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# **Formulation studies for eliminating saliva carcinogenic acetaldehyde with L-cysteine containing chewing gum**

ALMA KARTAL-HODZIC

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Pharmacy of the University of Helsinki, for public examination in Auditorium 2 at Viikki Infocenter, Viikinkaari 11, on 15 December 2012, at 12 noon.

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*To Nora and Dan*



## Abstract

Tobacco smoke is a major risk factor for the development of cancers in the upper parts of gastrointestinal tract. It has been estimated that the risk of oral cancer among smokers is 7–10 times higher than for never-smokers.

Acetaldehyde is formed during the tobacco smoking burning process and may be one of the most toxic compounds in tobacco smoke. According to the *International Agency for Research on Cancer* (IARC), in 2004 acetaldehyde was classified as a possible carcinogen in humans (Group 2B). In 2009, acetaldehyde was classified in Group 1, as a carcinogen to humans. A non-essential-amino acid such as L-cysteine, is able to bind acetaldehyde and form 2-methylthiazolidine-4-carboxylic acid (MTCA).

The general aim of this study was to through formulation studies to explore the ability of L-cysteine to eliminate carcinogenic acetaldehyde present in saliva. In addition, the aim was to develop user-friendly L-cysteine containing chewing gum to reduce acetaldehyde formed during tobacco smoking. The main variables were the chemical form of L-cysteine used (L-cysteine or L-cysteine hydrochloride) and the chewing gum preparation method (traditional and novel direct compression method). Furthermore, the aim was to obtain more information on the optimal formulation properties, using approaches such as stability studies and possible interactions between cysteine and used excipients. Caco-2 cell lines were used to access the ability of L-cysteine and MTCA to absorb from the gastrointestinal tract. A computational model was developed to analyse the effects of different physiological factors and effect of formulation parameters on tobacco smoke acetaldehyde.

The combined results of these studies suggested that tobacco smoke carcinogenic acetaldehyde can be successfully eliminated with prepared L-cysteine chewing gums. Compared to the traditional manufacturing process the directly compressed gum formulation can offer an alternative method to traditional chewing gum production. Due to the slower dissolution rate, better compatibility with excipients, and better stability under higher temperature and humidity, L-cysteine as a free base is a better candidate for chewing gum formulation than cysteine hydrochloride.

The Caco-2 permeability studies indicate no significant risk of the locally administered L-cysteine being absorbed before binding to acetaldehyde. Permeability results also indicated that MTCA is not absorbed locally from the gastrointestinal tract, which reduces the risk of systemic effects. An MTT assay, a widely used cytotoxicity test, demonstrated that neither L-cysteine nor MTCA was toxic to the Caco-2 cells.

A computational model that was developed was able to show how sensitive acetaldehyde is to changes in the amount of L-cysteine as well as in saliva excretion rates. The model can be used as a tool for the prediction of drug amount and the local effect in the mouth of water-soluble compounds, such as L-cysteine.

In conclusion, elimination of acetaldehyde, not only carcinogen, but also agent which possibly increases the addictive potential of tobacco, might help in the fight against smoking and make smoking cessation programs more efficient. L-cysteine, a non-essential amino acid, is able to prevent the harmful effects of acetaldehyde by binding acetaldehyde and forming MTCA. It should be kept in mind that acetaldehyde elimination does not make smoking completely harmless and tobacco smoke contains other carcinogens and

addictives. The best way to protect from tobacco induced diseases is to refrain from smoking. However, besides the fact that most smokers want to quit but most attempts fail and since tobacco smoke contains many carcinogenic compounds, in the future, developed computational models can offer a new view in eliminating or reducing not only one toxic compound from tobacco smoke but also many other compounds using only one formulation containing various active compounds.

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Helsinki, November 2012

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## List of original publications

*This thesis is based on the following publications, which are referred to in the text by their respective roman numerals (I-IV):*

**I**           **Kartal, A.**, Hietala, J., Laakso, I., Kaihovaara, P., Salaspuro, V., Säkkinen, M., Salaspuro, M., Marvola, M., 2007. Formulation and in-vivo evaluation of L-cysteine chewing gums for binding carcinogenic acetaldehyde in the saliva during smoking. *J Pharm Pharmacol* 59: 1353-1358.

**II**           **Kartal, A.**, Björkqvist, M., Lehto, V-P., Juppo, A.M., Marvola, M., Sivén, M., 2008. Compatibility of chewing gum excipients with the amino acid L-cysteine and stability of the active substance in directly compressed chewing gum formulation. *J Pharm Pharmacol* 60: 1131–1138.

**III**           **Kartal, A.**, Marvola, J., Matheka, J., Peltoniemi, M., Sivén, M., 2010. Computational prediction of local drug effect on carcinogenic acetaldehyde in the mouth based on in vitro/in vivo results of freely soluble L-cysteine. *Drug Dev Ind Pharm* 3: 715-723.

**IV**           **Kartal-Hodzic, A.**, Marvola, T., Schmitt, M., Harju, K., Peltoniemi, M., Sivén, M., 2012: Permeability and toxicity characteristics of L-cysteine and 2-methyl-thiazolidine-4-carboxylic acid (MTCA) in Caco-2 cells. *Pharm Dev Technol* (Accepted for publication).

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## Abbreviations

ADI	acceptable daily intake
ADME/T	absorption, distribution, metabolism, excretion and toxicity
ALDH	aldehyde dehydrogenase
CHO	Chinese hamster ovary
DMEM	Dulbecco's Modified Eagle Medium
DSC	differential scanning calorimetry
e.g.	exempli gratia
EU	European Union
FDA	Food and Drug Administration
FT-IR	Fourier transform infrared
GRAS	generally recognised as safe
HBSS	Hanks' balanced salts solution
HCl	hydrochloride acid
HEPES	4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid
HIFBS	heat inactivated fetal bovine serum
HPLC	high pressure liquid chromatography
HPV	human papillomavirus
IARC	International Agency for Research on Cancer
IMC	isothermal microcalorimetry
KH <sub>2</sub> PO <sub>4</sub>	potassium dihydrogen phosphate
LDH	lactate dehydrogenase
MAO	monoamine oxidase
MDCK	Madin Darby canine kidney
MES	2-(N-morpholino)ethanesulfonic acid
MTCA	2-methylthiazolidine-4-carboxylic acid
MTT	3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide
MW	molecular weight
NaCl	sodium hydrochloride
Na <sub>2</sub> EDTA	ethylenediaminetetraacetic acid disodium salt dehydrate
NaHCO <sub>3</sub>	sodium bicarbonate
Na <sub>2</sub> HPO <sub>4</sub>	dinatrium hydrogenphosphate
NEAA	non essential amino acids
NRT	nicotine replacement therapy
PAEM	Prediction of drug Amount and Effect in the Mouth
PAH	polycyclic aromatic hydrocarbons
PBPK	physiologically based pharmacokinetic (model)
pH	measure of the acidity and basicity of a solution
pKa	acid dissociation constant, a measure of the strength of acids and bases in solution
QSPR	quantitative structure-property relationship
RH	relative humidity
SBD-F	ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate

SCC	squamous cell carcinomas
Stella <sup>®</sup>	System Thinking Educational Learning Laboratory with Animation
TBAHS	tetrabutylammonium hydrogensulfate
TPP	target product profile
WHO	World Health Organisation

# 1 Introduction

According to the World Health Organisation (WHO, 2007), there are more than one milliard tobacco smokers in the world. About 80 % of smokers live in low and middle-income countries (Jha, 2009). Tobacco use kills 6 million people each year and by 2030 it will kill more than 8 million people each year (WHO, 2011). The dangers of smoking are well known and despite well documented health consequences of tobacco use, it continues to be a public health issue. Thus, there is an urgent need to inform smokers how to reduce their tobacco caused health risks, to encourage smokers to quit, to emphasise the importance of successful nicotine replacement therapy (NRT) and, most importantly, to inform young non-smokers of why they should not start smoking.

Oral cancer is the seventh most common cancer worldwide and has a poor prognosis. The five year survival rate for people diagnosed with late-stage oral cancer is approximately 16 % (Cawson and Odell, 2002). One of the main risk factors for the develop of oral cancer is tobacco smoking. This can be explained by the high levels of acetaldehyde formed during the burning process that may represent one of the most toxic compounds in tobacco smoke (Smith and Hansch, 2000; Seeman et al., 2002). According to IARC monograph from 2009, acetaldehyde was classified in Group 1, as a carcinogen to humans (Secretal et al., 2009). However, it should be mentioned that still in 2004, acetaldehyde was listed as a possible carcinogen in humans (Group 2B). Besides its carcinogenic properties, some studies have shown that acetaldehyde may increase the addictive potential of nicotine.

A non-essential-amino acid, such as L-cysteine, is able to bind acetaldehyde and form 2-methylthiazolidine-4-carboxylic acid (MTCA) (Sprince et al., 1974). Thus, high acetaldehyde concentrations found in the mouth during smoking can be reduced using L-cysteine containing products (Salaspuro et al., 2006). Taking into account that L-cysteine products are intended to be used by tobacco smokers, a dosage form may be a way to achieve good user compliance. To achieve the best possible user compliance, the design of a proper formulation is increasingly important. For example, compared to lozenges, chewing gum may allow better control of the release rate - since lozenges may be broken by biting, which again can result in rapid L-cysteine release from the lozenge.

In recent years, academic as well as drug development companies have paid more attention to the extremely high costs in the drug development process (DiMasi et al., 2003; Gobmar, et al. 2003; Vernon et al., 2010). One of the big issues is also the time required to get successful medicine to the market: typically a very time-consuming process. More effective methods such as computational methods as well as *in vitro* cell cultures are possible options to reduce costs. Computer modelling might be used in drug discovery and development from the early beginning to evaluate potential compounds for clinical trials (Leahy, 2003). Moreover, the model can be used to select proper drug candidates into early preformulation studies and to obtain answers on drug efficacy and safety in different patient populations. In the preformulation studies, the mean aim is to develop as safe and

efficient a product as possible for further clinical studies. Thus, the main questions are: manufacturing process, compatibility studies between active drug and excipients, factors affecting drug release, as well as benefits for patients such as formulation functionality, efficacy, convenience, and lifestyle.

Taking into account that over one milliard people smoke, and besides the fact that most smokers want to quit but most attempts fail, supportive means to help in fight against smoking are needed.

## 2 Review of the literature

### 2.1 Health effects of tobacco

Tobacco use is one of the main risk factors for a number of chronic diseases, including cancer, lung diseases, and cardiovascular diseases. Smoking is estimated to be the cause of 40–45 % of all cancer deaths, 90–95 % of lung cancer deaths, over 85 % of oral cancer deaths, 75 % of chronic obstructive lung disease deaths, and 35% of cardiovascular disease deaths (Johnson, 2001; Jha, 2009). It is by far the main contributor to lung cancer (Biesalski et al., 1998; Johnson et al., 2000), and it is an important cause of at least 15 different types of cancer in various organs including the oral cavity, oropharynx, esophagus, stomach, colon, liver, pancreas, kidney, urinary bladder, and breast (IARC, 2004).

The dangers of smoking are well known and despite well documented health consequences of tobacco use, it continues to be a public health problem. Efforts are needed to prevent and to reduce cancers caused by smoking and thus prevent one-third of cancer diseases. In the past decades, Western countries have been restricting tobacco advertising, and regulating who can buy and use tobacco products, and where people can smoke. In addition, public health organisations campaign openly against tobacco smoking emphasizing smoking prevention. Although, according to Gilpin et al. (1999) approximately 4800 adolescents (age 11–17 years) and 5500 youths (age 11–20 years) experiment with cigarettes for the first time each day, and up to almost 42 % of them develop smoking addiction. Whilst most smokers from industrialised countries would agree that their habit is dangerous, and the majority would want to quit, they often fail. Even after stopping, most relapse within 12 months, which is attributable to nicotine dependence (Croghan et al., 2003). The reasons for tobacco smoking addiction can be found not only in biochemical addiction but also in social behaviour. Acetaldehyde's role in smoking addiction will be discussed more in section 2.3.

