclosan was added as the surrogate standard. The sample was submitted to acid hydrolysis using sulphuric acid to release any triclosan bound in conjugates. The samples were then extracted using hexane/acetone, and cleaned up using sulphuric acid. Triclosan was converted to its pentafluorobenzoyl ester, and after final cleanup with concentrated sulfuric acid, the extract was analyzed using gas chromatography/electron capture negative ionization/mass spectrometry (GC/ECNI/MS).

Quantification

The triclosan concentration in the samples was quantified using isotope dilution. The limit of quantification (LOQ) was defined by the triclosan content of the blanks. The area ratio of triclosan to ^{13}C-labeled triclosan ($A_{triclosan} / A_{13C\ triclosan}$) in the blanks was consistent, and hence 4 x ($A_{triclosan} / A_{13C\ triclosan}$) in the blank was defined as the LOQ. Concentrations below the LOQ were also quantified, as this was considered superior to excluding the data. All samples were corrected for the blanks. Triclosan concentrations were determined as the sum of unchanged and conjugated triclosan (ng/g) on a fresh weight basis.

3.2 STUDY I AND STUDY II

Study population and sampling

Thirty-six mothers were recruited at a childcare center in the City of Stockholm between October 2003 and May 2004. The inclusion criteria were that the mother was a first time mother, healthy, and that at least half the infant food intake was breast milk. Milk and plasma were sampled on two occasions, approximately six and twelve weeks after delivery.

Monitoring of personal care products containing triclosan

On the first sampling occasion, the mothers' personal care products (here defined as any chemical product used for personal care, e.g. toothpaste, mouth rinse, soap, shampoo, deodorant, cosmetic, moisturizer etc.) labels were scrutinized for triclosan

content. Those who used products containing triclosan were denoted as "exposed", while those who did not were denoted as "controls". The study subjects ranked their knowledge about triclosan on a scale from "none" to "very good".

Chemical analysis

The triclosan analysis was performed as described in Study I.

3.3 STUDY III

Serum sample pools

The samples analyzed in this study were pools of human blood sera. Some were originally collected for the Australian National Dioxins Program (NDP) in 2002/03. Others were collected in 2004/05. The serum sample pools were selected to allow an evaluation of differences in triclosan concentrations between regions, ages, genders and sampling occasion. The following stratification criteria were applied for categorising the sample pools. Age: 0-4 years (2004/05 samples only), 5-15 years (2004/05 samples only), <16 years (2002/03 samples only), 16-30 years, 31-45 years, 46-60 years, > 60 years. Gender: female, male (2004/05 samples only). Region (four urban regions and one rural, female 2002/03 samples only): Northeast; Southeast; South; West; Rural region. For each sample category, 200 samples were retrieved, which were randomly divided into two pools.

Chemical analysis

The triclosan analysis was performed as described in Study I.

3.4 STUDY IV

Study population and exposure

Twelve healthy adults were recruited for the study. Exclusion criteria were use of prescription medication within the previous two months, use of prescription free drugs (e.g. St John's wort), illicit use of any drugs, and pregnancy. The study subjects agreed to avoid any other triclosan containing products for two weeks before as well as during the experimental exposure to triclosan toothpaste and to avoid intake of any products containing grapefruit, which is known to inhibit CYP3A4 activity. The study subjects were instructed to brush their teeth for 3 minutes with a 2 cm strain of a toothpaste (Colgate Total®) containing 0.3% (w/w) triclosan twice a day (morning and evening) for 14 days. No toothpaste was to be swallowed on purpose.

Chemical analysis

The triclosan analysis was performed as described in Study I.

CYP3A4 enzyme activity was analyzed indirectly by the plasma concentrations of 4β-hydroxycholesterol, an endogenous marker for induction of this enzyme in humans. 4β-hydroxycholesterol was determined by isotope dilution gas chromatography-mass spectrometry using a deuterium-labeled internal standard as described in Bodin et al. 2001 (Bodin et al., 2001).

Thyroid hormones (FT3, FT4 and TSH) were determined by commercial electrochemiluminescense immunoassays from Roche Diagnostics GmbH, Mannheim, Germany, run on a Roche/Hitachi Modular Analytics 170 instrument.

3.5 ETHICAL APPROVALS

Studies I and II were approved by the Regional Ethical Review Board, Huddinge University Hospital, Sweden (dnr: 395/03).

Study III was approved by the Medical Research Ethics Committee at the University of Queensland (Clearance Number 2002000656).

