Sheffield Hallam University

Novel analytical methods for the investigation of nicotine metabolism.

CHAMBERS, Kerry Louise.

Available from the Sheffield Hallam University Research Archive (SHURA) at:

http://shura.shu.ac.uk/19440/

A Sheffield Hallam University thesis
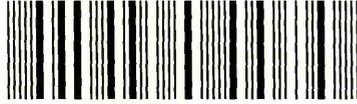
REFERENCE

Fines are charged at 50p per hour

2 9 APR 2003

8·46pm

Novel Analytical Methods for the Investigation of Nicotine Metabolism

Kerry Louise Chambers

A thesis submitted in partial fulfilment of the requirements of
Sheffield Hallam University for the degree of Doctor of
Philosophy

August 2002

Acknowledgements

Abstract

The thesis begins with a description of the history of tobacco usage, health effects related to tobacco consumption, the metabolism of nicotine and the analytical methods used for the determination of its metabolites.

The work described in this current study is related to the use of the König reaction for the colorimetric determination of nicotine and its metabolites. Two novel non-polar condensing reagents were prepared and evaluated as to their performance with an extensive range of individual nicotine metabolites. The work extends our basic knowledge of the analytical chemistry of this reaction particularly in relation to the molar absorptivity and partitioning of the coloured derivatives formed. It was found that each metabolite has its own unique molar absorptivity, a finding which has profound implications for the interpretation of this reaction when used quantitatively as a test for "Total Nicotine Metabolites" since it is increasingly recognised that nicotine metabolism may differ between individuals, ethnic groups and during different physiological states. In addition its possible utility as a colorimetric derivatisation procedure for the determination of nicotine concentrations by high performance liquid chromatography (HPLC) may also be compromised if cotinine alone is used for standardisation purposes.

The use of these novel derivatives was investigated in relation to their correlation with a specific radioimmunoassay (RIA) for cotinine and the ability of the test to discriminate between smokers and non-smokers. The use of more non-polar derivatives was found to result in a small enhancement of the discriminatory power of the reaction for the determination of smoking status. An excellent correlation was found between the RIA and the use of the König reaction. This work formed the basis of a paper published in the British Journal of Biomedical Science (K L Chambers, G A Ellard, AT Hewson and R F Smith (2001), "Urine test for the assessment of smoking status", *British Journal of Biomedical Sciences*, **58:61-65**).

There has been considerable debate as to the precise nature of the derivatives formed during the König reaction. Part of this work has been dedicated to the elucidation of the definitive structures of these compounds. The evidence presented here suggests that the structure proposed by Rustemeier et al (1993) is the more likely of the proposed structures (Klaus Rustemeier, Demetrios Demetriou, Georg Schepers and Peter Voncken (1993), "High-performance liquid chromatographic determination of nicotine and its urinary metabolites via their 1,3,diethyl-2-thiobarbituric acid derivatives", *Journal of Chromatography B*, **613:95-103**).

The work concludes with a consideration of the possible use of surfactants to enhance the procedure and enable the use of potassium thiocyanate rather than potassium cyanide during the reaction. Several combinations of anionic, cationic and neutral surfactants are investigated and their effects on the König reaction discussed.

Contents

Chapter 1

Introduction

Chapter 2

The König reaction

Chapter 3

Urine test for the assessment of smoking status

Chapter 4

Structural determination of the derivatives of nicotine and cotinine produced from the König reaction

Chapter 5

The effects of surfactants

Chapter 6
Conclusion

Chapter 7
References

Appendix 1

Chapter 1

Introduction

1.1 Historical aspects

Nicotine, in the form of tobacco products, has played a social and commercial part in human society for many centuries. The first recorded society believed to smoke tobacco were the Mayan tribes around 1000 BC. This is known through cave paintings, which the Mayan tribes left on the walls of ancient temples showing Mayan priests smoking through a pipe. During 470 to 630 AD, the Mayan tribes began to move throughout America, hence introducing tobacco to other parts of the continent, including the indigenous people of North America (http://www.bat.com and http://www.tobacco.org).

In 1492 Christopher Columbus was offered tobacco from the Arawak people of San Salvador, an island in the Bahamas, but it is believed Columbus did not fully understand the significance of the dried tobacco leaves presented to him and discarded them. Also in 1492, Rodrigo de Jerez and Luis Torres, two explorers returning from a trip into the interior of Cuba, stumbled upon villagers inhaling the smoke from burning dried tobacco leaves through a hollow Y-shaped piece of cane called tobago or tobaca. However, when Jerez returned to his home town, Ayamonte, he was accused by the holy inquisitors of "consorting with the devil" when they saw smoke coming from his mouth, and they imprisoned him for seven years, although by the time Jerez was released, smoking was common in Spain (http://www.bat.com and http://www.imperial-tobacco.com). However, it has also been reported that tobacco was not introduced to Spain until 1558, (some years later after Jerez was released from prison), when Francisco Fernandes, a physician, was sent by Phillip II of Spain to investigate the products of Mexico (http://www.tobacco.org). In 1570, Phillip II of Spain considered whether tobacco had any medicinal properties for possible commercial gain and

commissioned the royal physician, Francisco Hernandez, with making a study in to the plant's properties (http://www.bat.com).

In 1560 the French ambassador to Portugal, Jean Nicot de Villemain, was first reported to have sent tobacco and tobacco seeds to Paris from Portugal (Jackson 1941 and http://www.gallaher-group.com). Jean Nicot was believed to have recommended tobacco snuff to his royal patron, Catherine de Medici. The reason why Nicot recommended tobacco snuff is unclear, but it may have been used to treat her son Francis II's migraine headaches (http://www.tobacco.org) or it may have been used to cure Catherine's own severe migraines by making her sneeze (http://www.bat.com). In Nicot's honour the tobacco genus Nicotiana was later given its botanical name (Jackson 1941 and http://www.bat.com). However, there are reports that tobacco was introduced to France in 1556 by a revolutionary monk, Thevet. Thevet claims that he was first to transport Nicotiana tabacum from Brazil, but this has been disputed by many (http://www.tobacco.org).

Tobacco is thought to have been first introduced to England in 1564 by Sir John Hawkins and his ship's crew (http://www.tobacco.org and http://www.gallaher-group.com). In 1573, Sir Francis Drake returned from America with what was thought to be the first consignment of Nicotiana tabacum, and the potato, to the UK and in 1585, he introduced smoking to Sir Walter Raleigh. In 1586, Sir Walter Raleigh sailed to America where he met Ralph Lane, the first governor of Virginia who is believed to have taught Raleigh to smoke a clay pipe. Raleigh on his return also brought large amounts of tobacco from the "New World".

An Englishman, John Rolfe cultivated the first successful commercial crop of "tall tobacco" in 1612 (http://www.cnn.com and http://www.tobacco.org) and by 1619 the first Africans were brought as slaves to help with the tobacco crop on Rolfe's plantation; over the next two centuries, the growth of the tobacco trade largely fuelled the demand in North America for slave labour (http://www.cnn.com).

By 1800, tobacco was also being commonly used as an insecticide in England (Leistikow et al 1999). Today nicotine solution is still used as an effective insecticide to kill soft-bodied insects such as plant lice. The nicotine solution is usually sold as 40 percent nicotine sulphate solution to prevent volatilisation, and is widely used throughout the world as a relatively cheap and environmentally friendly insecticide. The threshold limit for commercial exposure to nicotine is at a concentration of $0.5mg/m^3$ (Gorrod and Jacob 1999).

In 1881, James Bonsack, a Virginian inventor, invented a cigarette machine that could produce 120,000 cigarettes a day and he was also granted the first cigarette machine patent. Also in 1881, James Buchanan "Buck" Duke entered the cigarette business in Durham, North Carolina, USA. By 1884 Buck Duke had decided to expand his tobacco business nationally and headed to New York. He also purchased two Bonsack cigarette machines, and was able to produce 120,000 cigarettes in 10 hours from a single machine by the end of the year (http://www.tobacco.org) and 21 years later he became the first chairman of British-American Tobacco, (BAT) (http://www.bat.com).

During World War II (1939-1945) servicemen and women were supplied with cigarettes as part of their rations and by the end of the war cigarette sales were at an all time high. This could be directly linked to the returning servicemen and women who became addicted to smoking during the war.

In 1965 cigarette advertisements on television were banned but it was not until 1991 that pipe tobacco and cigar advertising were withdrawn under a European Union Directive on broadcasting (Ash basic facts No 4, 2002).

The World Health Organisation, as part of their 40th anniversary set up the first World No Tobacco Day in 1988. The aim of the day was to promote global attention to the tobacco smoking epidemic and the preventable death and disease it causes

(http://www.5.who.int). They used the slogan "Tobacco or health: The choice is yours" to promote their campaign.

On 7th February 1999, the Royal family announced the removal of the Royal seal of approval from the tobacco company Gallaher Limited, makers of Benson and Hedges and Silk Cut cigarettes, ending a 122 year agreement (http://www.news.bbc.co.uk).

1.1.1 Historical health effects

The health effects associated with tobacco, whether beneficial or not have been reported for over four hundred years. At first tobacco was recommended by European doctors around 1577, as a cure for toothache, falling fingernails, worms, halitosis, lockjaw and cancer (http://www.tobacco.org). Smoking tobacco was even thought to have a protective effect from the great plague (1665-1666) and for this reason, smoking was made compulsory at Eton. It is said that every schoolboy was requested to smoke in school, every morning, under the supervision of a master, and the boys were whipped if they failed to do so (http://www.imperial-tobacco.com).