Tobacco smoke contains more than 4000 components, the main ones being nicotine, tar and carbon monoxide. From those 4000 components at least 69 are identified carcinogens in humans and/or animals (IARC, 2004). Carcinogens are divided into three groups according to their carcinogenicity. Of the 69, 11 belong to Group 1 and they are known as human carcinogens, 7 from Group 2A are probably carcinogenic in humans, and 49 from Group 2B of animal carcinogens are possibly also carcinogenic to humans. They belong to various classes of chemicals, as follows: polycyclic aromatic hydrocarbons (PAH) (10 compounds), N-nitrosamines (10), miscellaneous organic compounds (10), and metals and other inorganic compounds (9), heterocyclic amines (7), heterocyclic compounds (6), aromatic amines (4), volatile hydrocarbons (4), nitro compounds (3),

aldehydes (2), and other agents (6). Table 1 lists some of a total of 69 carcinogen agents in tobacco smoke and groups of agents.

It should be noted that levels of the compounds in tobacco smoke are playing an important role in their ability to induce tumours in laboratory animals (Pfeifer et al., 2002). For example, the concentration of acetaldehyde in tobacco smoke is more than 1000 times greater than that of PAHs and N-nitrosamines (Hoffmann et al., 2001). According to IARC monograph from 2004, acetaldehyde was classified as possible carcinogen in humans (Group 2B). In the late 2009, acetaldehyde was classified in Group 1, as a carcinogen to humans (Secretan et al., 2009). This new classification, highlights the importance of reducing carcinogenic acetaldehyde from tobacco smoke.

**Table 1.** *Some of a total 69 carcinogen agents in tobacco smoke and groups of agents (IARC, 2004; Secretan et al., 2009).*

<b>Agent</b>	<b>Group</b>
<b>PAH</b>	
Benzanthracene	2A
Benzopyrene	2A
Dibenzopyrene	2B
5-Methylchrysene	2B
<b>N-nitrosamines</b>	
N-Nitrosopyrrolidine	2B
N-Nitrosopiperidine	2B
<b>Miscellaneous organic compounds</b>	
Vinyl chloride	1
Acetamide	2B
<b>Metals and other inorganic compounds</b>	
Arsenic	1
Cadmium	1
Nickel	1
<b>Heterocyclic compounds</b>	
Furan	2B
Benzofuran	2B
<b>Aromatic amines</b>	
4-Aminobiphenyl	1
2-Toluidine	2B
<b>Volatile hydrocarbons</b>	
Benzene	1
1,3-Butadiene	2B
<b>Aldehydes</b>	
Acetaldehyde	1
<b>Other agents</b>	
Nitrobenzene	2B
2-Nitropropane	2B

Group 1: Human carcinogens

Group 2A: Probably carcinogenic to humans

Group 2B: Possibly carcinogenic to humans



## 2.2 Oral cancer

### Epidemiology

Oral cancer is one of the most common cancers in the world, with approximately 270 000 new cases and 120 500 deaths occurring each year (Parkin et al., 2005). More than 90 % of oral cancers are squamous cell carcinomas (SCC). Carcinomas of the oral cavity include cancers of tongue, tonsils, salivary glands, as well as the lips, the palate, and the cheek. For both genders, oral cancer ranks sixth overall in the world, behind lung, stomach, breast, colon and rectum, and cervix plus corpus uteri (Johnson, 2001). In industrialised countries, men are affected two to three times as often as women, and the reason for that may be attributed to their higher use of alcohol and tobacco. The incidence of oral cancer also shows geographical variation and incidence increase with age in all countries. 98 % of all patients are over the age of 40. The highest incidence rates for oral cancer are found in the South and Southeast Asia, especially in Sri Lanka, India, Pakistan and Taiwan, in Brazil, and in Central Europe ranking France, Hungary, and Czech Republic very high (Johnson, 2001; Nair et al., 2004). In addition, race is named as a risk factor (Silverman, 2001); where African people experience oral cancer twice as often as cases reported in the Caucasian population.

### Aetiology

As mentioned, tobacco smoke is a major risk factor for the development of cancers in the oral cavity and the pharynx (Blot et al., 1988; La Vecchia et al., 1997). It has been estimated that the risk of oral cancer among smokers is 7–10 times higher than for never-smokers (Warnakulasuriya et al., 2005). The risk increases with amount and duration of smoking. In a large population-based case control study, Blot et al. (2008) reported that those who were both heavy alcohol drinkers and heavy smokers have 38 times more risk of oral and pharyngeal cancer than those of non-smokers/non-drinkers. Although the reasons remain unclear, this can be partly explained by the fact that these factors interact synergistically, increasing each other's harmful effects. Findings from Blot et al. (2008) confirmed the results from several studies which have suggested the synergistic effects of smoking and drinking (Choi and Kahyo, 1991; Schlecht et al., 1999; Castellsagué et al., 2004). Based on all of these research results, it has been calculated that 75 % of all oral cancer deaths could be prevented, and risk of dying of this cancer could be reduced by eliminating smoking and excessive drinking (Walker et al., 2003; Ide et al., 2008).

Besides chemical factors, tobacco smoke, and alcohol, there are also some lifestyle and physical factors, as well as biological factors, which can have influence on the aetiology of these types of cancers. For example, there are studies which indicate a diet low in fruits and vegetables could be a risk factor and also exposure to ultraviolet radiation increases the risk of lip cancer (La Vecchia et al., 1997; Freedman et al., 2008). Furthermore, oral human papillomavirus (HPV) infection is also associated with oral cavity risks (Smith et al., 2004; Hansson et al., 2005; Pintos et al., 2008).

## Survival rates

Compared to other cancers, oral carcinoma has a poor prognosis (Franco et al., 1993; Sciubba, 2001; Shiboski et al., 2007). The quality of life in the late stages of the disease is poor. Factors such as delay in treatment, gender, age, ethnic group, tumour size, and therapy may influence oral carcinoma survival rates. Despite advances in surgery, radiation, and chemotherapy, the five-year survival rate for oral cancer has not improved over the past several decades (Silverman, 2001). Diagnosing oral cancer at an early stage significantly increases 5-year survival rates. Survival rates are presented in Table 2.

**Table 2.** *Relative 1-year, 5-year, and 10-year survival rates for male patients with oral cancer (Cawson and Odell, 2002).*

Disease stage	Survival rate (males, %)		
	First years	Five years	10 years
Early stage	90	65	55
Late-stage	45	16	12

## 2.3 Acetaldehyde as a carcinogen and addictive agent

Acetaldehyde (ethanal; CH<sub>3</sub>CHO) is a generally recognised as safe (GRAS) compound for use as a flavouring agent and adjuvant. It is still important chemical of food flavourings and is also added to milk products, fruit juices, desserts, and soft drinks. Considering that acetaldehyde is now classified as a carcinogen in humans (Group I) there is agreement that the GRAS classification of acetaldehyde should be reconsidered.

Acetaldehyde, volatile aldehyde present in tobacco smoke, is known as a carcinogen in animals (Swenberg et al., 1980; Woutersen et al., 1986). When administered by inhalation in rats, acetaldehyde increased the incidence of carcinomas in the nasal mucosa. Administration of acetaldehyde in drinking water to rats caused hyperplastic and hyperproliferating changes in the tongue and the forestomach (Homann et al., 1997). Some results indicate that acetaldehyde may also increase the addictive potential of nicotine, and thus has possible role in smoking addiction (Adriani et al., 2003; Belluzzi et al., 2005).

Theruvathu et al. (2005) showed that mutagenic and carcinogenic changes caused by acetaldehyde can occur at acetaldehyde concentration from 100 µM and above. A concentration of 100 µM can be found in saliva after moderate ethanol consumption (Homann et al., 1997). Some *in vivo* animal studies have also shown that administration of drinking water containing acetaldehyde to rats can cause hyperplastic changes in the tongue and in the stomach. In addition, there is increasing evidence that acetaldehyde, the first metabolite of non-carcinogenic ethanol, may be one important factor to explain the cocarcinogenic effect of alcohol (Boyle et al., 2003; Seitz et al., 2004). Furthermore, normally, after alcohol ingestion acetaldehyde is metabolised rapidly into harmless acetate by the aldehyde dehydrogenase (ALDH) enzyme. Väkeväinen et al. (2000) have shown that Asians with a mutant enzyme (ALDH2) have 2–3 times higher salivary acetaldehyde

levels after a moderate dose of ethanol than those Asians with normal ALDH enzyme. This genetic defect occurs in 30-50 per cent of Asian people. In Asian heavy drinkers, with this genetic inability to remove acetaldehyde, the risk of digestive tract cancers is markedly increased (Yokoyama et al., 1998).

High amounts of acetaldehyde are also found in tobacco smoke. Hoffmann et al. (2001) showed that the amounts of acetaldehyde found in tobacco smoke vary within the range of 400–1400 µg/cigarette.

### **2.3.1 Acetaldehyde from tobacco smoke**

Acetaldehyde is formed during the burning process and may be one of the most toxic compounds in tobacco smoke (Smith and Hansch, 2000; Seeman et al., 2002). The concentration of acetaldehyde in tobacco smoke is more than 1000 times greater than that of PAHs and N-nitrosamines. There is also some evidence that the concentrations of PAHs are too low to explain the carcinogenicity of the tobacco smoke (Pfeifer et al. 2002). Because the acetaldehyde from the smoke is dissolved into saliva, during the period of smoking only one cigarette, salivary acetaldehyde levels increase over 260 µM from the basal level of 0 (Salaspuro and Salaspuro, 2004). Since those acetaldehyde concentrations are clearly above the mutagenic level of 50 µM, acetaldehyde may provide one clear explanation for the increase risks of oral cavity, pharyngeal, and laryngeal cancers among smokers. Furthermore, it may also provide strong evidence for the increased risk of esophageal and stomach cancer among smokers (González et al., 2004). That can partly be explained by the fact that as a part of saliva, acetaldehyde is transported by swallowing to the mucous membranes of the esophagus and stomach, where it can affect as a local carcinogen.

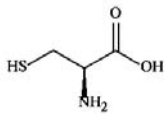
Acetaldehyde has been considered in some studies to be addictive, in addition to the main, and the most well-known addictive component, nicotine (Adriani et al., 2003; Belluzzi et al., 2005). As such, in self administration studies, rats have been shown to consume five times more acetaldehyde/nicotine mixture than nicotine or acetaldehyde alone (Charles et al., 1983; Belluzzi et al., 2005). This effect may be due to harman and salsolinol, condensation products of acetaldehyde, which inhibit monoamine oxidase (MAO). Some MAO-inhibitors are known to increase nicotine self-administration. Interestingly, results also showed that the youngest rodents, compared to adult rodents, reacted significantly more to acetaldehyde/nicotine mixture than to saline or either drug alone. Taken together, these results indicate that acetaldehyde may increase the addictive potential of nicotine and also partly confirmed hypothesis that human youth are more sensitive to tobacco addiction than adults.

## 2.4 L-cysteine as a potential agent for reducing acetaldehyde

### 2.4.1 Physicochemical properties and use of L-cysteine

Cysteine (2-amino-3-sulfanyl-propanoic acid) is a sulfur-containing amino acid. It is a non-essential amino acid, which means that humans can synthesise it in the body (Finkelstein, 1990). Although classified as a non-essential amino acid, in some rare cases, L-cysteine may be also essential, e.g. individuals who suffer from malabsorption syndromes. L-cysteine's chemical properties are listed in Table 3.