Study IV was approved by the ethics committee at Karolinska Institutet, Huddinge, Sweden; Dnr 2008/615-31.

4 RESULTS AND DISCUSSION

The first paper in this thesis describes a sensitive and precise method for analyzing triclosan in human body fluids. The performance of the presented method was satisfying and the limit of quantification was lowered by several orders of magnitude as compared to previously used methods.

Plasma (n = 72) and milk (n = 71) samples were fortified with ^{13}C-labeled triclosan and analyzed applying the final method described in paper I. The mean absolute recovery of the ^{13}C-labeled triclosan was 46% (CV = 23%) in plasma (85% in blanks) and 49% (CV = 18%) in milk (91% in blanks). The main loss of triclosan is believed to occur in the derivatisation step, in which varying amounts of precipitate formed in the sample extract, possibly hindering the derivatization of the triclosan. In the blanks, which were treated in the same way as the samples, but where there was no precipitation, the recovery was considerably higher. Introducing an additional cleanup step before the derivatization could probably improve the recovery. However, despite a high variability in recovery of the ^{13}C-labeled triclosan, the repeatability of the method was satisfactory. The coefficient of variation was 6% for the high concentration (mean 0.84 ng/g, n=7), 1% for the intermediate concentration (mean 0.31 ng/g, n=3), and 5% for the low concentration (mean 0.020 ng/g, n=3).

A key factor in increasing the method sensibility was the use of pentafluorobenzoylation to enhance the spectrometric properties of triclosan. Applying GC/ECNI/MS, the relative response factor for the triclosan pentafluorobenzoylate derivative was 5 and 28 times higher than for the acetylate and methylate derivatives, respectively. In addition, the pentafluorobenzoylate is stable towards cleanup with concentrated sulphuric acid, which is a means of reducing interferences during the instrumental analyses.

A major challenge during the development of the method was the highly variable presence of triclosan in blanks. The source of the triclosan contamination was found to be insufficient cleaning of reused test tube caps. Sealing the test tubes with a double layer of aluminium foil before capping almost completely eliminated the blank problem. Since the LOQ was defined by the triclosan content in the blanks, this was a requisite factor for the improvement of the method performance.

The capability to analyse triclosan at lower levels was central for elucidating the ubiquitous background exposure to triclosan in the general human population. As illustrated in Figure 6 in paper I, most of the triclosan concentrations in the Swedish plasma and milk samples would have been defined as censored data by using the methods applied by e.g. Bagley and Lin (2000). By using the herein presented method, the triclosan concentrations in all plasma and 45% of the milk samples were above the limit of quantification.

Promising high quality methods for the analysis of triclosan and other phenols in human samples have also been developed and evaluated by Ye and coauthors (Ye et al., 2008a; Ye et al., 2008b). They apply on-line solid phase extraction high performance liquid chromatography tandem mass spectrometry. An advantage of this method is that it requires a small volume of sample (0.1 ml), minimal sample pretreatment, and little solvent. However, a drawback is the comparably high LOD of 1 ng/ml, which, as a comparison, would have left all milk samples in Study II defined as censored. Dirtu et al., (2008) presented a method for analyzing triclosan in plasma, which combines solid phase extraction, derivatisation with pentafluoropropionic acid, and determination on GC/ECNI/MS. Their method provides a low limit of detection and a high degree of precision, comparable to the method presented in paper I.

In study II (paper II), the labeling of the mothers' personal care products brought to the first sampling occasion, indicated that nine of the 36 Swedish nursing mothers were exposed to triclosan via personal care products. Seven of the mothers used triclosan toothpaste, while one used soap and one used deodorant containing triclosan. These figures are in agreement with the information regarding the market shares of triclosan toothpaste sold in Sweden (Holmer O., personal communication).

The triclosan concentrations in plasma and milk were highly and significantly elevated in the group of mothers that used triclosan containing products. In the exposed group the lowest concentrations were found in the mothers using triclosan soap and deodorant. Corroborating the results from Sandborgh-Englund et al. (2006), these data also indicate a source of triclosan exposure in the control group, rendering a lower but ubiquitous presence of triclosan in human plasma and milk. This was also supported by the analyses of plasma samples collected before experimental exposure in study IV, in which the baseline triclosan plasma concentrations ranged between 0.009-0.81 ng/g.

The source of the background exposure to triclosan in Sweden has not been identified. Note that improper labeling of triclosan containing products or incomplete assessment of products used by the mother may have lead to incorrect grouping of some individuals as controls. However, the omnipresence of triclosan in plasma and milk points towards a diffuse source. There have been findings of triclosan in food and dust, but as discussed above the contribution of these sources are not likely to solely account for the background concentrations found in body fluids from Swedish residents.