However, by 1761, the tables had started to turn and the negative health effects associated with tobacco were starting to emerge. In 1761, Dr Percival Pott, a physician in England theorised, that there may be a connection between cancer and exposure to soot due to the number of chimneysweepers developing cancer of the scrotum. In the same year, another physician from London, John Hill published "cautions against the immoderate use of snuff". In this publication he warned snuff users that they were vulnerable to developing cancers of the nose and later, in 1791, he reported cases in which snuff had caused nasal cancers (http://www.tobacco.org) and Sammuel Thomas von Soemmering of Maine in 1795, reported that he had observed cancer of the lip in pipe smokers (http://www.tobacco.org). It was not, however, until as late as 1912 that Dr Isaac Adler first suggested a strong link between smoking and lung cancer (http://www.tobacco.org).

In 1908, legislation in England was brought in to prohibit the sales of tobacco to under 16s, based on the belief that smoking stunted childrens growth (http://www.tobacco.org). This law was then reinforced by the 1991 Childrens and Young Persons (Protection from tobacco) Act (Ash Basic Facts No 4, 2002).

In 1928, a study carried out by Lombard and Doering examined 217 cancer victims. They compared the age, gender, economic status, diet, smoking and drinking habits of these patients. Overall, their findings suggested that cancer rates were only slightly less for non-smokers but that 34 of 35 site-specific (lung, lips, cheek and jaw) cancer sufferers were heavy smokers (http://www.tobacco.org).

The first formal statistical evidence that lung cancer was related to tobacco was published in 1929 by Fritz Lickint of Dresden. Lickint also argued that since men smoke tobacco more than women, then this can explain why lung cancer is four or five time more likely to develop in men than women (http://www.tobacco.org). In 1930, another statistical correlation was made between cancer and smoking by researchers in Cologne, Germany (http://www.tobacco.org).

A British researcher, L M Johnston reported in 1942, that he had successfully substituted nicotine injections for smoking and concluded that the essence of tobacco smoking is the tobacco and not the smoking. He also concluded that the satisfaction of smoking could be obtained from chewing tobacco, from snuff taking and from administration of nicotine (http://www.tobacco.org).

During 1950, three important epidemiological studies linking smoking and lung cancer were published. Morton Levin published the first major study linking smoking to lung cancer. Ernst Wynder and Evarts Graham published, "Tobacco smoking as a possible etiologic factor in bronchiogenic carcinoma: A study of 684 proved cases" and found that 96.5 percent of lung cancer patients interviewed were moderate to heavy chain smokers and two British researchers, Richard Doll and Bradford Hill, published

findings that suggested heavy smokers were fifty times more likely to contract lung cancer than non-smokers (http://www.tobacco.org).

Richard Doll and Bradford Hill in 1952 reported the results of a four-year study comparing 1,465 lung cancer patients to an equal number of patients with other diseases of the same age and sex. They concluded that lung cancer patients are more likely to be smokers and even more likely to be heavy smokers (http://www.bat.com).

In 1956, Ernst Wynder reported that he applied 40 mg of tobacco tar to mice, three times a week and within 10 months, the first signs of cancer had started to appear. By the end of the second year, 59 percent of the mice had developed papillomas, and 44 percent had developed carcinomas, although none of the control animals that were painted with acetone (used as the solvent for the tobacco tar) developed any lesions within this time (Wynder 1956).

In 1957, Winea Simpson found that the incidence of premature births and of all the complications that go with prematurity was twice as great for smoking mothers as it was for non-smoking mothers. This report also confirmed that children of smokers are likely to weigh less and they are more likely to be either still born or die within one month of birth (http://www.tobacco.org).

During the 1960s, health warnings started to appear on the dangers of smoking and over the next forty years a variety of studies were carried out to determine the health effects associated with smoking and advancements in smoking cessation including nicotine replacement therapy. Such advances will be discussed in more detail in 1.4 and 1.5.

1.1.2 History of the chemistry associated with nicotine

Tobacco is known to have been available from around 1570, although it was not until the early 19th century that the chemical identification of nicotine was described. Cerioli in 1807 described the isolation of the "olio essenziale" of tobacco (Holmstedt 1988 and Gorrod and Jacob 1999) and in 1809 Vauquelin independently reported the same potent, volatile and colourless substance which he called "essence de tabac" (Jackson 1941, Holmstedt 1988 and Gorrod and Jacob 1999). Vauquelin's discovery was also confirmed by Hermbstadt in 1822.

Wilhelm Heinrich Posselt and Ludwig Reimann, two students at the University of Heidelberg, isolated nicotine from tobacco in 1828 and in 1843 Melsens first described the chemical empirical formula of nicotine as $C_{10}H_{14}N_2$ (Holmstedt 1988). Barral, in 1847, confirmed Melsens findings and determined the molecular weight of nicotine to be 162 (Holmstedt 1988). Nicotine was first synthesised in 1895 by Pictet and Crepieux (Holmstedt 1988) and in 1904 Pictet and Rotschy described the chemical isomerism of nicotine, although it was Auzies who obtained the French patent 425,370 (January 3 1911) for the synthesis of nicotine (Jackson 1941).

In 1978 Pitner et al suggested it would be desirable to obtain a more detailed conformational picture of nicotine to aid in the interpretation of its physiological activity in terms of structure-function relationship and proposed a spatial orientation of natural nicotine ((S)-nicotine) (Pitner et al 1978).

1.2 Chemical properties of nicotine and tobacco

The compound known to establish tobacco dependence is nicotine. The chemical properties of nicotine have been well documented (Merck 1996 and Jackson 1941). Nicotine is a natural liquid alkaloid produced from the dried leaves of the Nicotiana tabacum or Nicotiana rustica plant and usually consists of 2-8 percent of the dry weight of the cured leaf.

For many years nicotine was considered to be the only alkaloid in tobacco, although it is now known that other alkaloids, for example anabasine, anatabine, isonicoteine, 1-*N*-methylanabasine, 1-*N*-methylanatabine, nicotelline, nicotimine, nicoteine, nicotyrine and nornicotine can be isolated from tobacco and other plants (Jackson 1941).

Nicotine is a colourless to pale yellow oily liquid with a characteristic odour. However, on exposure to air and light or even on standing in the dark in a sealed bottle, the colourless or pale yellow liquid adopts the characteristic brown colour of stored nicotine.

Nicotine is a tertiary amine consisting of a pyridine and pyrrolidine and is chemically known as 3-(1-methyl-2-pyrrolidinyl)-pyridine, 1-methyl-2-(3-pyridyl) pyrrolidine or β-pyridyl-α-N-methylpyrrolidine. Nicotine has an empirical formula of $C_{10}H_{14}N_2$ and a molecular weight of 162.23 (figure 1.1). When (S)-nicotine is boiled at 250^0C with potassium tertiary butoxide, it racemizes. The active isomer is (S)-nicotine, which binds to nicotinic cholinergic receptors, and is the isomer present in tobacco, whereas (R)-nicotine is only a weak agonist of cholinergic receptors. During smoking, some racemization takes place, exposing the smoker to small quantities of (R)-nicotine (Zevin et al 1998).
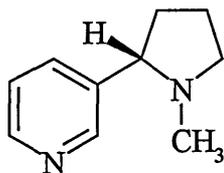
Figure 1.1: Molecular structure of (S)-nicotine

Tobacco smoke is made up of "sidestream smoke" from burning tip of the cigarette, and "mainstream smoke" which is inhaled by the smoker. Tobacco smoke contains thousands of different chemicals, which are released into the air as particles and gases and these chemicals are often found at a higher concentration in sidestream smoke, than mainstream smoke (Ash fact sheet no 12, 2001). Sidestream smoke accounts for nearly 85 percent of the smoke in a room, and approximately one-half of the smoke generated from a lighted cigarette is emitted from the smouldering cigarette between puffs (Ash fact sheet no 12, 2001 and Zhong et al 2000) Together both sidestream and mainstream smoke form the major components of environmental tobacco smoke (ETS).

Cigarette smoke is composed of two phases, the volatile and the particulate. The volatile gaseous portion comprises of about 95 percent of cigarette smoke by weight and consists of approximately five hundred gaseous compounds; these include nitrogen oxides, carbon monoxide, carbon dioxide, ammonia, hydrogen cyanide and benzene. The remaining 5 percent of the cigarette smoke makes up the particulate phase and is composed of approximately 3500 different compounds, which include the major alkaloid nicotine (Zevin and Benowitz 1999) (table 1.1). The particulate matter minus its alkaloids and water content is called tar. Cigarette tar is known to contain many carcinogens, such as polynuclear aromatic hydrocarbons, N-nitrosamines and aromatic amines (Zevin and Benowitz 1999).

VAPOUR PHASE	AMOUNT	PARTICULATE PHASE	AMOUNT
Carbon monoxide	10-23 mg	Particulate matter	15-40 mg
Carbon dioxide	20-40 mg	Nicotine	1-3 mg
Carbon sulphide	18-42 μg	Phenol	60-140 μg
Benzene	12-48 μg	Catechol	100-360 μg
Toluene	100-200 μg	Hydroquinone	110-300 μg
Formaldehyde	70-100 μg	Aniline	360 ng
Acrolein	60-100 μg	Benz[a]anthracene	20-70 ng
Acetone	100-250 μg	Benzo[a]pyrene	20-40 ng
Pyridine	16-40 μg	Quinolone	0.5-2 μg
Hydrogen cyanide	400-500 μg	2-Toluidine	160 ng
Nitrogen oxides	100-600 μg	Cadmium	100 ng
Acetic acid	330-810 μg	Polonium-210	0.03-0.5 pCi
Methyl chloride	150-600 μg	Benzoic acid	14-28 μg

Table 1.1 Major components of fresh, undiluted mainstream smoke of non-filtered cigarettes

The amount of nicotine contained in cigarettes is not specified by the tobacco manufacturers and because nicotine is obtained from tobacco plants, the nicotine content will vary among and within different species of tobacco plants. Benowitz et al (1983) measured the amount of nicotine present in 15 American cigarette brands. Their findings suggested that the average cigarette contained 1.5 percent nicotine by weight, and that low tar/nicotine (low tar) cigarettes tended to have a higher concentration of nicotine compared to "regular" cigarettes. However, low tar cigarettes contained less tobacco per cigarette and so low tar cigarettes are not low tar because they contain less nicotine per cigarette, but because of filtration and ventilation characteristics which remove tar and nicotine and/or dilute smoke with air (Benowitz 1988a).