**Table 3.** *Physicochemical properties of L-cysteine* (The Merck Index, 1996).

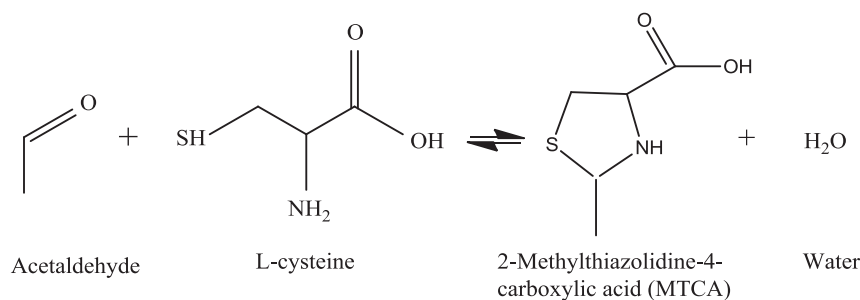
Properties of L-cysteine	
Chemical structure	
Systematic name	2-amino-3-sulfanyl-propanoic acid
Molecular formula	C <sub>3</sub> H <sub>7</sub> NO <sub>2</sub> S
Molar mass	121.16 g/mol
Appearance	White crystals or powder
Melting point	240°C
Solubility in water	Freely soluble in water and ethanol, 280 g/l
pKa	1.71, 8.33, 10.78

L-cysteine can be found from different natural sources, such as red peppers, garlic, onions, broccoli, and oats, with an estimated average intake of about 1 g/day. According to the EU Commission, L-cysteine is considered to be a food additive with an acceptable daily intake (ADI) that is “not specified” (European Commission, 2012). The industrial source of L-cysteine is human hair, as well chicken feathers, and pig bristles. At this moment, 80 % of all industrial L-cysteine is prepared in China, where it is extracted particularly from human hair and chicken feathers. As L-cysteine is used not only in the pharmaceutical industry but also in the food industry, it should be taken into account that some cultures and religions do not allow the consumption of any part of the human or animal body. According to the EU directive 2000/63/EC, for L-cysteine (E-code: E 920), human hair may not be used as a source for this substance in EU countries (European Commission, 2000). Generally, that might be a serious issue and the facts about L-cysteine sources should be seriously considered in formulating L-cysteine containing products.

As mentioned, L-cysteine is normally consumed as a food component and is considered as a safe component (Raguso et al., 2000). However, some studies have shown that administration of L-cysteine might act as a neuronal toxin in sites that lack the blood-brain barrier (Meister, 1989; Janaky et al., 2000). The toxic L-cysteine dose in a 70 kg human is 80 g (Meister, 1989).

## 2.4.2 L-cysteine as acetaldehyde binding agent

In the early 1970s, Sprince et al. (1974) presented a study in which protection against toxic acetaldehyde might be obtained with the thiol compound, L-cysteine. In the reaction, L-cysteine is able to protect from acetaldehyde toxicity by binding acetaldehyde and forming MTCA (Fig. 1). MTCA is suspected to be a non-toxic compound.



**Figure 1.** Chemical reaction between acetaldehyde and L-cysteine forming MTCA (Sprince et al., 1974).

## 2.4.3 Routes of administration and dosage form

In order to get the best possible influence on acetaldehyde, L-cysteine should be administered locally (Salaspuro et al., 2006). As a benefit of local administration, acetaldehyde is directly and immediately bound and the total amount of L-cysteine utilised should be as low as possible. Salaspuro et al. (2002) have shown that with a buccal tablet containing 100 mg of L-cysteine, it is possible to bind and thus to reduce acetaldehyde concentration formed during ethanol oxidation in the saliva (from  $162.3 \pm 34.2 \mu\text{M} \times \text{h}$  to  $54.3 \pm 11 \mu\text{M} \times \text{h}$ ). The same research group also showed that a locally administered 5 mg L-cysteine containing tablet can effectively eliminate acetaldehyde from the oral cavity during 5 minutes of smoking (Salaspuro et al., 2006).

Taking into account that L-cysteine products are intended to be used by tobacco smokers, a dosage form may be a way to achieve good user compliance. To achieve as good user compliance as possible, design of a proper formulation is an increasingly important consideration. For example, compared to lozenges, chewing gum may allow better control of the release rate - since lozenges may be broken by biting, which again can result in rapid L-cysteine release from the lozenge.

## 2.5 Intraoral drug delivery

### 2.5.1 Oral cavity as a route of administration

The main goal of local drug delivery (local effect) to the mouth is to treat conditions of the oral cavity, principally aphthous ulcers, fungal diseases, and dental care. In addition to local effect there is also a possibility of oral transmucosal delivery (systemic effect). In the case of systemic drug delivery, the aim is to achieve drug absorption through the mucosal barrier to reach the systemic circulation. The oral transmucosal route can be divided into two groups: sublingual (under the tongue) and buccal drug delivery systems. Sublingual drug delivery is used for the delivery of the drugs characterised by a high permeability across the mucosa. It is used in the treatment of acute disorders. Buccal drug delivery system is used in chronic disorders when prolonged release of drug and absorption is required. It is also an excellent route for the treatment of local mouth diseases.

Intraoral drug delivery system is associated with many advantages, such as rich blood supply, elimination of hepatic first-pass metabolism, low dose related side effects, ease to use, low metabolic activity for oral delivery of enzymatically labile drugs, and good patient compliance (Scholtz et al., 2003; Sudhakar et al., 2006).

Various types of formulations are used in intraoral drug delivery. Such dosage forms are liquids (*e.g.* sprays, syrups), semisolids (*e.g.* ointment pastes), and solid dosage forms (*e.g.* sublingual tablet, lozenges, chewing gums, patches) (Bukka et al., 2010). Since the experimental part of this thesis is more focused on chewing gum as a dosage form, it will be more discussed in section 2.6.

The main challenge of oral mucosal drug delivery comes from the relatively small absorption area ( $\sim 100 \text{ cm}^2$ ) and from the barrier properties of mucosa and saliva. For example, swallowing and speaking, can reduce drug exposure to impair absorption.

To fulfil therapeutic requirements, formulations designed for intraoral drug administration, should consider possible physiological changes in the oral cavity. The daily secretion of saliva normally ranges between 800 and 1500 ml but physiological and pathological changes can have an effect on saliva production, *e.g.* smell and taste stimulation, chewing, drugs, age, oral hygiene, and physical exercise (Chicharro et al., 1998; Walsh et al., 2004). Even if saliva contains several organic and inorganic components, in general, those levels are very low and the main compound of saliva is water (99.5 %). Normally saliva is colourless with a pH between 6.7 and 7.4, but it can temporarily drop below 2 when sweets, carbonated fruit drinks, and other dietary acids are consumed (Hall et al., 1999; Diaz-Arnold and Marek, 2002; West et al., 2003; Jensdoir et al., 2005). In addition, the temperature, which is normally around 37 °C, can vary between +5 and +55 °C for short times, when eating or drinking cold or hot meals or drinks (Scholz et al., 2008). Some drugs, such as beta blocking agents, nitrates, and diuretics, as well as tobacco smoking can also reduce salivary pH (Birkhed and Heintze, 1989).

## 2.6 Chewing gum as a drug delivery system

### 2.6.1 Use of chewing gum in drug treatment

The arrival of nicotine chewing gum on the market in the 1980s opened a new field and interest in drug research – the use of medicated chewing gum. Chewing gums are an alternative drug delivery system when considering intraoral or per oral administration of drug substances. There are several established medical chewing gums on the market, and the best well-known are the NRT nicotine gums. Examples of medical chewing gums available worldwide, with their indication, are summarised in Table 4. The most significant advantage is the fact that active components of medicated chewing gums can be absorbed through the buccal mucosa and also through the gastrointestinal tract if saliva is swallowed (Christrup et al., 1990a, b). As a result, a systemic effect can be obtained. Chewing gum is also an effective drug-dosage form for local treatment of diseases of the oral cavity and throat (Rassing, 1996). Other advantages and disadvantages are listed in Table 5.

**Table 4.** *Some medical chewing gums available on the market worldwide (Meteti et al., 2012).*

Active substance	Indication	Trade name
Acetylsalicylic acid	Analgesic	Aspergum <sup>®</sup>
Caffeine	Attentiveness	Stay Alert <sup>®</sup>
Dimenhydrinate	Travel sickness	Trawell <sup>®</sup>
Fluoride	Cariostatic	Flourette <sup>®</sup>
Nicotine	Smoking cessation	Nicorette <sup>®</sup> , Nicotinelle <sup>®</sup>
Xylitol	Prevention of dental caries	V6 <sup>®</sup>

**Table 5.** *Summary of advantages and disadvantages of chewing gum as a drug delivery system (Rassing et al., 2003).*

Advantage	Disadvantages
Fast release of active substance	Prolonged chewing on a gum may result in pain in facial muscles and earache in children
Advantageous for patients having difficulty in swallowing	Widely used excipients (e.g. sorbitol and xylitol) can cause diarrhea
Less risk of overdose	Can adhere to teeth denture
Acceptable by most age groups	
Avoids first pass metabolism	

## 2.6.2 Chewing gum preparation

Medical chewing gum consists of a tasteless gum base mixed with an active drug substance, softeners, flavour agents, and sweeteners. Medical chewing gum composition is listed in Table 6.

**Table 6.** *Components used in medical chewing gum (Pedersen and Rassing, 1990; Rowe, 2003; Maggi et al., 2005).*

Component	Function
Drug	Active compound
Elastomers: Synthetic: styrene-butadiene rubber Natural: smoked rubber	To provide the elasticity
Resins (polyvinyl acetate)	To improve texture; reduces the tendency of the gum to stick to the teeth
Waxes (paraffin)	Softening agents
Emulsifiers (PEG 6000, glycerol)	To promote the uptake of saliva
Sweeteners (sorbitol, xylitol)	To improve the taste and also as a non-cariogenic agent
Flavours (citrus, peppermint)	To improve the taste

The traditional gum process is the most frequent method for manufacturing chewing gums. The first step in the traditional production process is melting of a gum base in a mixer to which other excipients, together with active compound, are added to the melted phase (Maggi et al., 2005). The next steps are cooling and rolling into sheets and the final step is cutting the mass into small pieces. The high temperatures (40-50 °C) used during the melting process are considered a big disadvantage of this preparation method.

Recently, researchers have focused more on direct compression methods, which may avoid the high costs associated with traditional chewing gum production (Morjaria et al., 2004; Maggi et al., 2005). Using a direct compression manufacturing process, the gum base, together with other chewing gum ingredients, can be rapidly compacted into a gum tablet using standard tablet press equipment. The low temperature used during the direct compression process, compared to traditional processes, protects thermally unstable active substances.

Drug release from chewing gum is strongly dependent upon individual variability such as chewing frequency and intensity (Gavaskar et al., 2011). The release rate of an active substance is determined not only by the formulation of the chewing gum, but also by the properties of the physicochemical properties of active substance (Jacobsen et al., 2004). Thus, lipophilic substances, which many drug substances are, first adhere into the lipophilic gum base and then released slowly and incompletely. One method to achieve faster lipophilic substance release is increasing the amount of softeners. Thus, softer gum might offer faster drug release (Patel et al., 2011). Pedersen and Rasing (1990) have shown that the use of emulsifiers, such as PEG 6000, increased the release rate of miconazole. In contrast, hydrophilic active substances will show immediate release within a few minutes. Fast released drugs may be swallowed with saliva and thus, cause



unwanted side-effects in the gastrointestinal tract. Therefore, it might be necessary to slow down the release rate by increasing the amount of lipophilic gum base.

## 2.7 Drug development process

In 1878, Rudolf Buchheim wrote in his 1<sup>st</sup> Manual of Pharmacology “*Long before a drug is used to cure an illness it must be our effort to receive the greatest possible amount of knowledge regarding the illness to be treated as well as the drug to be used. Equipped with this knowledge the observation of the effect on the patient must then lead us to the correct use of the drug*”. Until now, that has remained unchanged (Kuhlmann, 1997).