On an individual basis, the triclosan concentrations were higher in plasma than in milk in all but two cases. Triclosan is a phenol and a weak acid (p*K*a=7.9). Thus the differences in acid ionization equilibrium for triclosan in plasma (pH=7.5) and milk (pH=6.5) will favor the ionized form in plasma compared to milk. Assuming that the neutral form of triclosan equilibrates across the mammary membrane, the total concentration of triclosan in milk will be lower in milk than in plasma. In paper II we assumed that conjugated triclosan would not be transferred from plasma to the milk. This assumption was later supported by Ye et al., who found a majority of the total triclosan content in human breast milk to be in its unconjugated form (Ye et al., 2008b).

The total intake of triclosan via breast milk was calculated to <0.11-570 ng/day. Hence, the exposure of the breastfed infant to triclosan via milk was considered to be a negligible risk. This conclusion was also supported by a risk assessment study on triclosan in human milk from US nursing women (Dayan, 2007). In the US milk study the upper decile of the sample concentrations ranged from 14-40 ng/g, as compared to the 0.64 – 0.95 ng/g range in the upper decile of the Swedish samples. Nevertheless, based on a NOAEL=50 mg/kg/day in weaning rats, the margin of safety for the worst case scenario was estimated to be ~7000 for the breast-fed human infant in the US. However, it may be of higher importance for the child that triclosan is present in plasma of the pregnant mother. In a study of cord blood from mothers in the Netherlands, triclosan was present at concentrations of 0.5-5 ng/g, indicating that triclosan is transferred to the developing fetus from the exposed mother (Peters, 2005). Bearing in mind that triclosan may possess endocrine disruptive properties that affect functions related to neurological development, the *in utero* exposure to this chemical should be regarded as an issue of concern. In this context it would be interesting to analyze the triclosan content in individual plasma samples from pregnant US women, where the milk concentrations in many cases were markedly high. In study II in this

thesis, the milk to plasma ratio for triclosan was <1 and decreased with increasing plasma concentrations. Extrapolating to the US mothers, their plasma triclosan concentrations could be expected to be much higher than in the plasma from Swedish mothers, where the highest triclosan concentration in plasma, 38 ng/g, resulted in a milk concentration of 0.70 ng/g.

An interesting finding from study II was that none of the individuals in the exposed group were aware of their triclosan use. This indicates that the explicit labeling of triclosan toothpaste in Sweden, which aims at helping the customer in making an informed choice, is not effective. More recently, the Swedish Society for Nature Conservation made a Gallup survey which confirm that the use of triclosan containing toothpaste is random and mostly unwitting (SSNC, 2007). This is most probably a universal feature not only restricted to Sweden. Thus, since both independent researchers (Edwardsson et al., 2007) and dental organizations in the Nordic countries explicitly advise against general use of triclosan toothpaste, and even propose a regulation through the Medical Products Agency (Nordic Dental Associations, 2006-2007), there is good reason to question if it should be sold to the general population in retail stores.

In the Australian pooled serum samples analysed in study III, there were no differences in serum triclosan concentrations between regions. Some age variations were observed; the 31–45 year group had significantly higher serum concentrations than all other age categories. A similar age distribution pattern for triclosan was also observed in the human population in the US, where urine samples from different age groups were analyzed for triclosan (Calafat et al., 2008). However, when the Australian data were assessed on a gender basis, there were no statistical differences among the different female age categories. In the male sample pools, the 31–45 year category had significantly higher concentrations than all groups except for the 16–30 year old males. The 16–30 year old males, however, had significantly higher triclosan concentrations than the 0–4 year old males. As a group, the males had significantly higher concentrations than the females. The differences between genders on an age category basis was, however, not significant. Apart from the small gender differences and a small variation with age, the most notable finding from the study was that the exposure was relatively homogenous throughout the population.