1.3 The metabolism of nicotine

Nicotine is a weak base with a pKa of 8.0 (Benowitz 1988b), and consequently its absorption, excretion, pharmacology and toxicology is pH dependent. The pH of tobacco used for most American cigarettes, is acidic (pH 5.5). Nicotine at this pH is mostly ionised (positively charged) and therefore very little nicotine is absorbed through the buccal mucosa, even when it is held in the mouth. However, the pH of tobacco smoke from air-cured tobaccos, such as that used for cigars and pipe tobacco is alkaline (pH8.5), and therefore nicotine at this pH is mostly unionised and consequently is readily absorbed from the mouth (Benowitz 1988a and Zevin et al 1998). Chewing tobacco, snuff and nicotine gum are also buffered to an alkaline pH to facilitate nicotine absorption (Benowitz 1988a).

Nicotine is known to be readily absorbed through the skin, mucous membranes and lungs and is known to have a variety of pharmacological actions on various systems within the body. Once nicotine from cigarette smoke reaches the small airways and the alveoli of the lung, it is buffered to physiological pH (Zevin et al 1998), where approximately 31 percent of nicotine is in the unionised form and can therefore cross the cell membranes (Benowitz 1988b). As nicotine is rapidly absorbed, it enters the blood circulation and is distributed to various tissues, including the brain. Nicotine is thought to the reach the brain approximately 10 to 20 seconds after smoking and at the same time arterial levels of nicotine are thought to exceed venous levels by two to six-fold. The brain and plasma levels then decline very rapidly due to the distribution of nicotine to the peripheral tissues or elimination from the body (Zevin et al 1998).

Tobacco may be smoked, chewed or ingested intranasally as snuff and approximately 80-90 percent of ingested nicotine is altered in the body. Metabolism primarily occurs in the liver but also can occur in the kidney and a significant degree of inhaled nicotine may be metabolised by the lung (Benowitz et al 1994). It is known that approximately 70-80 percent of all nicotine is converted to cotinine. The remaining nicotine is converted to various nicotine metabolites via first phase or second phase metabolism (figure 1.2)
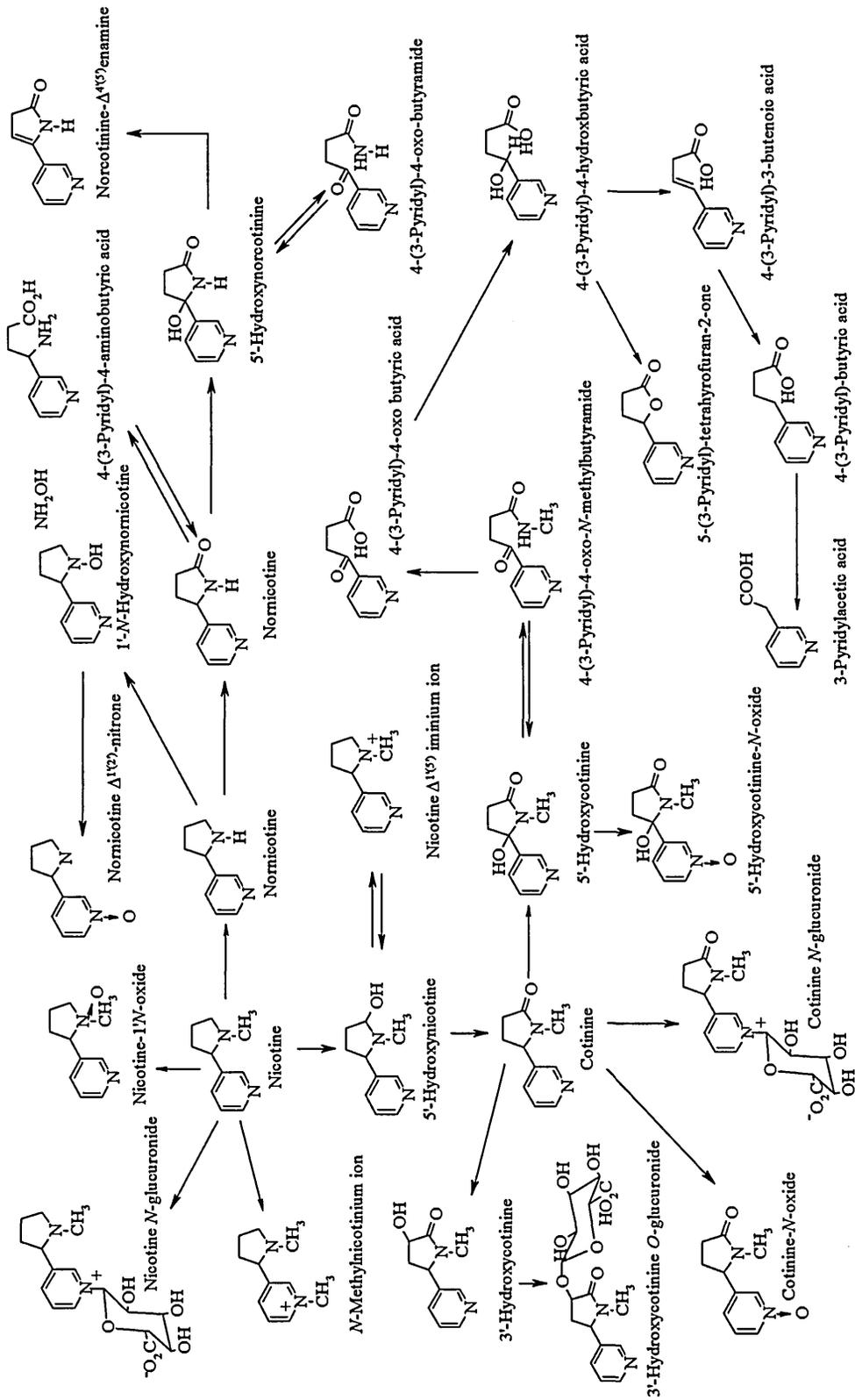
Figure 1.2 Proposed pathway for the metabolism of nicotine

1.3.1 Phase I metabolism of nicotine

Nicotine is primarily metabolised to cotinine or nicotine $1'$-N-oxide, by C- and N-oxidation respectively. The metabolic transformation of nicotine to cotinine in mammals consists of a two step reaction. The first step involves the microsomal cytochrome P450 system which catalyses nicotine to nicotine-Δ-$^{1'(5')}$-iminium ion (McCracken et al 1992, Nakajima et al 1996 and Messina et al 1997).

Nicotine-Δ-$^{1'(5')}$-iminium ion was first reported to act as an intermediate in the metabolic pathway between nicotine and cotinine (Murphy 1973). Murphy (1973) reported that the formation of nicotine-Δ-$^{1'(5')}$-iminium ion is catalysed by an enzyme system that has the properties of a mixed function oxidase and that the initial product of the oxidase reaction is likely to be $5'$-hydroxynicotine, although it may be possible that the formation of the nicotine-Δ-$^{1'(5')}$-iminium ion could occur via direct dehydrogenation, but this pathway is less likely.

Gorrod and Hibberd (1982) confirmed that nicotine-Δ-$^{1'(5')}$-iminium ion acted as an intermediate in the metabolic pathway between nicotine and cotinine. The conversion of the iminium ion to cotinine is then mediated by cytosolic aldehyde oxidases to form cotinine (McCracken et al 1992 and Nakajima et al 1996). Various enzymes involved in the conversion of nicotine to cotinine, have also been suggested and include, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2D6, CYP2E1, CYP2F1 and CYP2C8 (Flammang et al 1992, and McCracken et al 1992). McCracken et al (1992) concluded that it is possible that CYP2B6, CYP2D6 and CYP2A6 can mediate the conversion of nicotine to cotinine, although CYP2B6 is not constitutively expressed in human liver and therefore will be relatively unimportant in the metabolism of nicotine in most individuals. However, Messina et al (1997) concluded from their study that CYP2D6 is also not important in nicotine metabolism in humans and suggested that CYP2A6 was the principal cytochrome P450 involved in nicotine metabolism.

Nicotine may be further metabolised by microsomal flavin monooxygenases to form nicotine 1′N-oxide or by N-demethylation via nicotine- $\Delta^{1'(6')}$ iminium ion to form nornicotine (Nguyen et al 1976 and Pacific et al 1992). Nicotine-1′N-oxide can also be reduced back to nicotine in man by the bacteria in the gastrointestinal tract (Gorrod and Jacob 1999).

Cotinine can be excreted unchanged or be further oxidised at either the carbon or nitrogen centres to form other metabolites. Providing that the cytochrome P450 enzyme, CYP2A6 is present and active (Nakajima et al 1996), hydroxylation at the 3′-position can occur to form either cis- or $trans$-3′-hydroxycotinine, although in humans the trans isomer is predominant (>98 percent) (Jacob et al 1990). Hydroxylation can also occur at the 5′-position to form 5′-hydroxycotinine (Dagne et al 1974), and under physiological conditions 5′-hydroxycotinine predominates over its open tautomer 4-(3-pyridyl)-4-oxo-N-methylbutyramide. Both cotinine and 5′-hydroxycotinine can be further converted to the corresponding N-oxides by oxidation of the pyridyl nitrogen (Dagne et al 1972 and Schepers et al 1999).