The development of a new therapeutic drug is a very long process and the costs are extremely high not to mention a high risk that it will not succeed. From highly potent candidates, only 8 % reach the market and it can take approximately 10–15 years and over 800 million dollars to bring a drug from an idea to the market (DiMasi et al., 2003; FDA, 2004; Vernon et al., 2010). Traditional drug development, from the idea to the product, may be divided into several steps: compound discovery, preclinical studies, clinical development, approval, and market (Kuhlmann, 1997). To make sure that a drug will be safe to humans, during preclinical development the drug has to be first tested under laboratory circumstances, *in silico*, in cells *in vitro*, and in animals *in vivo*. Therefore, results from the studies are utilised by preformulation experts to determine how to best formulate the drug to the next step, for clinical testing. Clinical trials in healthy volunteers and ill patients are essential for the development of a successful drug therapy (Kuhlmann, 1997). This is due to the evident differences between animals and humans, and for example many diseases in humans do not occur in animals. Until now, a suitable animal model, not to mention *in silico* or *in vitro* cell model, has not yet been found for human diseases. However, animal studies, as well as *in silico* or cell models, can significantly minimise the risk for human patients and improve the drug development process.

Clinical trials are classified into four steps, called phases (phase I-IV). Usually, the drug will be approved for use by national regulatory authorities if the drug successfully passes through Phases I-III. In the past (data from 1991), poor biopharmaceutical properties of compounds of (40 %) have been considered to be the primary reason that many potential drug candidates fail from the clinical studies (Kola and Landis, 2004). However, due to the success rate on drug biopharmaceutical properties during 2000, nowadays, the main reason of drug failure in Phase II and III is considered to be insufficient efficacy of the drug (51% and 66 % correspondingly) (Table 7) (Arrowsmith, 2011a, b; Khanna, 2012).

**Table 7.** The reasons for drugs failure in Phase II and III during the years 2007-2010 (Khanna, 2012).

	Phase II (%)	Phase III (%)
Efficacy	51	66
Financial and/or Commercial	29	7
Safety	19	21
Biopharmacokinetics	1	-
Not disclosed	-	6

Taking into account the costs, time, and need for new medicines, it is clear that there is an urgent need to speed up the drug development process (FDA, 2004; Hunter, 2011). As such the traditional drug development process requires changes and a new way of operating is characterized by multidisciplinary approaches. Translational research builds the bridges from the early beginning between different science fields, pharmaceutical industry, and regulatory authorities. The main idea of translational research is to bring effective research from the “laboratory bench to the hospital bedside” and thus to cut costs and reduce the time required to reach the market. Some typical tools used in translational drug development are pharmacogenomics (personalised medicine), biomarkers, imaging tools, and modelling.

The importance of different drug development tools used the in experimental part of this thesis will be discussed further in the text.

## 2.7 1 Cell cultures as a tool in drug development

### Use of cell cultures

Since *in vivo* experiments in laboratory animals and in humans are expensive and time consuming, *in vitro* methods are needed as part of the drug development process. The use of cell lines in the early stages of drug discovery might reduce cost and time typical of *in vivo* animal and human studies (Allen et al., 2005). Over the last twenty years, the human intestinal cell lines, such as Caco-2, HT29, and T84 have been used as an effective *in vitro* tool for estimation and prediction of human drug absorption (Huet et al., 1987; Dias and Yatscoff, 1994; Artursson et al., 2001; Andersson et al., 2012). Animal cell lines have also been used as *in vitro* models in drug development for intestinal absorption. Such cell lines are MDCK (Madin Darby canine kidney), 2/4/A1 (rat), and CHO (Chinese hamster ovary). Furthermore, human cell lines, such as HepaRG, are used also as *in vitro* tools for understanding drug metabolism and toxicology in humans (Andersson et al., 2012). Recently, tumour-derived human cell lines have been used to evaluate the therapeutic efficacy of cancer medicine candidates (Sharma et al., 2012).

From another point of view, cells cultures are also used as well as a model for drug-drug, drug-excipient, and for drug-food interactions during the absorption process (Rege et al., 2001; Laitinen et.al. 2003; Laitinen et al., 2004). Cell cultures are also widely used as an excellent tool to investigate cellular damage caused by different concentrations of a

drug and thus, to get the first view of possible human toxicity without expensive *in vivo* studies (Fotakis and Timbrell, 2005; Weyermann et al., 2005). The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), the lactate dehydrogenase (LDH), the protein, and the neutral red (NR) assays are the most commonly used tests for the detection of toxicity of a drug. To increase the reliability of the results, it is suggested to use more than one assay.

### **Caco-2 cells**

Caco-2 monolayers have been widely accepted by pharmaceutical companies, and also by regulatory authorities, as a standard *in vitro* model system, together with *in vitro* dissolution studies, to identify drugs with potential absorption problems and possibly to predict permeability and absorption of compounds *in vivo* (Artursson and Borchardt, 1997; FDA, 2000; Artursson et al., 2001; Allen et al., 2005). Originally isolated from a colorectal carcinoma in the 1970s, Caco-2 cell monolayers express morphological and functional characteristics of mature small-intestinal enterocytes (Fogh et al., 1977; Pinto et al., 1983). However, because the tightness of the monolayer resembles more colonic than small intestinal tissue, it is not possible to mimic the exact situation in the small-intestine.

It is well known that the transport of drugs across the intestinal epithelium may occur by one or more of four different routes: the passive transcellular and paracellular routes, the carrier mediated route, and by transcytosis. Comparison of drug transport in Caco-2 monolayers with intestinal drug transport *in vivo* indicates that these monolayers can be used for drug absorption prediction by all four routes (Artursson et al., 1993; Artursson et al., 2001). However, the best correlation to the *in vivo* situation is obtained for drugs that are transported by the passive transcellular route, which is known as the most common drug penetration route (Mandagere et al., 2002). Variations in culture conditions between different laboratories, and also different Caco-2 cell lines, still pose problems, resulting in different permeability results. Therefore, in permeability studies it is necessary to use one or several compounds, with well-known permeability properties as references.

## **2.7.2 Dosage form in preformulation studies**

### **Considerations behind dosage form development**

As mentioned, the research and development of a new drug is an expensive and lengthy process. Therefore, to increase the productivity of drug development and the efficiency of new product it is extremely important to have studies a proper dosage form in early clinical, which, depending on results, can be further developed during the whole clinical process. For pharmaceutical companies, it is obvious that a new product should be well defined before any serious product development. The rush to get a final product from the laboratory to the market might be fateful to pharmacologically effective drug. Thus, to have as ideal a formulation as possible, there are several critical questions for formulation experts. These include the manufacturing process, compatibility studies between active drug and excipients and benefits for patients such as formulation functionality, efficacy,

convenience, and lifestyle. Thus, it is necessary to seriously observe product design from the early beginning of the dosage form development.

It is well known that the physicochemical properties of raw material might a strong high influence on the kind of chosen manufacturing process. One good example of that is, as already mentioned, selection of chewing gum manufacturing process for temperature sensitive compounds. Successful formulation must be such that all components are physically and chemically compatible, including the active compound(s), excipients, and also the packaging materials (Verma and Garg, 2005; Mora et al., 2006). Thus, choice of proper excipients and packaging material for the final formulation is essential in formulation studies. The final formulation must also be well protected from microbial contamination as well from high temperature, light, and moisture. Furthermore, since most drugs have an unpleasant taste, during the whole development process, good acceptability by patients must be kept in mind (Rassing et al., 2003). It is especially important in develop of intraoral drug delivery systems.

Since the experimental part of this thesis is also focused on the compatibility of active ingredient with excipients, it will be discussed further in following sections.

### **Compatibility studies between drug and excipient(s)**

When the active compound is in contact with one or more excipients, and if potential physical and chemical interactions take place between drug and excipient(s), drug stability and bioavailability of the drug can be affected thereby affecting their efficacy and/or safety (Mura et al., 1998; Rahkola et al., 2000; Verma and Garg, 2005; Mora et al., 2006).

Studies of drug-excipient(s) compatibilities are an important process in the development of a stable and proper formulation (Verma and Garg, 2005). Compatibility studies at an early stage helps in the selection of excipients to increase the probability of developing a stable dosage form; this actively improves the characteristics of formulations. It is essential to avoid incompatibilities, since it has been shown that interactions can result in therapeutic response of final product (Rahkola et al., 2000). Despite the fact that excipients can alter the stability and bioavailability of the final product, the general principles of selecting proper excipients are not well-defined.

### **Methods used in compatibility studies**

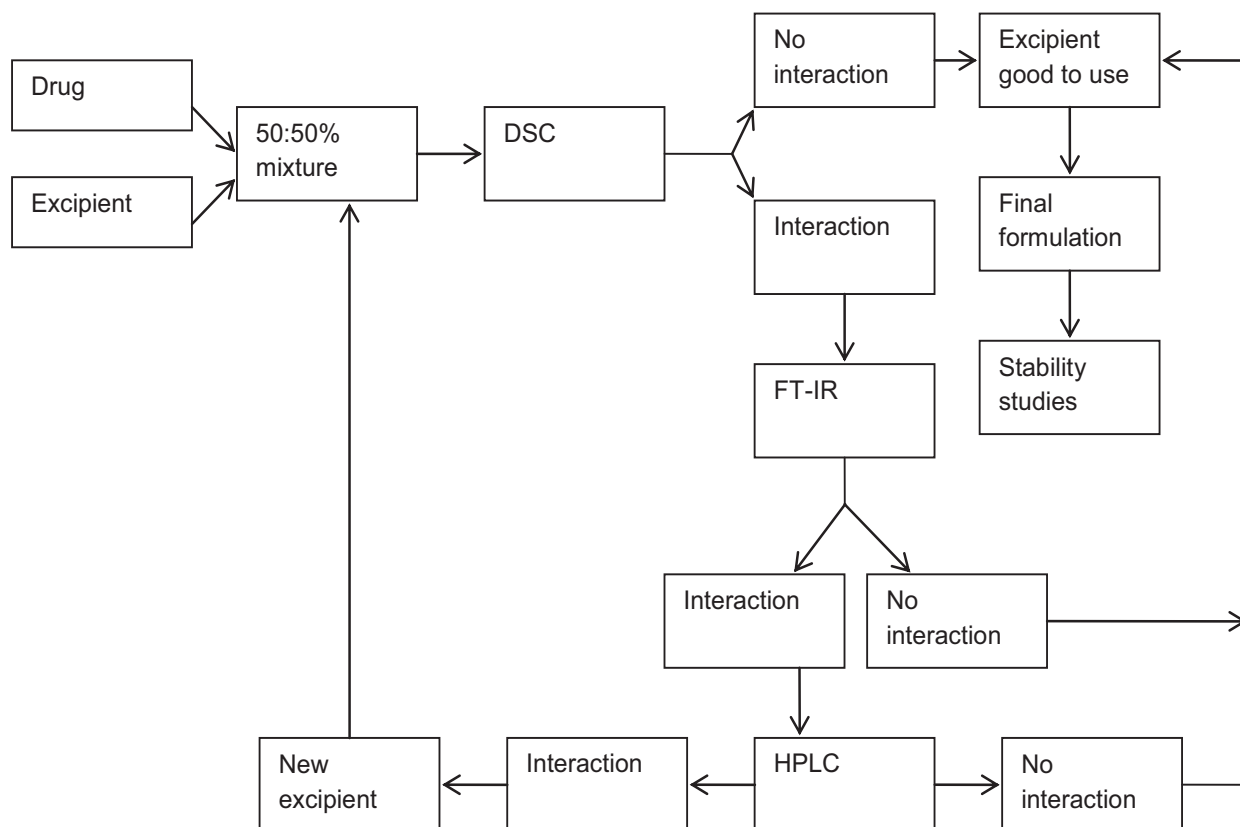
There are many techniques which can be used to indicate interactions between a drug and excipient(s). Due to that, it is extremely important to choose methods for the evaluation of the solid state stability that give fast and correct information about existing incompatibility problems.