Due to the high turnover rate of triclosan in human plasma, the results from study III describe the exposure to triclosan containing products in Australia at the time of sampling. The observed homogeneity is possibly due to marketing of triclosan containing product types, for example soaps, that impact usage independently of region, sex and age. The implied peak in triclosan exposure in middle-aged males may be due to a more frequent use of, for example, deodorants containing triclosan by this group. It is noteworthy that the distribution of exposure between individuals within each category may range widely depending on the market shares of triclosan containing products within a certain product type. This was observed in Study II where the distribution of triclosan concentrations in human plasma samples was highly skewed and widely ranging. A comparison between the triclosan concentrations in plasma from Swedish nursing mothers in Study II with the corresponding group of females in the Australian sample set indicated that the levels were higher in the Australian population. It is noted that the strength of any conclusions to be drawn from such comparison is limited. However, in Sweden there is an active debate cautioning against unnecessary use of antibacterial compounds such as triclosan. One may therefore speculate that the human exposure to triclosan is lower in Sweden, than in countries where the consumer advisories against the use of these types of substances are less common. Triclosan containing soaps have never been commonly available on the Swedish market, and since the SSNC report on triclosan usage in Sweden, triclosan containing toothpastes are no longer on sale in many of the Swedish supermarket chains.

As mentioned above, several *in vitro* and *in vivo* animal studies have shown that triclosan is able to interfere with biological systems. In study IV, two toxicological aspects of triclosan related to exposure in humans were examined. The exposure to triclosan via triclosan containing toothpaste did not induce CYP3A4 enzymes or cause changes of thyroid hormone concentrations in humans. The reason was most probably that the triclosan concentrations in plasma were too low to trigger a response. It may also be, at least for the disruption of thyroid homeostasis observed following triclosan exposure in rats, that the effect is species-specific. This investigation included a relatively small population exposed to triclosan for the short time of 14 days. Hence the conditions do not ideally reflect the prolonged systemic exposure to triclosan that may proceed for years or even decades in large groups of human populations. Albeit, the results are good indices that the normal use of triclosan toothpaste, and the nM plasma triclosan concentrations resulting from the exposure, is not likely to alter metabolism of

drugs via CYP3A4 induction or cause thyroid hormone disturbances and thereto related adverse events in humans.

4.1 FUTURE PERSPECTIVES

Animal *in vivo* studies show dose-dependent effects from triclosan exposure and *in vitro* receptor assays give responses at low uM levels of triclosan (Ahn et al., 2008; Chen et al., 2007; Kumar et al., 2009; Zorrilla et al., 2009). The clinical implications of triclosan exposure should hence be further examined in order to assess whether the widespread use and chronic exposure to triclosan in humans are connected to any adverse effects. Furthermore, in light of the frequently disputed claims of beneficial effects from general use of triclosan in both toothpaste and soaps, it is motivated to further question if it is wise to spread and use this chemical in the unrestricted manner we see today (Aiello et al., 2007; FDA, 2005; Nordic Dental Associations, 2006-2007; SSNC, 2007).

5 CONCLUSIONS

The analytical method developed in this thesis is sensitive and precise, and it enables the analysis of background concentrations of triclosan in human body fluids.

Triclosan is ubiquitous in plasma and milk from Swedish nursing women, and the levels are highly and significantly elevated when the women are exposed to triclosan containing products.

On an individual basis, the triclosan concentrations are lower in milk than in plasma.

The exposure of breast-fed infants is low and regarded as of negligible risk.

Although triclosan toothpastes in Sweden are explicitly labeled for their content, the use thereof is not based on informed choice.

The distribution of triclosan exposure between subcategories in Australian population was homogenous.

Short-term exposure to triclosan via toothpaste does not alter CYP3A4 enzyme activity or thyroid hormone homeostasis in humans.

6 SWEDISH SUMMARY

Triclosan (2,4,4´-trikloro-2´-hydroxydifenyleter) är en fettlöslig kemisk substans med bakteriedödande egenskaper. Användningen av triclosan har ökat på grund av en stark marknadsföring av antibakteriella produkter. I Sverige används 4 ton triclosan årligen, varav 2 ton når konsumenter som tillsats i tandkräm och 0.3 ton i kosmetika. En fjärdedel av all tandkräm som säljs i Sverige innehåller triclosan. En undersökning av flytande tvålar tillgängliga för konsumenter i USA visade att 75 % av tvålarna innehöll triclosan. Ämnet förekommer även i tyg och plast. En stor del av befolkningen exponeras dagligen för ämnet när de borstar tänderna eller använder andra egenvårdsprodukter. Man har visat att triclosan absorberas via hud och slemhinna, men att det också utsöndras relativt snabbt vid orala doser. Utsöndringen sker i huvudsak som glukuronid- och sulfatkonjugat via urin.