Neurath et al (1987) reported that although $trans$-3′-hydroxycotinine had been known since 1963, it had not been measured quantitatively and they found that $trans$-3′-hydroxycotinine was excreted the most in urine compared to cotinine, nicotine, nicotine-1′N-oxide and nornicotine. Results obtained by Benowitz et al (1994) also confirmed that $trans$-3′-hydroxycotinine is the major urinary metabolite of nicotine, accounting for an average of 38 percent of all metabolites, whereas cotinine and nicotine account for 15 and 10 percent of urine metabolites, respectively. They also suggested that the pattern of metabolism is generally similar when nicotine is absorbed following inhalation of tobacco or is absorbed transdermally.

Norcotinine can be formed either by N-demethylation of cotinine (Dagne and Castagnoli 1972 and Schepers et al 1999), or by oxidation of nornicotine (Wada et al 1961 and Aislaitner et al 1992).

Both cotinine and norcotinine can be converted into metabolites that possess a pyridyl moiety attached to a modified butyric acid residue. 5′-Hydroxycotinine is further metabolised to 4-(3-pyridyl)-4-oxo-*N*-methylbutyramide, which is further converted to 4-(3-pyridyl)-4-oxobutyric acid by a single enzymatic hydrolytic reaction rather than by *N*-demethylation followed by hydrolysis of the amide (Gorrod and Jacob 1999). 4-(3-Pyridyl)-4-oxo-butyramide is derived from norcotinine and can be hydrolysed to 4-(3-pyridyl)-4-oxobutyric acid (Eldirdiri et al 1997), which can be derived from either cotinine or norcotinine.

Sai and Gorrod (1995) carried out in vitro studies to determine if 4-(3-pyridyl)-4-oxobutyric acid is further metabolised and their results showed that it is converted by reduction to the corresponding carbinol 4-(3-pyridyl)-4-hydroxybutyric acid which cyclises to 5-(3-pyridyl)-tetrahydrofuran-2-one. Bowman (1968) showed that when 5-(3-pyridyl)-tetrahydrofuran-2-one was administrated to rats, five König positive metabolites were detected in their urine samples; these included 4-(3-pyridyl)-4-hydroxybutric acid and 3-pyridylacetic acid. 3-Pyridylacetic acid has been detected in the urine samples taken from animals or man after cotinine (Bowman et al 1964 and McKennis et al 1964), nicotine (Kyerematen et al 1988 and Schepers et al 1993) or 4-(3-pyridyl)-4-oxobutyric acid (McKennis et al 1964) was administered and it is now accepted that 3-pyridylacetic acid is an end metabolite of nicotine phase I metabolism in mammalian systems.

1.3.2 The role of the cytochrome P450, CYP2A6

Cytochrome P450 is a superfamily consisting of haem thiolate enzymes, which are involved in the metabolism of numerous endogenous and exogenous compounds that include steroid hormones, fatty acids and a variety of clinical drugs (Oscarson et al 1998). This superfamily consists of 74 families (CYP1 to CYP118), however only CYP1, CYP2 and CYP3 are mainly involved in the metabolism of foreign compounds in humans (Yokoi and Kamataki 1998) and it is thought that the CYP2 family and in particular, CYP2A6 is the principal cytochrome P450 involved in nicotine metabolism (Messina et al 1997). CYP2A6 is also responsible for coumarin-7-hydroxylase activity in humans and is involved in the metabolic activity of several procarcinogens including aflatoxin B1 and 4-(methylnitrosamine)-1-(3-pyridyl)-1-butanone (NNK) (Kitagawa et al 1999).

Genetic polymorphisms of CYP2A6 have been identified as CYP2A6*1A, CYP2A6*1B, CYP2A6*2, CYP2A6*3, CYP2A6*4 and CYP2A6*5. Initially three CYP2A6 alleles were discovered, a wild type CYP2A6*1, CYP2A6*2 and CYP2A6*3 (Yamano et al 1990, Fernandez-Salguero et al 1995). Yamano et al (1990) reported that the CYP2A6*2 allele consisted of a single amino acid substitution (Leu160His) and encodes for an unstable and inactive enzyme. Fernandez-Salguero et al (1995) suggested that the CYP2A6*3 allele may encode for an inactive protein because it appears to be generated by multiple gene conversions between CYP2A6 and CYP2A7 (an allele that also encodes for an inactive protein) in exons 3, 6 and 8.

Individuals with the CYP2A6*2/3 and CYP2A6*3/3 genotype have no CYP2A6 mediated metabolism. Tyndale et al (2001) reported that livers taken from heterozygous Caucasians (CYP2A6*1/2 and CYP2A6*1/3) have 50 percent of the CYP2A6 mediated metabolism when using nicotine as a substrate in vitro. This suggests that if two active forms of the gene are inherited, normal nicotine metabolism will occur and an inheritance of one or more defective genes will then reduce their ability to metabolise nicotine. Tyndale et al (2001) looked at a group of individuals who were tobacco dependent smokers who had one defective allele (CYP2A6*2 or

16

CYP2A6*3) and one active allele (CYP2A6*1) and found that they smoked significantly fewer cigarettes per day and per week than tobacco dependent smokers without a defective allele (i.e. homozygous for the wild type allele CYP2A6*1)

A further three alleles were then discovered, CYP2A6*4, which deletes the whole CYP2A6 gene (Nunoya et al 1999 and Oscarson et al 1999a), CYP2A6*1B and CYP2A6*5 (Oscarson et al 1999b). The CYP2A6*1B allele consists of a gene conversion with CYP2A7 in the 3′-untranslated region but the effect of this allele on CYP2A6 activity is unclear, whereas the CYP2A6*5 allele encodes for an unstable enzyme due to a point mutation in exon 9 leading to a single amino acid substitution (G479V), as well as the gene conversion in the 3′-untranslated region (Oscarson et al 1999b).

Pianezza et al (1998) suggested that a group of individuals, lacking the genetically variable enzyme CYP2A6, have an impaired nicotine metabolism and therefore are significantly protected against becoming tobacco dependent smokers. Pianezza et al (1998) also suggested that individuals carrying CYP2A6-null alleles might have a decreased risk of developing tobacco-related cancers and other medical complications. This could be due to one of two factors. The main factor is that these individuals have a decreased risk of becoming a smoker, although, if they do become a dependent smoker, they are more likely to smoke less than those without impaired nicotine metabolism. The second factor is that tobacco smoke contains nitrosamines, which can be activated to carcinogens by CYP2A6, so individuals who carry CYP2A6-null alleles may also be less efficient at activating tobacco-smoke procarcinogens to carcinogens.

Ethnic differences in the metabolism of nicotine have been reported (Messina et al 1997, Pérez-Stable et al 1998 and Benowitz et al 1999). Pérez-Stable et al (1998) reported that serum cotinine concentration per cigarette smoked is significantly higher and the intake of nicotine per cigarette tends to be higher in black smokers, compared to white smokers. They also suggested that blacks metabolise cotinine significantly more slowly, compared to whites. Therefore, as the intake of nicotine is known to be highly correlated to the exposure of tar and oxidant gases, Pérez-Stable et al (1998) put

17

forward the suggestion that this observation may explain why blacks are more likely to have higher smoking related risks of lung cancer and reproductive disorders compared to whites.

Caraballo et al (1998) have also reported that nicotine metabolism varies between populations, especially between non-Hispanic blacks and non-Hispanic whites or Mexican Americans. Their results suggest that serum cotinine levels are higher among non-Hispanic black smokers compared to non-Hispanic whites or Mexican American smokers. These differences in serum cotinine levels cannot be explained by reporting the number of cigarettes smoked or environmental tobacco smoke exposure, although they may be influenced by group-specific patterns of smoking behaviour and by differences in nicotine pharmacokinetics and brand mentholation, especially since more blacks (76 percent), compared to whites (23 percent) prefer mentholated cigarettes (Caraballo et al 1998). Mentholated cigarettes contain enough menthol to stimulate cold receptors, which simulates a "cool sensation". This result in individuals unconsciously holding their breath, which allows for a greater transfer of inhaled tobacco smoke into the lung and pulmonary blood stream (http://www.goodhealth.freeservers.com).

Benowitz et al (1999) also reported that there were ethnic differences in the glucuronidation of nicotine and cotinine. Their findings suggested that blacks excrete significantly less nicotine as nicotine-N-glucuronide and cotinine as cotinine-N-glucuronide compared to whites, although there was no difference in the excretion of 3′-hydroxycotinine-O-glucuronide

Nicotine metabolism has been reported to alter during pregnancy (Rebagliato et al 1998 and Dempsey et al 1998). Dempsey et al (1998) reported that CYP2A6 activity is enhanced during pregnancy, and this could lead to a greater tobacco consumption during pregnancy. Therefore, any nicotine therapy given to an expectant mother to help with smoking cessation would need to be altered to allow for the change in CYP2A6 activity. Dempsey et al (2002) also investigated nicotine metabolism in newborn babies. They collected blood samples during the first day of life for cotinine and nicotine analyses

from 13 babies and daily urine samples from 9 of the 13 babies for up to 7 days to analyse for nicotine, cotinine, 3′-hydroxycotinine and their glucuronides. All babies enrolled on the study were at risk of maternal cocaine use, but biological samples were only taken from babies whose umbilical cord plasma was negative for cocaine or metabolites but positive for cotinine.

The elimination half-life for nicotine and cotinine was 11.2 hours and 16.3 hours based on whole blood analysis, and 9.0 hours and 22.8 hours based on the urine analysis. The elimination half-life for nicotine glucuronide was 13 hours, for cotinine glucuronide 19.8 hours and 18.8 hours for 3′-hydroxycotinine glucuronide. Overall, the half-life for nicotine was three to four times longer in newborn babies than adults, whereas the half-life for cotinine was similar. They suggested that if the same enzymes were primarily responsible for the metabolism of nicotine, then it is unclear why the half-life should be different in newborn children and adults. However, it might be possible for a foetal-newborn form of CYP2A6 that has an altered affinity for nicotine but not cotinine (Dempsey et al 2002).