Thermal analytical method, differential scanning calorimetry (DSC), which is one of the well-developed techniques, has been used to evaluate compatibility between drug and excipient(s) for over 30 years. The most important advantages of DSC are small sample amounts and rapid measurements. However, interactions observed at high temperature, close to melting point, may not be relevant under the real storage conditions (Rahkola et al., 2000). As a result, understanding of the results is often difficult and the possibility of making incorrect conclusions does exist (Tomassetti et al., 2005). Accordingly, it has

been recommended that one method should be used in combination with another method. Fourier transform infrared (FT-IR) spectra can also offer fast information about interactions of a drug with different excipients. Additionally, standard high pressure liquid chromatography (HPLC), spectrophotometric analysis, as well as dissolution studies have been widely used in compatibility studies (Phipps and Mackin, 2000). Lately, isothermal microcalorimetry (IMC) is shown as a very useful technique. Compared to HPLC it is much faster and more sensitive.

In 50:50 mixture compatibility studies with different methods, it is important to notice the real excipient concentration in the product. For example, magnesium stearate is incompatible with several active substances in 50:50 blends (Kerc et al., 1992; Mora et al., 2006). However, those interactions are not too relevant as magnesium stearate was generally present in low amounts, typically within 0.5–2 % (w/w) range.

A schematic example of preformulation screening of drug-excipient interactions in a 50:50 mixture of drug with the excipient is shown in Fig. 2. In all cases, the basic method is the same – mix together active compound with one or more excipient(s) and monitor any interactions (Schmitt et al., 2001).



**Figure 2.** Schematic example of preformulation screening of drug-excipient interactions in a 50:50 mixture of drug with the excipients (modified from Wells and Aulton, 1998).

### 2.7.3 Computational modelling as a tool in drug development

During the last decade both academics and pharmaceutical companies have paid more and more attention to modelling and simulation as a tool to reduce development cycle time and costs. Computer modelling might be used in drug discovery and development from the early beginning to evaluate potential compounds for clinical trials (Leahy, 2003). The challenge is to have as optimal formulation as possible at the beginning of clinical trials. In addition, the FDA (Food and Drug Administration) has recognised modelling and simulation as a valuable tool in drug development (Gobburu and Marroum, 2001). Computer modelling is an essential part of almost all scientific fields, and is widely used not only in pharmaceutical research, but also in biology, mathematics, physical, and social sciences.

#### Model creating

The first simulation model to predict pharmacokinetics was introduced by Bischoff and Dedrick in 1970, and as mentioned, nowadays predicting oral drug absorption in humans is a major goal. To create as useful a model as possible, it is important to answer the

critical questions: what do we want to know, what data should be obtained (Grass, 1997). Another important task of simulation models is to be able to take variability in physiological factors into account, such as bodyweight, gender, age, race background, food intake, or smoking habits. When necessary and preliminary data is collected, it is possible to start establishing a model (Burman et al., 2005). Usually at the beginning, data is sourced from preclinical data, expert opinions, or combined data from published research papers. Furthermore, developed models can be updated and refined, depending on the results and new data.

### **Use of simulation models**

Since animal drug experiments are not only an expensive tool, but also painful for animals, *in silico* methods, such as computational modelling, are needed to reduce animal studies in the early drug discovery, which is an extremely important issue. Drug discovery can benefit from modelling using fast and simple computational methods in early drug discovery stages to predict the absorption, distribution, metabolism, excretion and toxicity (ADME/T) properties of molecules (Khan, 2010). The model can be used to select proper drug candidates into early preformulation studies and for obtain answers on drug efficacy and safety in different patient populations. Thus, developed models can aid the design of clinical trials, in finding the population that benefits from the drug and furthermore, the running of successful clinical trials. As a result, modelling might facilitate the selection of the right drug candidate and thus reduce the risk of failure in clinical trials.

Physiologically based pharmacokinetic (PBPK) simulation models are widely used in modelling the pharmacokinetics of drugs. Edginton and Willmann (2008) have developed a PKPB model, which can be used to predict drug pharmacokinetics in patients with liver cirrhosis. The model compared physiological differences (blood flow and reduced hepatic function) between healthy individuals and patients with liver cirrhosis. PBPK models are also widely used in paediatric medicine to minimise difficulties in clinical paediatric studies without compromising the well-being of paediatric patients in clinical studies (Bellanti and Pasqua, 2011; Khalil and Læer, 2011).

Quantitative structure-property relationship (QSPR) models have also been developed to predict the ADME/T properties of drug candidates (Zhao et al., 2002; Linnankoski et al., 2006). Linnankoski et al. (2008) showed that intestinal absorption prediction of 178 drugs is possible using computational modelling. As a result, models have shown that prediction of passive intestinal absorption is equal to *in vivo* prediction using Caco-2 and the 2/4/A1 cell lines. The same research has also shown that using simulation models, it was possible to predict the absorption of 65 selected drugs almost equally to absorption studies with rats.

### 3 Aims of the study

Elimination of acetaldehyde, not only carcinogen, but also agent which possibly increases the addictive potential of tobacco, might help in the fight against smoking and make smoking cessation programs more efficient. The ultimate aim of this study was to, through preformulation studies investigate, L-cysteine's ability to eliminate carcinogenic acetaldehyde from saliva with developed chewing gums.

The specific aims were:

1. To develop user-friendly L-cysteine containing chewing gum to reduce tobacco smoking carcinogenic acetaldehyde using two alternative chewing gum manufacturing methods, namely the traditional method and much simpler and cost effective direct compression method. The main three variables were: (1) chemical form of cysteine (L-cysteine or L-cysteine hydrochloride), (2) the amount of active ingredient in the gum and (3) manufacturing procedure (traditional or direct compression method) (I).
2. Since changes in temperature and humidity during chewing gum manufacture and product storage might affect product stability, it was important to determine whether temperature or humidity could affect L-cysteine stability. The aim was also to determine possible interactions between L-cysteine and some excipients which are commonly used in direct compression (II).
3. To analyse the roles of different physiological factors and the effect of formulation parameters on acetaldehyde in tobacco smoke using a computational model. Physiological factors studied were changes in salivary excretion rate. Formulation variables were changes in L-cysteine amount (III).
4. To determine whether L-cysteine or MTCA, the compound formed in reaction with acetaldehyde and L-cysteine, could be absorbed through the Caco-2 cell lines. The study was done to get more information on the ability of the compounds to absorb from the gastrointestinal tract. The aim was also to determine safety of L-cysteine and MTCA to the cells. Since stable MTCA in different parts of the gastrointestinal tract is wanted, the aim was also to determine the stability of MTCA (IV).



## 4 Experimental

### 4.1 Chemicals (I-IV)

The chemicals used in the studies are listed in Table 8.

**Table 8.** *Summary of the chemicals used in the publications I-IV.*

Chemical	Producer/Supplier	Publication
1% NEAA (non essential amino acids)	Euroclone, Pero, Italy	IV
<sup>14</sup> [C]-mannitol	Amersham-Pharmacia Biotech UK Ltd, Amersham, England	IV
Acetaldehyde	Sigma, St. Louis, MO, USA	IV
Ethylenediaminetetraacetic acid disodium salt dehydrate (Na <sub>2</sub> EDTA)		III, IV
HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)		IV
MES (2-(N-morpholino)ethanesulfonic acid)		IV
MTT (3-[4,5]-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium)		IV
Tetrabutylammonium hydrogensulfate 97% (TBAHS)		IV
10% trichloroacetic acid		III, IV
Tri-n-butylphosphine		III, IV
Acetonitrile	Rathburn, Walkenburgh, Scotland	IV
Ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBD-F)	Fluka Chemicals, Buchs, Switzerland	III, IV
Boric acid		III, IV
Dinatrium hydrogenphosphate (Na <sub>2</sub> HPO <sub>4</sub> )		IV
L-cysteine		I-IV
Black currant flavoring	Quest International, Naarden, the Netherlands	III
Calcium dichloride (CaCl <sub>2</sub> )	Sigma- Aldrich, Seelze, Germany	III
37% hydrochloride acid (HCl)		III
Potassium dihydrogen phosphate (KH <sub>2</sub> PO <sub>4</sub> )		III
Sodium bicarbonate (NaHCO <sub>3</sub> )		III

**Table 8.** (Continued).

Sodium hydrochloride (NaCl)		III
Sodium hydroxide (NaOH)		III, IV
DMEM (Dulbecco's Modified Eagle Medium)	Gibco Invitrogen Corporation, Carlsbad, CA, USA	IV
1 % L-glutamine		IV
HIFBS (Heat inactivated fetal bovine serum)		IV
HBSS 10x concentrate (Hanks' balanced salts solution)		IV
Penicillin (100 IU/ml)		IV
Streptomycin (100 µg/ml)		IV
0.25 % trypsin, Every T Toco	Gum Base Company S.p.A., Italy	I, II
Smily 2 Toco		I, II
Ferric sulfate	J. T. Baker, Netherlands	I,II,III
Ferrozine	Sigma-Aldrich Chemie GmbH, USA	I,II,III
L-cysteine hydrochloride	Quimicos, Gonmisol S.A., Barcelona, Spain	I,II
Lemon flavour	Quest International, Netherlands	I,II
Magnesium stearate	Merck, Darmstadt, Germany	I, II, III
Mannitol		III, IV
Orthophosphoric acid		III
Potassium chloride (KCl)		III, IV
Methanol	Rathburn, Walkenburg, Scotland	III, IV
N,N-Dimethylformamide	Sigma-Aldrich, Steinheim, Germany	III, IV
Pharmagum S	SPI Pharma, New Castle, USA	I,II
Sodium perchlorate	Sigma-Aldrich Chemie GmbH, USA	I, II,III
Sorbitol	Roquette, Lestrem Cedex	II
Xylitol	France	I, II

## 4.2 Methods

### 4.2.1 Study formulations

#### Direct compression method (I, II, III)

Two different chewing gum formulations were prepared. One formulation (A) contained 7.7 mg of L-cysteine and the other (B) 10.0 mg of L-cysteine hydrochloride, corresponding to 7.7 mg of L-cysteine. Pharmagum S was used as a gum base and lemon flavour was used as a flavouring agent to disguise the unappealing taste of L-cysteine. All

components of these two formulations, except for magnesium stearate, were mixed for 20 min in a Turbula shaker mixer (T2C Willy A. Bachofen A6 Maschinenfabrik, Switzerland). Magnesium stearate was added to the formulations at the end of the mixing and then it was mixed for two more minutes. The total weight of chewing gum was 1080 mg. Chewing gums were compressed with an instrumented eccentric tablet machine (Korsch EK-0, Erweka Apparatebau, Germany) using flat faced punches with a diameter of 13 mm. The applied compression force was 7–8 kN.

Tablets used in publication III containing 5 mg of L-cysteine were prepared as above. Mannitol (725 mg) was used as a filler and black currant (20 mg) as a flavouring agent.

#### **Traditional method (I)**

Three formulations were prepared using the traditional gum process by Fennobon Oy, Karkkila, Finland. The first formulation contained 10 mg of L-cysteine hydrochloride (equivalent to 7.7 mg L-cysteine) and the second one contained 6.5 mg L-cysteine hydrochloride (equivalent to 5.0 mg of L-cysteine). The third chewing gum formulation was a placebo containing no L-cysteine. Each chewing gum contained gum base and sweeteners such as xylitol and sorbitol as the major ingredient components. The other excipients present in small amounts in formulations were: flavours, thickener, humectant, emulsifier, artificial sweeteners, food colour, and glazing agent. The total weight of chewing gum was 1080 mg. In preparation, the gum base of chewing gums was heated at a temperature between 40 and 50 °C for melting. After that, L-cysteine hydrochloride was added along with the other components. After mixing, the homogenous chewing gum mixture was cooled, cut into squares, and hardened at room temperature. The pieces were then coated with xylitol in a coating drum, and finally polished. All formulations were shaped to be similar in weight, colour, and size.