Triclosan har påvisats i human plasma och bröstmjölk i den allmänna befolkningen. Mätbara basala nivåer av triclosan har påvisats i plasma från personer som medvetet undvikit triclosaninnehållande varor, vilket pekar på att vi ofrivilligt exponeras för triclosan från andra källor än de direkt uppenbara. Att triclosan påvisas i bröstmjölk är oroande då det innebär att spädbarn exponeras för triclosan via födan till följd av moderns exponering. Hälsoriskerna för människa vid lång exponering är inte fullständigt utredda. *In vitro*-studier och djurstudier *in vivo* har dock visat att triclosan kan störa biologiska system genom påverkan på metabolism och hormonbalans.

Syftet med den här avhandlingen är att bidra till en bättre förståelse av konsekvenserna av att använda triclosan i produkter för dagligt bruk. I den första studien, Study I, utvecklades en känslig och precis metod för analyser av triclsoan i människa. Metoden innebar en klar förbättring avseende känslighet jämfört med tidigare metoder, och möjliggjorde analys av bakgrundshalter av triclosan i människa.

I den andra studien, Study II, kartläggs svenska nyblivna mödrars exponering för triclosan via hygienprodukter och spädbarns exponering för triclosan via bröstmjölk. Triclosan förekom i samtliga prover, även från mödrar utan uppenbar exponering via hygienprodukter, något som styrker de tidigare indikationerna på en diffus källa till triclosan. Resultaten visar också att triclsoaninnehållande hygienprodukter är den dominerande enskilda källan till triclosan i ammande svenska mödrar.

Koncentrationerna av triclosan i plasma och mjölk var signifikant och mycket förhöjda i mödrar som var exponerade för dessa triclosanprodukter. På individnivå var triclosankoncentrationen högre i plasma än i mjölk, dessutom minskade överföringskvoten från plasma till mjölk med ökande plasmakoncentration. Därför drogs slutsatsen att det ammande spädbarnet exponeras för en försumbar mängd triclosan via modersmjölk. Ingen av de exponerade mödrarna var medveten om sitt bruk av triclosan.

Det tredje delarbetet, Study III, visar triclosankoncentrationer i poolade serumprover från olika grupper av den Australiensiska befolkningen. Män var som grupp mer exponerade än kvinnor, och hos män fanns en signifikant högre exponering i gruppen 31-45-åringar. I övrigt var triclosanexponeringen märkbart homogen; inga större skillnader mellan grupper av olika ålder, kön och regionstillhörighet kunde utläsas.

Den fjärde delstudien, Study IV, syftade till att undersöka om triclosanexponering via tandkräm påverkar det läkemedelsomsättande enzymet cytochromP450 3A4 och/eller stör thyroidhormonbalansen i människa. Av den relativt korta exponering om 14 dagar som försöket innebar, kunde man inte se några effekter på dessa parametrar. Detta trots att triclosankoncentraionerna i plasma i försöksgruppen efter exponering låg i det högre intervallet av vad som kan förväntas vid normal exponering via tandkräm.

Triclosan är allestädes närvarande i människors blod och mjölk. I human plasma ligger nivåerna runt ng/g (färsk vikt). Studier har visat att triclosan kan påverka flera olika biologiska system. Vi har undersökt två av dessa i människa och inte kunnat konstatera någon effekt. Dock finns det skäl att vidare studera andra tänkbara negativa effekter, såsom hormonstörningar, i människa. Användningen av triclosanprodukter i den allmänna befolkningen i flera länder är utbredd och dessutom till synes omedveten. Dock är många oberoende forskare och myndigheter samstämmiga om att en sådan generell användning saknar positiva effekter på människors hälsa. Alltså; med tanke på de – om än små – riskerna som påvisats med triclosanexponering i människor, så talar en risk-nyttokalkyl för att en generell användning av triclosan bör ifrågasättas.

7 STATEMENT

I, Mats Allmyr, made the following contributions to the studies presented herein:

Study I

I performed the methodological development and was responsible for the chemical and instrumental analysis as well as the data analysis and validation of the method. I was the lead author of the paper.

Study II

I was involved in the writing of the application for ethical approval as well as coordinating the recruitment of, and sampling the blood from the study subjects. I analysed the samples, conducted the statistical evaluation of the data and was the lead author of the paper.

Study III

I was responsible for the analysis of triclosan in the samples as well as the statistical evaluation of data. I was the lead author of the paper.

Study IV

I launched the idea and took the initiative to analyse the effects of triclosan on CYP3A4 activity and thyroid homeostasis in humans *in vivo*. I was also active in planning the project design and writing the application for ethical approval. I analysed the triclosan content in the samples, kept correspondence with the collaborative laboratory that analysed the other variables, and I evaluated the data. I was the lead author of the paper.

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