1.3.3 Phase II metabolism of nicotine

Phase II metabolites, are metabolites that have been further metabolised by an enzymatic transfer to a molecule or molecular substructure from an endogenous compound thus forming a metabolite with a higher molecular weight. These metabolites are often highly polar, water-soluble compounds which can aid the urinary excretion of foreign compounds and phase I metabolites. *N*-Methylation, catalysed by methyltransferases and the formation of glucuronides are especially important phase II pathways in nicotine metabolism (Gorrod and Jacob 1999).

Curvall et al (1989) reported that the concentrations of nicotine and its metabolites, cotinine and *trans*-3′-hydroxycotinine in urine samples taken from smokers and snuff users increased on average by a factor of 2.3 and 1.4, respectively when the urine samples were treated with β-glucuronidase. They interpreted this to mean that the

observed increase in the concentration of nicotine and metabolites was due to the enzymatic cleavage of the respective glucuronides. They also reported that the glucuronides of cotinine and *trans*-3'-hydroxycotinine were found in urine samples taken from non-smokers after oral administration of cotinine. Schepers et al (1992) reported that *trans*-3'-hydroxycotinine glucuronide was the major phase II nicotine metabolite present in urine samples analysed in their study. Byrd et al (1992) confirmed that only nicotine, cotinine and *trans*-3'-hydroxycotinine are excreted as glucuronides in smokers' urine and that the glucuronides can account for up to 29 percent of the total metabolites detected in urine from the individuals they studied. However, their results suggested that cotinine glucuronide was the major glucuronide excreted in urine.

The formation of the glucuronides of nicotine, cotinine and 3'-hydroxycotinine is catalysed by UDP-glucuronosyltransferases (UGT), although the individual isozymes involved in *N*-glucuronidation of nicotine and cotinine and *O*-glucuronidation of *trans*-3'-hydroxycotinine have not been identified (Gorrod and Jacob 1999). However, Benowitz et al (1994) suggested that the same UGT isozymes might be involved in the *N*-glucuronide formation of nicotine and cotinine, whereas a different UGT isozyme may be used for *O*-glucuronidation of *trans*-3'-hydroxycotinine.

1.4 Health effects associated with smoking

Inhaling tobacco smoke causes several immediate responses within the body. Once nicotine from the inhaled tobacco smoke reaches the small airways and the alveoli of the lung, it is buffered to physiological pH, where it can cross the cell membrane. Nicotine is rapidly absorbed into the blood stream, reaching the brain within approximately 10 to 20 seconds after smoking (Zevin et al 1998). Nicotine initially causes the release of adrenaline, an increase in blood pressure and heart rate. This effect may cause the heart rate to increase by as much as 30 percent during the first 10 minutes of smoking (Ash facts sheet no 6, 2002). Glucose is then released into the blood stream because of the increased levels of adrenaline. Nicotine can also block the release of the principal hyperglycaemic hormone, insulin, which prevents the excess glucose in the bloodstream from entering muscle and fat tissues. The smoker can then become hyperglycaemic, which may enable the brain to detect the excess sugar levels and down regulate hormones and other signals, causing an impaired appetite (http://www.howstuffworks.com).

Nicotine can cause a slight increase in the basal metabolic rate, enabling smokers to burn more calories. However, smokers tend to have an increased level of blood cholesterol compared to non-smokers, due to a lower ratio of high-density lipoproteins to low-density lipoproteins. Cigarette smokers have also been shown to have raised levels of fibrinogen and platelets, which may result in the smoker developing various forms of atherosclerotic disease. This may result in the arteries becoming more rigid and narrowed and it is more likely that the blood may form a thrombosis. The sudden blockage of an artery may lead to a fatal heart attack, a stroke or gangrene of the leg (Ash fact sheet no 6, 2002).

Tobacco smoke also contains chemicals like carbon monoxide which reduces the blood's ability to carry oxygen as carbon monoxide can attach itself to haemoglobin much more readily than oxygen, thus reducing the overall amount of oxygen available to the tissues. The smoker can rapidly become tolerant to some of the side effects that occur as result of smoking, such as, dizziness, nausea and vomiting. The tolerance to

subjective effects and acceleration of heart rate can develop within a day in regular smokers (Benowitz et al 1998).

1.4.1 Diseases attributed to smoking

Smoking has been associated with a variety of health problems (Table 1.2), at least 80 percent of all deaths from lung cancer, around 80 percent of all deaths from bronchitis and emphysema and 17 percent of all deaths from heart disease are caused as a result of smoking (Table 1.3) (Ash fact sheet no 2, 2001).

In 1995, there were 46,000 cancer deaths (approximately a third of all cancers deaths) in the UK because of smoking (Ash fact sheet No 4, 2001). Cigarette smoking is the predominant cause of cancer of the lung, larynx, pharynx, oesophagus, bladder, kidney and pancreas and a 1996 review of cancers related to smoking concluded that cigarette smoking is also a cause of cancer of the lip (Ash fact sheet no 4, 2001).

Enzymes induced by tobacco smoking may increase the risk of cancer by enhancing the metabolic activation of carcinogens (Zevin and Benowitz 1999). The risk of dying from lung cancer increases with the number of cigarettes smoked per day (Doll and Peto 1976) (Table 1.4) and it has been more recently reported to increase proportionally with the number of cigarettes smoked daily and the total number of smoking years (Thompson and Hunter 1998). Heavy smokers are at risk of up to 30 percent cumulative lifetime risk of developing cancer, compared to 1 percent for non-smokers (Thompson and Hunter 1998).

INCREASED RISK FOR SMOKERS	
Acute necrotizing ulcerative gingivitis (gum disease)	Muscle injuries
Angina (20 x risk)	Neck pain
Back pain	Nystagmus (abnormal eye movement)
Buerger's disease (severe circulatory disease)	Ocular Histoplasmosis (fungal eye infection)
Duodenal ulcer	Osteoporosis (in both sexes)
Cataract, posterior subcapsular (3 x risk)	Osteoarthritis
Colon Polyps	Penis (inability to have an erection)
Crohn's disease (chronic inflamed bowel)	Peripheral vascular disease
Depression	Psoriasis (2 x risk)
Diabetes (Type 2, non-insulin dependent)	Skin wrinkling (2 x risk)
Hearing loss	Stomach ulcer
Influenza	Rheumatoid arthritis
Impotence (2 x risk)	Tendon injuries
Optic Neuropathy (loss of vision, 16 x risk)	Tobacco Amblyopia (loss of vision)
Ligament injuries	Tooth loss
Macular degeneration (2 x risk)	Tuberculosis
FUNCTION IMPAIRED IN SMOKERS	
Ejaculation (volume reduced)	Sperm count reduced
Fertility (30% lower in women)	Sperm motility impaired
Immune System (impaired)	Sperm less able to penetrate ovum
Menopause (onset 1.74 years early on average)	Sperm shape abnormalities increased
SYMPTOMS WORSE IN SMOKERS	
Asthma	Graves' disease (over-active thyroid gland)
Chronic rhinitis (chronic inflammation of the nose)	Multiple Sclerosis
Diabetic retinopathy	Optic Neuritis
DISEASE MORE SEVERE OR PERSISTENT	
Common cold	Pneumonia
Crohn's Disease	Tuberculosis
Influenza	

Table 1.2 Summary of health problems associated with smoking (Ash fact sheet no 2, 2001)

ESTIMATED PERCENTAGES AND NUMBERS OD DEATHS ATTRIBUTED TO SMOKING IN THE UK (BASED ON 1995 MORTALITY DATA)					
Condition	Percentage of deaths from the condition attributable to smoking in 1995		Number of deaths from the condition attributable to smoking in 1995		
	Men	Women	Men	Women	Total
CANCERS:					
Lung	90	73	21,100	9,500	30,600
Throat and mouth	74	47	1,500	400	1,900
Oesophagus	71	62	2,900	1,600	4,500
Bladder	48	17	1,700	300	2,000
Kidney	41	5	700	100	800
Stomach	35	10	1,700	300	2,000
Pancreas	21	25	600	800	1,400
Unspecified site	34	7	2,400	500	2,900
Leukaemia	19	10	200	100	300
Total					**46,400**
HEART and CIRCULATION:					
Ischaemic heart disease	23	11	18,700	7,600	26,300
Aortic aneurysm	62	48	4,000	1,800	5,800
Myocardial degeneration	24	10	300	300	600
Atherosclerosis	17	5	100	100	200
Stroke	13	9	3,400	3,900	7,300
Total					**40,200**
OTHERS:					
Bronchitis and emphysema	86	79	15,100	9,300	24,400
Pneumonia	25	11	5,800	4,100	9,900
Ulcer of the stomach and duodenum	47	41	1000	1000	2,000
Total					**36,300**
Sub total			**81,200**	**41,700**	**122,900**
PREVENTABLE BY SMOKING*:					
Parkinson's			900	300	1,200
Cancer of the endometrium				100	100
Sub total			**900**	**400**	**1,300**
TOTAL			**80,400**	**41,300**	**121,600**

* Studies have shown that smoking appears to have a protective effect against the onset of some diseases such as endometrial cancer. However, the positive effect is so small in comparison with the overwhelming toll of death and disease caused by smoking that there is no direct public health benefit.

Deaths caused by smoking are six times higher than the 20,170 deaths arising from: road accidents (3,444); poisoning and overdose (2,663); other accidental deaths (8,986); murder and manslaughter (503); suicide (4,379); and HIV infection (195) in the UK during 1998 (Ash fact sheet 2, 2001).

Table 1.3 Estimated number of deaths attributed to smoking (Ash fact sheet 2, 2001).