#### **4.2.2 Solid-state stability (II, IV)**

Active compounds, in addition to a 1:1 (w/w) physical mixture of active compound and excipients ( $n = 6$ ), were prepared by gently mixing the components with a spatula, and stored in stability test chambers (KBF 115, Binder GmbH, Germany) as follows: in tightly closed and open amber glass vials at 25 °C/60 % RH (relative humidity) and in tightly closed and open amber glass vials at 45 °C/75 % RH. The compatibility of L-cysteine with xylitol, sorbitol and two gum bases, Every T Toco and Smily 2 Toco, were also studied. The remaining amounts of L-cysteine were analysed spectrophotometrically immediately after mixing and then at regular intervals (1, 2 and 3 weeks and 1, 2 and 3 months) by the method described in Eid (1998).

Solid-state stability studies have also been done for pure MTCA at the same temperature and humidity conditions as mention above. The remaining MTCA was analysed by HPLC method immediately and after 1, 2, 3, 4, 8, and 12 weeks.

## **FT-IR (II)**

The L-cysteine and 1:1 (w/w) physical mixture of active compound and excipients was prepared by gently mixing the components with a spatula, and was stored in stability test chambers at 25 °C/60 % RH for over 10 days. Pellets of samples were scanned over a wavenumber range of 4000 cm<sup>-1</sup> to 550 cm<sup>-1</sup>. Differential spectra were evaluated and interactions were determined by comparing differential spectra with that of pure L-cysteine as either a free base or a salt.

## **IMC (II)**

A 2277 Thermal Activity Monitor (TAM) (ThermoMetric AB, Sweden) microcalorimeter was used. The measurements were carried out at 25 °C and 45 °C. At both temperatures, measurements were carried out without an extra moisture source and with a controlled relative humidity atmosphere. The relative humidity inside the sample ampoule was controlled with a saturated salt solution in a miniaturised ampoule. At 25 °C, a saturated salt solution of NaBr was used to maintain about 60 % RH, and at 45 °C, NaCl was used to maintain about 75 % RH. Before measuring started under the regulated humidity atmospheres (25 °C/60 % RH and 45 °C/75 % RH), pure components were stored under measuring conditions for at least 10 days. In each case, the samples of pure L-cysteine (as a free base or salt) and excipients, in addition to the binary mixtures, were prepared and measured in duplicate. Samples were weighed into glass ampoules and sealed with teflon-coated discs of rubber and aluminium caps. An empty sealed glass ampoule was used as a reference. The ampoules were inserted into the pre-equilibrium state of the calorimeter (referred as t=0 s) for 20 min, after which they were lowered into the final measurement position. The electrical calibration was performed each time after the temperature was changed. Data were collected using the dedicated Digitam (ThermoMetric AB, Sweden) software.

### **4.2.3 Solution-state stability of MTCA (IV)**

The stability of the MTCA in the buffer solutions used in permeability studies, HBSS buffer containing 10 mM HEPES (pH 7.4) and HBSS containing MES (pH 5.5), was measured with an HPLC method as described below. Samples were taken immediately and at 15, 30, 45, 60, 90 min, and at 2, 3, 4, 5, 6, and 24 h and analysed immediately.

### **4.2.4 L-cysteine stability in chewing gum (II)**

Chewing gums were stored in stability test chambers (over a period of three months in the same conditions as mentioned in 4.2.2 paragraph (n = 6). The remaining amount of L-cysteine was analysed spectrophotometrically as described in Eid (1998) immediately after chewing gum preparation and then at regular intervals (1, 2 and 3 weeks, and 1, 2 and 3 months).

#### **Drug compressed on a hydraulic press (II)**

A L-cysteine sample of 250 mg ( $n = 4$ ) was weighed and compressed on a hydraulic press (Carver model C laboratory press; Menomonee, WI) at 185 MPa to study the influence of compaction on the pure L-cysteine stability. Uncompressed powder was used as a control. The remaining amounts of L-cysteine were immediately analysed spectrophotometrically as mentioned above.

#### **4.2.5 Dissolution studies (I, IV)**

Dissolution tests of pure L-cysteine were carried out with 20 mg of L-cysteine powder, using the basket method described in USP 24 in 500 ml of distilled water and also in artificial saliva, pH 7.4 and 4.7, at  $37 \pm 0.5$  °C. The artificial saliva was prepared as described by Duffó (2004): 4.201 g NaCl, 0.151 g KCl, 0.149 g CaCl<sub>2</sub> and 0.104 g NaHCO<sub>3</sub> were dissolved in pure water (500 ml), pH adjusted with HCl. The speed of rotation was 50 rpm. The drug concentrations were determined by spectrophotometry as described in Eid (1998). Dissolution test was statistically analysed with Microsoft Excel 2002 using two-way ANOVA.  $P < 0.05$  was considered statistically significant.

#### **4.2.6 Cell culture studies (IV)**

##### **Cell cultures (IV)**

Caco-2 (wild type) cells obtained from the American Type Culture Collection (Rockville, MD, USA), were maintained at +37 °C in an atmosphere containing 5 % CO<sub>2</sub> at 95 % RH. The cells were grown in a medium containing DMEM, 10% HIFBS, 1% NEAA, 1% L-glutamine, penicillin (100 IU/ml), and streptomycin (100 µg/ml). The medium was changed three times per week. The cells were harvested weekly from plastic flasks (75 cm<sup>2</sup>) with 0.25 % trypsin. The cells were seeded at  $6.8 \times 10^4$  cells/cm<sup>2</sup> in 12-well Transwell<sup>®</sup> insert plates.

##### **MTT toxicity test (IV)**

Caco-2 cell viability was determined by MTT assay after test compound treatment (90 min), to measure the mitochondrial activity of the cells. The tested concentrations of both compounds were: 75, 150, 300, 600, and 1200 µg/ml. The cells were exposed to L-cysteine and MTCA for 3 h at 37 °C. The intensity of the developed color was measured at a wavelength of 590 nm, using a Varioskan Flash spectral scanning multimode reader (Thermo Electron Corporation, Vantaa, Finland).

#### **Permeability studies (IV)**

Transport of L-cysteine and MTCA across the Caco-2 monolayers was studied both in the apical to basolateral and basolateral to apical directions at pH 7.4 and 5.5. The cell monolayers were first washed twice with HBSS buffer containing 10 mM HEPES (pH 7.4) or MES (pH 5.5) and then equilibrated for 30 min in the washing buffer solution. The apical solution was then changed to HBSS buffer containing the test compound (L-cysteine or MTCA for their respective permeability studies). The used concentrations for the test compounds were 150, 300, and 600 µg/ml. The samples were taken from the receiver compartment at 15, 30, 45, 60, 90, and 120 min and replaced immediately with the buffer. The concentrations of L-cysteine and MTCA were analysed, using the HPLC method as described below.

#### **4.2.7 Analytical methods**

##### **Determination of acetaldehyde by gas chromatography (I)**

Acetaldehyde levels were measured by headspace gas chromatograph (Perkin Elmer, Norwalk, CT, USA) as described by Salaspuro et al. (2006) with slight modifications. A sample of saliva (500 µL) was immediately transferred into a headspace vial and stored at 5–8 °C for less than 1 h before assay. Gas chromatography conditions were as follows: column 60/80 Carbowax B/5% Carbowax 20 M, 2 m x 3 mm (Supelco, Inc., Bellefonte, PA, USA), oven temperature 37 °C, transfer line and detector temperature 150 °C. Each sample was made in duplicate.

##### **Determination of L-cysteine and MTCA by HPLC (III, IV)**

L-cysteine was determined by the methods described by Zappacosta et al. (1999) and Frick et al. (2003). For MTCA, the method was slightly modified from that described in Alary et al. (1989).

The samples were analysed with a Waters HPLC apparatus (Waters Millennium, USA). The samples were done in triplicate. L-cysteine with a Waters 486 fluorescence detector, 717 autosampler, and the 510 pump, MTCA with a Waters 484 tunable absorbance detector at 210 nm. The mobile phase for L-cysteine was: 0.1 M KH<sub>2</sub>PO<sub>4</sub> with 5% methanol, adjusted to pH 2.7 with 85 % orthophosphoric acid. The mobile phase for MTCA was acetonitrile with Na<sub>2</sub>HPO<sub>4</sub>-TBAHS solution (5:95 v/v), adjusted to pH 6.5. A SunFire™ C-18 column was used (150 mm x 4.6 mm; Waters, Ireland) with flow rate of 1.5 ml/min for L-cysteine and 1 ml/min for MTCA. The injection volume for L-cysteine was 10 µl and total analysis time 7 min (retention time ~ 4 min). For MTCA, the injection volume was 5 µl and total analysis time 10 min (retention time ~ 1 min). For L-cysteine the fluorescence intensities were measured with excitation at 385 nm and emission at 515 nm. In both cases, the standard curves were linear over the concentration range used (50-600 µg/ml).

#### **4.2.8 *In vivo* studies (I, III)**

##### **Effect of L-cysteine chewing gums on salivary acetaldehyde (I)**

Six volunteers took part in the studies: five active smokers (> 10 cigarettes per day) and one habitual smoker (< 10 cigarettes per week) (4 men, 2 women; mean age  $32 \pm 12$  years). The study was approved by the Coordinating Ethics Committee, Hospital District of Helsinki and Uusimaa (Finland).

##### **Effect of saliva pH on L-cysteine in saliva (III)**

Six volunteers took part in the studies (three males and three females, mean age  $31 \pm 8$  years). The volunteers sucked 4 mg of pure L-cysteine or a tablet containing 4 mg of L-cysteine for 5 min. Saliva was collected continuously for 2.5 min before the test, during the 5 min of the test and for 5 min after the test. Saliva for each 2.5 min interval was collected into separate collection tubes, yielding five samples altogether. All volunteers had normal, healthy saliva pH (mean pH  $7.4 \pm 0.3$ ). To lower the saliva pH to a mean of  $4.7 \pm 0.4$ , the volunteers sucked a small amount of citric acid for 1 min before the test started.

##### **The chew-out study (I)**

The *in vivo* release of L-cysteine from chewing gums was studied by chew-out studies. Each volunteer chewed one piece of each chewing gum formulation at a rate of 12–15 and 30–35 chews in 1 min. At the beginning of chewing, the gum crumbles into small fragments, which tongue movements bring together to form a gum. Gum that had been chewed for 1 min was frozen overnight at  $-40$  °C and then ground in a grinder to obtain a fine powder. The concentration of L-cysteine was determined as described in Eid (1998).

##### **Effect of L-cysteine chewing gums on salivary acetaldehyde (I)**

The effect of chewing gums containing L-cysteine as free base or as hydrochloride salt was tested in five active and one habitual smoker (4 males and 2 females; mean age  $32 \pm 12$  years). In each test, one cigarette was smoked in five minutes. During the first smoking period, volunteers chewed a placebo chewing gum. For the remaining four smoking periods, each volunteer chewed one piece of each kind of four chewing gum formulations at 12-15 chews/min for five minutes. After five minutes smoking was stopped and chewing gum spat out. Saliva samples were collected as mentioned above. All volunteers were instructed not to swallow any saliva. All subjects refrained from drinking, eating, or smoking half an hour before saliva collection. The results of *in vivo* studies were analysed by two-way analysis of variance using Microsoft Excel.  $P < 0.05$  was considered as statistically significant.