NUMBER OF CIGARETTES SMOKED PER DAY	ANNUAL DEATH RATE PER 100,000 MEN
0	10
1-14	78 (8 times that of a non-smokers)
15-25	127 (13 times that of a non-smokers)
25 or more	251 (25 times that of a non-smokers)

Table 1.4 A study of male British doctors, deaths from lung cancer in smokers and non-smokers (Doll and Peto 1976)

Tobacco smoke contains a number of tobacco specific procarcinogen nitrosamines; for example N-nitrosodiethylamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N'-nitrosonornicotine (NNN). These compounds are termed pre- or procarcinogens because they are activated by the body to carcinogens. Carcinogens found in cigarette smoke through direct exposure, absorption, or transport through the body are also known to cause cancer of the pancreas, stomach, cervix, kidney and liver in addition to causing leukaemia (Thompson and Hunter 1998).

Each year, tobacco smoke accounts for around 26,000 deaths from coronary heart disease (CHD) in the UK, which is approximately 17 percent of all heart disease deaths (23 percent of deaths in men and 11 percent in women) (Ash fact sheet no 6, 2002). The risk of a cigarette smoker of having a heart attack is two to three times that of a non-smoker. Doll and Peto (1994) reported than men under the age of 45 who smoke 25 or more cigarettes a day, were 15 times as likely to die from CHD as non-smokers of the same age. Even light smokers have an increased risk of developing CHD, and it has been reported that women who smoked 1-4 cigarettes a day had a 2.5-fold increase risk of fatal CHD (Bartecchi et al 1994).

1.4.2 Smoking and pregnancy

Women who smoke may have reduced fertility. A study by Baird and Wilcox (1985) found that 38 percent of non-smokers conceived in their first cycle, compared to 28 percent of smokers and that smokers were 3.4 times more likely than non-smokers taken more than one year to conceive. A recent British study found that smoking by men and both active and passive smoking by women were associated with delayed conception (Hull et al 2000). Fukuda et al (2002) found that the offspring ratio (male to female) was lower when either one or both parents smoked more than 20 cigarettes per day compared to couples were neither one smoked. The reason for the reduction in males is unclear, but it might be due to chronic exposure to toxic environmental agents, such as the toxins in cigarette smoke, that predominantly affect males and the male reproductive system. They also suggested that smoking reduced the offspring ratio around the time of conception, rather than imposing a selective disadvantage on male foetuses during later pregnancy.

Sasco and Vainio (1999) suggested that there is evidence that genotoxic and carcinogenic components of tobacco smoke can pass through to the placenta and the developing foetus. This is important since in western countries the highest or second highest group of smokers among women are of reproductive age (18-34 years old), and this may explain the increased awareness of the health effects linked to smoking in pregnancy (Sasco and Vainio 1999). About one-third of pregnant smokers quit smoking, the vast majority during the first trimester, although smoking cessation is dependent on several social factors and lone mothers, under-educated and less privileged ones are more unlikely to quit than better off women. A study carried out by Fingerhut et al (1990) found that 70 percent of women who quit smoking during pregnancy resumed smoking within 12 months. Their results also suggested that of the individuals, who resumed smoking, 67 percent resumed smoking within 3 months and 93 percent started smoking within six months of delivery.

26

Babies born to women who smoke are usually lighter, shorter and have a smaller head circumference. A study by Wang et al (1997) found that babies born to continuously smoking mothers were on average, 257 g lighter, 1.2 cm shorter and 0.5 cm smaller in head circumference, compared to babies born to non-smoking mothers. Babies born to intermediate smoking mothers were on average, 56 g lighter in weight but they had similar birth length and head circumferences as babies born to non-smoking mothers.

Becker et al (1999) measured urinary cotinine levels in 507 infants during the first two weeks of life and found that cotinine levels of infants from smoking mothers were significantly increased. Also breast-fed infants had higher cotinine levels than non-breast-fed infants, but it was only significant (P<0.05) if the mother smoked. When they compared urinary cotinine levels of breast-fed infants whose mother smoked and infants whose mother smoked but did not breast-feed, the urinary concentrations were 5 times higher in the breast-fed infants. This study highlighted the importance of encouraging mothers who smoke to give up, rather than discouraging them from breast-feeding (Becker et al 1999).

A study carried out at Nottingham City Hospital compared data from 15,000 children born during one week in 1970. Their findings were that almost 40 percent of children either exposed to tobacco smoke in the womb or soon afterwards displayed asthma symptoms by the age of 16. This compared to 29 percent of children who developed asthma but were not exposed to tobacco smoke (Ash asthma and smoking, 2002).

1.4.3 Environmental tobacco smoke

Environmental tobacco smoke (ETS) or passive smoking in the UK is thought to cause several hundred cases of lung cancer and several thousand cases of heart disease in non-smokers every year (Ash basic fact no 2, 2002). It has also been reported that people, who are exposed to other people's tobacco smoke, have suffered irritation of the eye, nose and throat, reduced lung function in adults with no chronic chest problems and an increase in the prevalence of cardiovascular disease. However, the health risks

associated with ETS are small, compared to the risks associated with active smokers (Cummings et al 1990).

Biological samples taken from non-smokers have been shown to contain the constituents of ETS, which have been inhaled or absorbed by the non-smokers. A review of the literature by Zhong et al (2000) found that life-time non-smoking men and women experience a 48 percent and 20 percent excess risk of lung cancer respectively after being exposed to ETS from their spouses, and a 29 percent and 15 percent excess risk of developing lung cancer due to exposure to ETS at work. However, they also concluded that there was no evidence that exposure to ETS during childhood conferred a higher risk of lung cancer. Sasco and Vainio (1999) also carried out a review of the literature previously and suggested that there was no strong association between maternal smoking in pregnancy or exposure to ETS and cancer risk in childhood, although they did point out that their findings do not imply that tobacco use is not an issue.

Passive smoking also effects babies and children, and more than 17,000 children under the age of five are admitted to hospital every year because of the effects of passive smoking (Ash basic fact no 2, 2002). In children passive smoking can cause an increased risk of lower respiratory tract infection, increased severity of asthma symptoms, more frequent occurrence of chronic coughs, phlegm and wheezing, and increased risk of cot death and chronic middle ear effusion (glue ear).

Nafstad et al (1995) analysed nicotine concentrations in hair samples taken from children aged between 12 and 36 months. Questionnaires were also taken to enable information on the child's ETS exposure to be determined. Compared to children registered as unexposed to ETS by the questionnaire, nicotine levels were 12.4 times higher among children who were exposed to more than 10 cigarettes per day and 3.4 times higher among children exposed to between 1 and 10 cigarettes per day.

Oddoze et al (1999) analysed urinary cotinine of children between 4 and 14 years old who reported a confirmed diagnosis or symptoms of asthma. Their results suggested that urinary cotinine concentrations and cotinine to creatinine ratio in children are highly correlated with the number of cigarettes the parents smoked. Their results also suggested that children had a higher concentration of cotinine if the mother only smoked compared to if the father only smoked. This may be linked to the mother taking on the responsibility of childcare.

1.5 Nicotine replacement therapy

Cigarette smoking is a highly addictive habit, involving both pharmacological and psychological addiction and the dependence on nicotine is reflected by the high failure rate among smokers who try to quit. Over 80 percent of smokers express a desire to quit, only 35 percent try to stop each year, and fewer than 5 percent are successful in unaided attempts to quit (Goodman and Gilman 1996).

The cost to the NHS for treating diseases caused by smoking is approximately £1.5 billion a year. This amount includes the cost of hospital admissions, GP consultations and prescriptions. In 1997-98, 364,200 people were admitted to an NHS hospital to be treated for such diseases, occupying an average of 9,500 beds every day (Ash basic facts no 3, 2002).

Health care intervention to help with smoking cessation is very cost effective compared with many other treatments. For example the cost to the NHS of prescribing statins, drugs to lower cholesterol levels, is estimated to be between £5,400 and £13,300 per life year gained, whereas the cost of smoking cessation advice is estimated to range from £212 to £873 per life year gained (Ash basic facts no 3, 2002).

1.5.1 Smoking cessation

The health benefits for smokers who stop smoking compared to non-smokers have been reported to include a reduction in the overall risk of mortality 10-15 years after quitting, a reduction in the likelihood of suffering from coronary heart disease 2-4 years after smoking cessation and also a reduction in the possibility of developing all types of cancer after 10 years of abstaining from smoking (Thompson and Hunter 1998). Other health benefits include a decline in respiratory symptoms such as wheezing, coughing and phlegm production, which can be observed within 3 to 9 months after smoking cessation (Table 1.5) (Ash fact sheet no 11, 2001).

TIME SINCE QUITTING	BENEFICIAL HEALTH CHANGES THAT TAKE PLACE
20 minutes	Blood pressure and pulse rate return to normal.
8 hours	Nicotine and carbon monoxide levels in blood reduced by half, oxygen levels return to normal.
24 hours	Carbon monoxide will be eliminated from the body. Lungs start to clear out mucus and other smoking debris.
48 hours	There is no nicotine left in the body. Ability to taste and smell greatly improved.
72 hours	Breathing becomes easier. Bronchial tubes begin to relax and energy levels increase.
2-12 weeks	Circulation improves
3-9 months	Coughs, wheezing and breathing problems improve as lung function is increased by up to 10%.
1 year	Risk of a heart attack falls to about half that of a smoker.
10 years	Risk of lung cancer falls to half that of a smoker.
15 years	Risk of heart attack falls to the same as someone who has never smoked.

Table 1.5 Beneficial health changes that take place after abstaining from smoking

For smoking cessation to work successfully, both non-pharmacological and pharmacological treatments are required, especially as the smoker can often associate smoking with certain activities, such as smoking at social events and after meals with coffee or alcohol (Thompson and Hunter 1998). Non-pharmacological treatments can include education, behavioural counselling, support groups, self-management, self-monitoring, hypnosis and acupuncture (Thompson and Hunter 1998). However, White et al (2002) suggested that there is no clear evidence that acupuncture, acupressure, laser therapy or electrostimulation are effective for smoking cessation.

Nicotine replacement therapy is often used to alleviate nicotine withdrawal symptoms. Studies carried out by Henningfield et al (1995) have shown that this type of therapy can increase cessation rates by approximately two fold compared to placebo studies. The mechanism on how nicotine replacement therapy enhances smoking cessation is not completely understood, although it is believed that it enables relief of withdrawal symptoms by allowing the absorbed nicotine to bind to and desensitise nicotinic cholinergic receptors in the brain (Benowitz et al 1998). Benowitz (1993) reported that approximately 80 percent of individuals who typically smoke 10 or more cigarettes per day, exposing their body organs, including their brain, to nicotine 24 hours per day, who later abstain from smoking will experience withdrawal symptoms. Such withdrawal symptoms tend to occur as one or more symptom complexes; psychological distress, including irritability, anger, impatience and anxiety; difficulty concentrating, often associated with impaired cognitive performance, increased appetite, weight gain and tobacco craving (Table 1.6) (Ash fact sheet no 11, 2001).

WITHDRAWAL SYMPTOMS	DURATION	PERCENTAGE OF INDIVIDUALS WHO ARE TRYING TO QUIT BUT ARE AFFECTED
Irritability/ aggression	Less than 4 weeks	50
Depression	Less than 4 weeks	60
Restlessness	Less than 4 weeks	60
Poor concentration	Less than 2 weeks	60
Increased appetite	Greater than 10 weeks	70
Light-headaches	Less than 48 hours	10
Night-time awakenings	Less than 1 week	25
Tobacco craving	Greater than 2 weeks	70

Table 1.6 Percentage of individuals who are trying to quit but are affected by withdrawal symptoms (Ash fact sheet no 11, 2001).

The main reason for nicotine replacement therapy is to aid an individual in breaking their addiction by relieving withdrawal symptoms; this then enables the individual to modify their life style in order to achieve permanent smoking cessation. Benowitz (1993) carried out a literature review into the chances of an individual quitting smoking, comparing the nicotine replacement patch to a placebo patch. The results from this review suggested that 39 percent to 71 percent of the individuals studied were able to quit smoking at 4 to 6 weeks, compared with 13 percent to 41 percent who used a placebo patch. By 6 months the number of individuals who were still not smoking were 22 percent to 39 percent for the nicotine patch and 8 percent to 26 percent for the placebo patch. After one year the quit rates in the studies Benowitz (1993) looked at were 18 percent to 26 percent for nicotine patch versus 5 percent to 23 percent for placebo patch.

Bohadana et al (2000) published a double blind, placebo-controlled trial using nicotine inhaler and nicotine patch as a combined therapy for smoking cessation. A combination therapy was chosen because they believed that it might increase cessation rates, compared to using a single replacement therapy. The trial was split into two groups, each consisting of 200 subjects who had smoked 10 or more cigarettes per day for 3 years or longer. Group 1 subjects received the nicotine inhaler (10 mg) plus nicotine patch (delivering 15 mg of nicotine per 16 hours) for 6 weeks, then nicotine inhaler plus placebo patch for 6 weeks, then nicotine inhaler for 14 weeks. Group 2 subjects received the nicotine inhaler and placebo patch for 12 weeks, then the nicotine inhaler for 14 weeks. The inhaler could be used at a rate of 6 to 12 cartridges per day, *ad libitum* for 3 months before being tapered off.

Complete abstinence rates were then determined by carbon monoxide concentrations of less than 10 ppm and self reported smoking status. Complete abstinence rates at 6 weeks were 60.5 percent for group 1 and 47.5 percent for group 2. By 12 weeks, the abstinence rates had dropped to 42.0 percent and 31.0 percent for groups 1 and 2 respectively. At 6 months, the abstinence rates were 25 percent and 22.5 percent, dropping to 19.5 percent and 14 percent by 12 months for group 1 and 2 respectively. Overall, the results suggested that a therapy that combined the use of the nicotine inhaler and nicotine patch increased cessation rates, than if the inhaler was used alone.

Therefore, these results suggest that an individual is more likely to quit smoking with the aid of nicotine replacement therapy than unaided (Table 1.7). Even if an individual is unable to quit smoking altogether than nicotine replacement therapy can significantly decrease the number of cigarettes smoked.

1.5.2 Nicotine replacement products

Nicotine replacement therapy is marketed as a variety of products, which include nicotine gum, patches (transdermal nicotine), nasal sprays and nicotine inhalers. However, nicotine cannot be taken orally, due to possible side effects that include indigestion and also it will be largely metabolised in the liver before reaching the systemic circulation (Tang et al 1994). Therefore, all nicotine replacement therapies are designed to enable nicotine to be directly absorbed into the systemic circulation through the buccal or nasal mucosa, the alveoli or skin (Tang et al 1994).

The first nicotine replacement product to be marketed was nicotine gum. Nicotine is attached in a loose association with the ionic bonding agent polarcrilex, to enable the controlled release of nicotine. After about 20 minutes of chewing, approximately 90 percent of the available nicotine is released. Most of the nicotine is absorbed through the buccal mucosa and on average about a quarter is swallowed as saliva and metabolised but there is much variation between individuals (Tang et al 1994).

Nicotine replacement therapy is aimed at replacing nicotine that was previously consumed from their daily intake of tobacco, although for most individuals who are heavy smokers the amount of nicotine usually obtained from tobacco is not replaced by the therapy. Benowitz (1993) reported that for smokers who are more dependent on tobacco, then the average systemic intake of nicotine from cigarettes is approximately 30 mg per day (comparable to approximately 30 cigarettes per day). However, if these individuals used nicotine gum on a fixed schedule of 10 to 12 pieces per day, then their plasma nicotine concentration averaged one to two thirds less than those seen in higher dependent cigarette smokers. However, some individuals would be able to match the nicotine concentrations achieved through smoking, although most do not. Therefore, smokers that are more dependent may require nicotine gum that has a higher level of nicotine for optimal results.

34

QUITTING STRATEGY	INCREASE IN QUITTING PERCENTAGE
Counselling and support	
One to one advice (average 5 minute duration)	2
Counselling with follow-up (written, telephone, visit)	5
Minimal intervention strategy (MIS)	10
Group therapy	0-5
Behaviour therapy	
General	2
Aversion	No proven effect
Sensory deprivation	No proven effect
Hypnosis	No proven effect
Cutting down (reducing, changing brand)	Little or no effect
Drug therapy	
Nicotine substitutes (chewing gum, skin patches, inhalant, spray)	3-13
Zyban	11-15
Alternative therapy	
Acupuncture	No proven effect

Table 1.7 Overview of the most popular aids to quit smoking with their effectiveness in the long term (≥ 6 months). Results based on several studies, mainly biochemical monitoring (Berkmortel et al 2000)

Nicotine skin patches release nicotine into the blood at a slow but constant rate. Three brands are licensed in Britain and are available over the counter. Two (Nicotinell and Nicabate) are worn for 24 hours, with three strengths corresponding to patch areas of 30, 20 and 10 cm^3, delivering 21, 14 and 7 mg of nicotine into the circulation over 24 hours. One, Nicorette, is a 16 hour patch, removed at night, with three strengths, 15 mg, 10 mg and 5 mg. Courses of about three months are recommended, beginning with a higher dose of patch and reducing at intervals.

The levels of nicotine in venous blood produced by transdermal nicotine patches, consisting of a dose of 21 mg/day, are 15 to 20 ng/ml, which is equivalent to the venous levels found in light smokers. Transdermal nicotine delivers nicotine to the systemic circulation in doses of 7 to 21 mg per day, depending on the patch and because nicotine is delivered over 16 to 24 hours, the peak concentration of nicotine during this time is considerably less than that seen while smoking (typically 20 to 40 µg/mL), although nicotine concentrations with the patch may exceed those seen in cigarette smokers overnight. However, the intake of nicotine from nicotine gum, nicotine nasal spray and nicotine inhalers on average delivers an even lower dose of nicotine than that observed with nicotine patches (Benowitz et al 1998).

Lawson et al (1998) carried out a study to determine the urinary excretion rates of cotinine and nicotine, to determine if a given dose of nicotine using nicotine replacement patches achieves 100 percent replacement. They concluded that for many individuals the amount of nicotine delivered using a standard 22 mg patch was significantly less than the amount obtained while smoking. Using nicotine excretion rate to calculate nicotine replacement showed that only 17 percent of subjects using the 22 mg nicotine replacement patch achieved at least 100 percent replacement and when using cotinine excretion rates only 25 percent of subjects achieved 100 percent replacement (Lawson et al 1998).

Nicotine nasal spray is absorbed through the nasal mucosa. This product might satisfy craving more effectively as it produces steady state plasma nicotine concentration similar to that from smoking, but the amount of plasma nicotine achieved is only over half that obtained from smoking a cigarette. The nicotine inhaler absorbs nicotine through the alveoli, as with cigarette smoking, but plasma nicotine levels achieved are even lower than the nasal spray (Tang et al 1994).

Recent advances have resulted in an alternative nicotine replacement therapy, bupropion chloride, otherwise known by its tradename, Zyban, which works by de-sensitising brain nicotine receptors. Bupropion chloride is a monocyclic antidepressant, structurally related to amphetamine, and was originally introduced as an antidepressant under the trade name, Wellbutin (Berkmortel et al 2000).

Gonzales et al (2001) carried out a double blind, placebo-controlled study in 450 smokers who had previously used bupropion in a smoking cessation. Their study consisted of a screening phase, a 12 week treatment phase and a follow up after 6 months. The primary aim of the study was to achieve continuous abstinence from smoking from weeks 4 through to 7. Their results suggested that 27 percent of participants who received bupropion SR remained abstinent throughout the four week period (weeks 4- 7), compared to 5 percent of participants who received a placebo. After 6 months 12 percent of participants who received bupropion SR abstained from smoking from week 4 to the follow up period after 6 months, compared to only 2 percent of participants who received the placebo.