#### 4.2.9 PAEM (Prediction of drug Amount and Effect in the Mouth) model (III)

The computational model presented here was built using Stella<sup>®</sup> modeling software (Stella v9.0.2, isee systems, Inc.). The model was based on results described in publication III as well as previous results from our research group described in Salaspuro et al. 2006. In *in vivo* studies, salivary acetaldehyde increased rapidly during smoking to  $228 \pm 115 \mu\text{M}$  from the basal level (0) and declined rapidly after 5 minutes of smoking. The use of a L-cysteine containing tablet during the 5 minutes of smoking binds and thus eliminate acetaldehyde. Acetaldehyde production in the saliva during smoking was modelled as a zero-order process of 5 min duration. Release of L-cysteine from the formulation to the saliva was modelled to mimic *in vitro* data. The reaction kinetics of acetaldehyde binding and swallowing of the saliva was also mechanistically modelled. A simulation model for the prediction of drug amount and effect on carcinogenic acetaldehyde in the mouth was named PAEM.



## 5 Results and discussion

### 5.1 *In vivo* characteristics of L-cysteine

#### 5.1.1 Effect of saliva pH on L-cysteine (III)

The *in vivo* results, where it was shown that saliva pH changes did not affect the remaining amount of L-cysteine, were in good accordance with the *in vitro* dissolution studies (III, Fig. 2, 3). As a result, it is possible to conclude that the stability of L-cysteine is not pH dependent and is stable in saliva. This is an important result, since it is well known that some diseases, consumption of acidic drinks and tobacco smoking can reduce saliva pH (Parvinen, 1984). Thus, the L-cysteine product developed is effective for not only users with normal, healthy saliva pH but also for those with lower saliva pH.

#### 5.1.2 The chew-out study (I)

The results of the *in vivo* chew-out study indicated, that the active ingredient both as hydrochloride salt and also as base form, releases fairly quickly taking into account that smoking a cigarette takes about 5 minutes (I, Fig. 2). These findings are understandable, because the physicochemical properties of an active ingredient plays an important role in the release of drug from chewing gum formulations (Shrinivares et al., 2005). This might be explained on the basis that L-cysteine, as water soluble ingredient, released within few minutes, but lipid soluble drugs for example, are released first into the gum and then released slowly into the mouth.

Another important finding from this study was that release of L-cysteine from traditionally prepared chewing gum was about one-third slower than that of the directly compressed chewing gum ( $P < 0.001$ ) (I, Fig 2). This result was expected since directly compressed gum first broke down into smaller fragments, which increase surface area and cause a rapid initial release of the active ingredient (“crumbing effect”). To achieve better user compliance, this characteristic should be avoided. This study also indicated that a lower chewing frequency ensures more prolonged release for the L-cysteine from the directly compressed formulation (I, Fig 2). In addition, chewing frequency did not have an effect on drug release from traditionally prepared formulation. Since *in vivo* chew-out studies include disadvantages, such as lack of chewing and variations in the flow and compositions of volunteer’s saliva (Runwal, 2008), to increase the reliability of the results it would also be essential to examine if there is a correlation in release rate between the *in vivo* chew-out results and the *in vitro* dissolution study.

In general, it might be concluded that formulation such as chewing gum might be a proper drug delivery system for fast onset due to the rapid release of L-cysteine in the oral cavity. Compared to traditional manufacturing processes, the directly compressed chewing gum method is much simpler and cheaper than that of the traditional chewing gum.

### 5.1.3 Effect of L-cysteine on salivary acetaldehyde (I)

From the results in Fig. 3 (I), it is evident that chewing a placebo gum in itself does not have an effect on saliva acetaldehyde levels during smoking. All tested L-cysteine containing chewing gum formulations reduced salivary acetaldehyde levels during five minutes of smoking to below the *in vitro* mutagenic level (50  $\mu$ M). However, measurable acetaldehyde levels in saliva samples collected between 2.5 and 5 minutes were found with two formulations: directly compressed formulations containing 10 mg of L-cysteine hydrochloride, and traditional chewing gum containing 6.5 mg of L-cysteine hydrochloride. An explanation for that might again be found in the manufacturing process as well as the physicochemical properties of the active ingredient. It is also evident from the results that after five minutes of smoking, low saliva acetaldehyde levels were found in all study experiments. Since acetaldehyde levels are high only during smoking (for about 5 min) and L-cysteine effect is needed during that time, it is important to conclude that it is a wanted effect. However, it should be noted that this kind of formulation is not ideal for longer smoking periods, *e.g.* during cigar smoking (Salaspuro et al., 2006). As it was mentioned in the literature part of this thesis, the toxic dose of L-cysteine in a 70 kg human is 80 g. That is much higher than the maximum dose suggested by these *in vivo* studies.

The *in vivo* studies of Salaspuro et al. (2006) also showed that tobacco smoke carcinogenic acetaldehyde could be totally eliminated in the saliva during five minutes smoking by sucking a tablet containing 5 mg of L-cysteine. However, the disadvantages of sucking a tablet (lozenge), *e.g.*, dry mouth, when lozenges can stick to the oral mucosa and/or tongue and might also, in some cases cause local irritation by sucking, that might impact user acceptability (Sreebny et al., 1992; Codd and Deasy, 1998). If so, and because L-cysteine containing product is purposed to use during every tobacco smoking, L-cysteine chewing gum might be more acceptable to users.

As already mentioned, acetaldehyde is classified by IARC in Group 1, as a carcinogen to humans, but it is also an agent which possibly increases the addictive potential of tobacco. Thus, reducing carcinogenic acetaldehyde from tobacco smoke is needed. In addition, the fact that over one milliard people smoke, and that most smokers want to quit but most attempts fail (Croghan et al., 2003), make this importance of great worth. In conclusion, elimination of tobacco smoke carcinogenic acetaldehyde by L-cysteine containing chewing gum can open a new method, not only to lower the risk of development of upper gastrointestinal track cancers, but it may also play a significant role in fighting against tobacco addiction. It should also be remembered that acetaldehyde might be swallowed with saliva and carried further to the oesophagus and stomach, where it can induce cancer. Still, it should be kept in mind that acetaldehyde elimination does not make smoking completely harmless and tobacco smoke contains other carcinogens and

addictives. In the future, more clinical trials are needed to support the thesis findings of L-cysteine reducing effect on carcinogenic acetaldehyde.

## **5.2 *In vitro* characteristics of L-cysteine**

### **5.2.1 MTT cytotoxicity (IV)**

*In vitro* MTT-cytotoxicity of L-cysteine was examined prior to permeability studies. MTT-test demonstrated that L-cysteine, as was expected for an amino acid, was not toxic to the Caco-2 cells (IV, Fig. 5). The results showed *in vitro* that the formulation used to bind acetaldehyde might possibly also be safe *in vivo*.

### **5.2.2 Caco-2 permeability studies (IV)**

Caco-2 cells were used to evaluate the permeability of L-cysteine at concentrations 150, 300, and 600 µg/ml at pH 7.4 and 5.5 (IV). The permeability studies showed that the L-cysteine concentrations in the receiver chamber were lower than the quantitation limit (50 µg/ml) of the HPLC method used. Thus it can be concluded that, L-cysteine was not absorbed at pH 7.4 as well as 5.5 and at all tested concentrations through the Caco-2 monolayer. These *in vitro* results indicate that when administered locally to the mouth, stomach, or colon, where no LAT2 (which is known to be responsible for the absorption of L-cysteine in the small intestine; del Amo et al., 2008) is present, only a minimal amount of L-cysteine is absorbed.

According to the permeability studies, it is possible to conclude that the L-cysteine dissolved from products for local use in the mouth will probably leave the site of administration only by binding to acetaldehyde, or if L-cysteine is being swallowed with saliva, it may also be considered as a dietary amino acid in the gastrointestinal tract.

### **5.2.3 L-cysteine stability (II)**

#### **Pure L-cysteine (II)**

Under tested circumstances it was found that pure L-cysteine, as free base or as a salt, was stable. Compared to the initial amount, there were no significant differences in the remaining L-cysteine amount after three months (II, Table 1).

Since this thesis is partly focused on the chewing gum manufacturing process the compression effect on pure L-cysteine was also studied (II). Compared with the remaining amounts of L-cysteine before and after compaction on a hydraulic press, it is possible to make a conclusion that compaction affected the remaining amounts of L-cysteine

(Table 8). One explanation for this phenomenon might be found in that at elevated temperatures temperature-sensitive thiol, such as L-cysteine, is oxidised to cystine (Tan et al., 2003).

Thus, to minimize unwanted active compound degradation in manufacturing planning processes should include not only the stability of the drug in powder mixtures with excipients, but also the effect of the manufacturing process (*e.g.* compression). For such temperature sensitive compounds unwanted degradation during the manufacturing process might be avoided by choosing a proper manufacture process, *e.g.* cooling process before compaction (Athanikar and Gubler, 1999).

**Table 8.** *Remaining amount of L-cysteine (%) before and after compaction on a hydraulic press of 185 MPa (n=4).*

	Remaining amount (%)	
	L-cysteine free base	L-cysteine hydrochloride
Uncompressed powder	100±0.6	100±0.8
Compressed powder	88.5±3.8	89.8±4.7

#### **L-cysteine in 1:1 mixture with excipients (II)**

Some of the results from the compatibility of chewing gum excipients with L-cysteine at a 1:1 (w/w) ratio are showed in Table 9. Xylitol and sorbitol were chosen because of their wide use in chewing gum products, and Pharmagum S and magnesium stearate are the mean excipients of the directly compressed chewing gum used in those studies. The 1:1 (w/w) ratio was chosen to maximise the likelihood of observing any interaction (Mora et al., 2006).

The incompatibility results, using either a solid-stage method, FT-IR or IMC, indicated that L-cysteine as a free base is more stable with different excipients than its salt form. In both cases, L-cysteine was incompatible with magnesium stearate. According to the literature magnesium stearate is incompatible with several active substances in 50:50 blends (Kerc et al., 1992; Mora et al., 2006). Since magnesium stearate is generally present in low amounts (0.5–2 % (w/w)), those interactions are not too relevant.

L-cysteine as a salt was incompatible with all used excipients and results with all used methods were in agreement (II, Table 1; Table 2). In addition, free base form with different excipients was sensitive to temperature and humidity, and as a result, the remaining amount of L-cysteine decreased (II, Table 1; Table 2). An explanation for that might be found in the hygroscopic characteristics of excipients (Airaksinen et al., 2005). If so, when they are exposed to humidity, the absorbed moisture works as a source of water solvent for L-cysteine. As a result, L-cysteine could have been dissolved and degraded.

The compatibility studies were useful in determining the properties of developed chewing gum formulations and aided further development of the gum. Incompatibilities between drug and excipient(s) can affect the drug stability and bioavailability of the drug, and therefore, affect their efficacy and/or safety (Mura et al., 1998; Rahkola et al., 2000;

Verma and Garg, 2005; Mora et al., 2006). According to the results, the final product must be well protected from temperature and humidity during its shelf-life. Since the purpose of the product is to be used during tobacco smoking, that might impact user's acceptability.

**Table 9.** *Interaction occurrence in binary mixture 1:1 (w/w) of L-cysteine two forms with excipients determined with three different methods: solid-stage method, FT-IR and IMC at 25 °C/60 % RH closed/opened vials.*

Mixture components	3 months solid-stage at: 25 °C/60 % RH closed/opened vials	FT-IR	IMC at: 25 °C/60 % RH closed/opened vials
<b><u>L-cysteine:</u></b>			
Xylitol	No	Yes	No
Sorbitol	No	Yes	No
Pharmagum S	No	No	No
Magnesium stearate	No	Yes	No
<b><u>L-cysteine hydrochloride:</u></b>			
Xylitol	Yes	Yes	No
Sorbitol	Yes	Yes	Yes/Not measured
Pharmagum S	Yes	Yes	Yes/Not measured
Magnesium stearate	Yes	Yes	Yes/Not measured

#### **L-cysteine stability in chewing gum (II)**

In both chewing gum formulations, containing L-cysteine as a free base or as a salt, the remaining amount of L-cysteine at initial were found to be less than 90 % of that originally added . As mentioned, this might be explained on the basis that temperature can increase during compression and therefore tableting can have an effect on temperature-sensitive compounds (Picker-Freyer and Schmidt, 2004; Turner et al., 2006). This finding possibly explains why L-cysteine alone, or as a constituent of mixtures with different excipients, was more stable than the L-cysteine in gums immediately after gum preparation. If so, as already mentioned, manufacturing process might have a big role in L-cysteine stability.

Otherwise, at normal temperature and humidity after three months storage, L-cysteine salt-containing formulation was less stable than formulation containing base form. High temperature and humidity circumstances (75 % relative humidity and 40 °C) effected both formulations, and as a result the remaining amount of L-cysteine after three months was zero. Again, the study results make it obvious that high temperature and humidity are important factors for L-cysteine stability in the final chewing gum formulation. Beside those factors, incompatibility between L-cysteine and excipients should also be kept in mind. According to the results, it is easy to conclude that for temperature sensitive compounds, traditional gum preparation should be avoided because during the manufacturing process, the gum base, together with all ingredients, is heated to high temperature (40-50 °C). As mentioned, the low temperature used during the direct

compression process, compared with traditional processes, protects thermally unstable active substances.

In general, it was concluded from *in vitro* stability studies that the formulated chewing gum in this study containing L-cysteine should be properly closed during its shelf-life and stored under lower temperature and humidity conditions. In addition, for more stable product, the preformulation studies should include analysis of the effect of the manufacturing method.

#### **5.2.4 L-cysteine dissolution properties (I, III)**

*In vitro* dissolution tests in distilled water were carried out first with a pure L-cysteine as a free base and as a hydrochloride form to investigate which L-cysteine chemical form will be a better candidate for chewing gum formulations (I, Fig.1). The first overall finding is that the active substance, both as hydrochloride salt and also as base form, dissolves fairly quickly. However, the statistical difference between the two L-cysteine forms was highly significant ( $P < 0.001$ ) and as a result L-cysteine as a base would be a better candidate for chewing gum compared to the more water-soluble hydrochloride salt form. Changing dissolution fluid from distilled water to artificial saliva with different pH, 7.4 to mimic normal saliva and 4.7 to mimic lower saliva pH, did not show changes in dissolution rates (III, Fig.2).

Dissolution studies with tablets containing 4 mg of L-cysteine were also in the same lines as the above mentioned pure L-cysteine studies (III, Fig. 2). At 5 minutes, approximately 83% of the L-cysteine was released and the tablet was completely dissolved at 10 minutes.

The conclusion from the results of the dissolution studies is that L-cysteine might release fairly quickly (for about 5 min) also in *in vivo* studies, which is in this case an expected effect, since smoking a cigarette takes about 5 minutes. Furthermore, the conclusion that the stability of L-cysteine is not pH dependent is an extremely important finding. Thus, L-cysteine products might be effective for users with normal, healthy saliva pH, but also for those with lower saliva pH.

### **5.3 PAEM model (III)**

The physiologically based simulation model, PAEM, for the prediction of the drug amount and the effect on carcinogenic acetaldehyde in the mouth was built using Stella<sup>®</sup> computer programme (III, Fig. 3). The model is based on the *in vivo* results of six volunteers, and it was not validated for a larger population.

In our groups previous study, it was suggested that tablet formulation containing theoretically 5 mg of L-cysteine is sufficient to totally eliminate tobacco smoke acetaldehyde from oral cavity (Salaspuro et al., 2006). Due to problems caused by L-cysteine sensitivity to high pressure induced by compression, the amount found in the formulation after preparation was actually 4 mg. Using PAEM model a good correlation

was found between acetaldehyde and L-cysteine levels *in silico* and concentration levels measured *in vivo* (III, Fig. 3, 5). Thus, it might be concluded that the selected 4 mg L-cysteine amount is enough to drop carcinogenic acetaldehyde levels to below the harmful level of 50  $\mu\text{M}$ .

Further, the model was tested for sensitivity for changes in the lower and higher L-cysteine amount (III, Fig. 6). Tested amounts were 3 mg and 5 mg. The simulation results showed that a drop in the L-cysteine amount from 4 mg to 3 mg might bring an *in vivo* unwanted second acetaldehyde peak between 4 and 5 minutes. In contrast, it was not found that an increase in L-cysteine from 4 mg to 5 mg would promote any significant changes in acetaldehyde levels. If so, it can be concluded, that a formulation containing 4 mg of L-cysteine, which was also tested in *in vivo* studies, is well justified.

In some cases, local drug delivery to the mouth has been proven to be unsatisfactory because of the changing nature of the oral cavity (Seals et al., 1989). Because of that, the model was further tested to find out how sensitive acetaldehyde levels are to the changes in saliva excretion rate (III, Fig. 7). Simulated results showed that an increase in saliva excretion from 2 ml/min to 3 ml/min brought changes in acetaldehyde peaks. As a result, an unwanted second peak appears after 3 minutes with a larger AUC than the first peak. According to the model, it is therefore suggested that, in this extreme case of high saliva excretion, more than 4 mg of L-cysteine is needed to bind tobacco smoke acetaldehyde.

Since PAEM model is built for L-cysteine, a freely water-soluble compound, it might be used to predict the drug amount and local effect in the oral cavity of similar compounds or tablet formulations. For example, PAEM model can offer a new possibility in eliminating or reducing some other toxic compound using only one formulation containing various active compounds. Since the developed model is simple and easy to use, PAEM model might be used in the early phase of drug development, when L-cysteine-like molecules are chosen for further studies. This could provide an alternative tool for obtaining answers on drug efficacy and safety more rapidly, with more certainty and at lower cost (Rajman, 2008). It should be noted that validation of the model requires further simulation runs based on larger *in vivo* data for different formulations or subject groups. For example, the model can be used to predict the effect of the drug in different patient populations that benefit from the drug (Burman et al., 2005). The model can also be extended, for example, to predict drug release during the chewing of a gum. For that, *in vitro* dissolution studies with the European Pharmacopoeia accepted chewing gum dissolution apparatus are needed.

## 5.4 *In vitro* characteristics of MTCA (IV)

### 5.4.1 MTCA stability (IV)

#### Solid-stage stability

Solid-stage stability results suggested that humidity (60 % RH) as well as higher temperature and humidity (45 °C/75 % RH) decreased the remaining amount of synthesised MTCA (IV, Fig. 3b). The reason for that might be found in the extra moisture which was a source of water solvent for MTCA and thus over time MTCA was dissolved and further degraded. If this is the case, in order to obtain reliable results, before any experiments the synthesised MTCA should be used within four weeks and properly sealed and stored under 25 °C/ 60 % RH.

#### Solution-state stability

According to the results, MTCA was stable at a neutral pH of 7.4. However, results also showed that MTCA is pH-dependent, and degradation into acetaldehyde and cysteine possibly occurred. At pH 5.5 after 45 min, the remaining amount was only 32 % of the initial amount and 15 % after 24 h (IV, Fig. 4). Since stable MTCA is wanted in different parts of the gastrointestinal tract, the result is worrying. After all, unstable MTCA at lower pH raises concern that acetaldehyde will release again. To increase the reliability of the results, it would be essential to examine whether acetaldehyde is released from MTCA at a low pH. If so, acetaldehyde, might have a carcinogenic effect on those parts of the gastrointestinal tract where MTCA is deteriorated. Overall, at lower pH, L-cysteine is not able to bind effectively and thus to eliminate carcinogenic acetaldehyde.

### 5.4.2 MTT cytotoxicity (IV)

As for L-cysteine *in vitro* MTT-cytotoxicity of MTCA was examined. MTT-test demonstrated that only the highest tested MTCA concentration of 1200 µg/ml had a negative effect on mitochondrial activity of the cells (IV, Fig. 5). Thus further, it will be essential to investigate *in vivo* saliva concentrations of MTCA during smoking and chewing L-cysteine containing gum.

### 5.4.3 Caco-2 permeability studies (IV)

According to the permeability studies, minimal amounts of MTCA are absorbed from the lower parts of gastrointestinal tract. Thus, the risk of systemic effects is minimal. Since Caco-2 cells resemble intestinal enterocytes, but tighter junctions and carrier proteins are similar to those in the upper gastrointestinal tract, permeability results might indicate that minimal amounts of MTCA are also absorbed from the oral cavity.



## 6 Conclusions and future perspectives

The following conclusions can be drawn from the results:

- According to the results, active ingredient both as a hydrochloride salt and as a base, released fairly quickly from the developed chewing gum (I). Since cigarette smoking time is a short period (about five minute), fast release of L-cysteine from the formulations is a desired property. Still, the compressed gum requires further evaluation to decrease the “crumbing effect” for better user-friendly formulation. Crumbing of the gum into small fragments at the beginning of chewing is more pronounced for gums prepared by direct compression than for chewing gum manufactured by the traditional method.
- The *in vivo* results showed that carcinogenic acetaldehyde of tobacco smoke might be eliminated during five-minute smoking, below mutagenic acetaldehyde levels, with all developed L-cysteine chewing gum formulations (I). Formulation containing 7.7 mg of L-cysteine was able to eliminate all of the acetaldehyde from the oral cavity during smoking.
- It was found that under different temperature and humidity conditions, L-cysteine as a pure and as a component of binary mixture with different excipients is more stable than L-cysteine hydrochloride (II). However, taking together the results from the L-cysteine interactions with tested excipients and results from L-cysteine stability in chewing gum, it might be concluded that high temperature and humidity are important factors for the stability of the final L-cysteine-containing products. Thus, the final product should be well protected from high temperature and humidity. Due to better stability at normal temperature and humidity in a final formulation, the use of L-cysteine as a base form rather than its salt form is well justified.
- The PAEM model developed based on *in vitro* and *in vivo* results may be a valuable tool for the prediction of drug amount and the local effect of a water-soluble compound in the mouth, such as L-cysteine is (III). The model shows how sensitive acetaldehyde is to changes in L-cysteine amount, as well as sensitivity to changes in salivary excretion rate.
- The Caco-2 permeability results showed that there is minimal risk of the locally administered L-cysteine being absorbed before binding to acetaldehyde and that the risk of local side effects while using these formulations is minimal

(IV). The Caco-2 permeability results also indicated that MTCA, the final product of reaction between L-cysteine and acetaldehyde, is not absorbed locally from the lower parts of gastrointestinal tract, which reduces the risk of systemic effects. MTT test showed that both L-cysteine and MTCA are non-toxic to the Caco-2 cells. Thus, results indicated that the L-cysteine, at the concentrations used in this study, seems to be safe in humans.

- Solution-state stability results showed that MTCA stability is pH-dependent, and possibly at lower pH (< 5.5) L-cysteine is not able to bind and eliminate carcinogenic acetaldehyde (IV). That raises the consideration about L-cysteine efficacy in reducing the risk of cancer caused by acetaldehyde at lower pH in the gastrointestinal tract.

In conclusion, elimination of tobacco smoking carcinogenic acetaldehyde by L-cysteine containing chewing gum may possibly lead to lower the risk of development of upper gastrointestinal tract cancers. With chewing gum prepared by direct compression method it would be possible to avoid the high costs associated with traditional chewing gum production. Since acetaldehyde is also an agent which possibly increases the addictive potential of tobacco, reducing acetaldehyde levels in the mouth may play a significant role in fighting against tobacco addiction. Despite encouraging results for both hypotheses, more data is required from experimental studies, and the most efficient way to protect from smoking damage is still to refrain from smoking altogether. However, it should be kept in mind that besides the fact that most smokers want to quit, most attempts fail, and smoke contains many carcinogenic compounds. In the future, developed computational models can offer a new view in eliminating or reducing not only one toxic compound from tobacco smoke, but also many other compounds by using only one formulation containing various active compounds.

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