The results from clinical trials look promising that Zyban could be used as an alternative nicotine replacement therapy. However, for some adults it may not be safe to use as the estimated risk of the most serious side effect, seizures, is less than 1 in 1000 and less serious side effects have also been reported, which include, insomnia, dry mouth and headaches (Ash fact sheet no 11, 2001). A recent publication by Tracey et al (2002) reported that in the initial 6 months after Zyban had been introduced to Ireland, there had been 12 overdose cases reported to The Nation Poisons Information Centre, 8 patients

developed symptoms of toxicity, whose symptoms included tachycardia, drowsiness, hallucinations and convulsions and 2 patients developed severe cardiac arrhythmias. One of the patients who developed a severe cardiac arrhythmia had to be resuscitated following a cardiac arrest. Tracey et al (2002) then further report that the potential toxic effect should be considered when prescribing Zyban as a smoking cessation aid.

Nicotine replacement therapy is not recommended by manufacturers to be used during pregnancy, due to the possible health risks to the foetus. However, smoking during pregnancy is harmful to the foetus and it is believed that nicotine may be one of the toxic components in tobacco that may contribute to foetal hypoxia and growth retardation through a reduction in placental blood flow (Tang et al 1994). Therefore, nicotine replacement therapy could have potential health risks to the unborn child, but they are likely to be less hazardous than moderate smoking, as nicotine replacement therapy produces a slower increase in plasma nicotine concentration, does not yield carbon monoxide or other noxious substances, and, if successful, does not expose the foetus to nicotine throughout pregnancy. Ideally, it would be in the interests of the unborn child, if the mother stopped smoking during the pregnancy without the aid of nicotine replacement therapy, but nicotine replacement may be justified if other methods fail.

1.6 Analytical methods for the determination of nicotine and its metabolites

For many years individual smoking status has been determined using a variety of analytical methods to measure nicotine and its metabolites. The simplest method to determine if a person is a smoker or non-smoker, and to derive information regarding their smoking habits, is to use a questionnaire (Jarvis et al 1984, Cope et al 1996, Caraballo et al 1998, Kharrazi et al 1999). The first questionnaire devised to measure nicotine addiction was the Fagerström tolerance questionnaire (FTQ) (Fagerström 1978) which was altered to become the Fagerström test for nicotine dependence (FTND) (Table 1.8) (Heatherton et al 1991). The FTND questionnaire consists of 6 questions and a series of predetermined optional answers. Each answer is awarded a number of points, the sum total of these forms a score, which can determine the level of nicotine dependence.

For many years, questionnaires have provided information on individual smoking habits, and the information given has often been found to be incorrect compared to measurements taken in a laboratory (Sillett et al 1978, Wilcox et al 1979, Gillies et al 1982, Jarvis et al 1984, Peach et al 1985 and Nafstad et al 1995). Measurements using biological markers to determine the exposure to nicotine and multiple interviews regarding smoking habits over several years, suggest that 3 to 5 percent of smokers deny ever having smoked (Zhong et al 2000). A possible reason for this difference could be due to the attitude society has towards smoking; smokers are increasingly likely to underestimate their consumption of tobacco or even deny smoking altogether (Cope et al 1996). It is important, therefore, to be able to monitor patients attending a particular health clinic, such as smoking cessation or antenatal clinics, using quantitative or qualitative results. However, the only way to establish long-term exposure to nicotine is via questionnaires, as all other biological markers known are unable to account for long-term exposure to tobacco smoke (Zhong et al 2000).

QUESTION	ANSWER	POINTS
1. How soon after you wake up do you smoke your first cigarette	Within 5 minutes	3
	6 to 30 minutes	2
	31 to 60 minutes	1
	After 60 minutes	0
2. Do you find it difficult to refrain from smoking in places where it is forbidden, for example in church, at the library, in the cinema?	Yes	1
	No	0
3. Which cigarette would you hate most to give up?	The first one in the morning	1
	All of them	0
4. How many cigarettes per day do you smoke?	10 or less	0
	11 to 20	1
	21 to 30	2
	31 or more	3
5. Do you smoke more frequently during the first hours after waking than during the rest of the day?	Yes	1
	No	0
6. Do you smoke if you are so ill that you are in bed most of the day?	Yes	1
	No	0

SCORING		
	0 to 2	Very low dependence
	3 to 4	Low dependence
	5	Medium dependence
	6 to 7	High dependence
	8 to 10	Very high dependence

Table 1.8 Fagerström test for nicotine dependence

Nicotine is known to be the compound that establishes tobacco dependence, but this product only has a half-life of approximately two hours in serum, compared to cotinine which has a half-life of about seventeen hours in serum (Wall et al 1988). For this reason, nicotine is rarely used as a biological marker. Over the years alternative biological markers have been employed to determination smoking status, such as cotinine or compounds that are related to tobacco smoking, which have included thiocyanate (Hee et al 1995), carboxyhaemoglobin and carbon monoxide (Cope et al 1996 and Taniguchi et al 1999). However, the majority of analytical procedures use cotinine as the preferred biomarker of choice, due to its long half-life and it is also the primary derivative of nicotine. Other biological markers that have been used do not just represent an individual's smoking habit as they can be detected in non-smokers. For example, thiocyanate is found in leafy green vegetables and carbon monoxide is found in traffic fumes. Therefore, it may be difficult to distinguish a light smoker and a non-smoker who is exposed to high levels of traffic fumes, resulting in a false positive result.

Vesey et al (1982) reported that self-reported cigarette consumption does not correlate to intake of nicotine since smokers can vary their intake of smoke by regulating the length and depth of inhalation or by the number and size of puffs and this is another reason why questionnaires differ from laboratory measurements. From their data they concluded that the measurement of either carboxyhaemogloblin or plasma thiocyanate concentrations is superior to self-reported cigarette consumption as an index of exposure to tobacco smoke.

Techniques used to measure smoking status have included colorimetric (Peach et al 1985, Barlow et al 1987 and Smith et al 1998), radiommunoassay (Langone et al 1973 and Knight et al 1985), high performance liquid chromatography (HPLC) (Kyerematen et al 1982 and Moore et al 1990) and gas chromatography (GC) (Jacob et al 1981), using a samples taken from a variety of biological fluids, which include serum, saliva, urine and hair. Ideally samples used can be taken non-invasively, such as saliva, urine and hair, although hair samples have to be washed and pre-treated with sodium hydroxide overnight before cotinine analysis can be undertaken (Klein and Koren 1999). However, the advantage of using hair

samples is that it is possible to determine tobacco exposure over a longer period than if other biological markers were used (i.e. serum, saliva and urine).

Each of the above techniques has it own advantages and disadvantages. The colorimetric technique is simple, cheap (less than 10 pence per test), and if determined qualitatively, then no laboratory equipment is required. However, this technique is has limited sensitivity and can not be used to measure small quantities or individual amounts of nicotine and its metabolites. The advantage of using radioimmunoassay is that it is an established method, and is often referred to as the "gold standard" for analysis of cotinine. This technique is more sensitive than the colorimetric methods and can detect serum nicotine levels of 6-8 ng/mL of serum. However, the disadvantage is that it uses radioactive isotopes, it can only measure one metabolite, usually cotinine, but cross over from other metabolites, such as nicotine and 3′-hydroxycotinine, can occur.

Techniques like HPLC and GC can be more time consuming and require specialised equipment that can be expensive and is often only found in larger laboratories. Both HPLC and GC methods are more sensitive than the colorimetric technique, but to determine the small amounts of nicotine and its metabolites in environmental tobacco smoke then these techniques may be required to be coupled to a mass spectrometer. However, the advantage of HPLC and GC is that individual metabolites can be separated out, allowing metabolic profiles of the metabolism of nicotine to be studied.

1.6.1 High performance liquid chromatography techniques

HPLC techniques used to measure nicotine and its metabolites have included both UV (Zuccaro et al 1995 Nakajima et al 2000) and colorimetric methods of detection (Smith et al 1989, Moore et al 1990, Ubbink et al 1993, and Vindatiche et al 2000) using either pure nicotine or its metabolites or samples taken from biological fluids (for example urine, plasma or saliva).

The investigation into nicotine pharmacokinetics requires a rapid, sensitive and reliable technique. Kyerematen et al (1987) reported such a technique using a radiometric HPLC assay for determining the concentration of nicotine and twelve of it metabolites in plasma and urine. Initial metabolic studies were performed on male Sprague-Dawley rats and each rat received a single 0.1 mg/kg dose of nicotine ([2-^{14}C]pyrrolidine) containing 5 µCi. The mobile phase used was a binary gradient system consisting of solvent A: water: methanol: 0.1 M acetic acid buffer (pH 4.0): acetonitrile (187.5:11:1:0.5 v/v) and solvent B: water: methanol: 0.5 M acetic acid buffer (pH 4.0): acetonitrile (187.5:11:1:0.5 v/v) adjusted to pH 5.0 with triethylamine (0.10 percent, v/v).

Demetriou et al (1992) reported two radiochromatographic methods based on ion-pair HPLC and cation exchange HPLC. The reason why they chose radiometric detection was it enables the quantification of the known as well as the unknown nicotine metabolites on a molar basis. The two methods were then applied to investigate the metabolism of 2'-^{14}C-nicotine in rats' urine. The ion-pair HPLC method determined more than 40 different radioactive peaks, 18 of which were identified by comparison with reference compound, and the ion exchange HPLC method detected the major nicotine metabolites.

Determination of nicotine and its metabolites using UV detection mainly consisted of isocratic HPLC techniques. Metabolites separated out successfully using this technique include nicotine, cotinine and 3'-hydroxycotinine from urine samples (Zuccaro et al 1995), nicotine and cotinine from meconium samples (Baranowski et al 1998) and nicotine and cotinine either from urine samples (Oddoze et al 1998) or plasma samples (Nakajima et al 2000). Ghosheh et al (2000) also used plasma samples but were able to separate cotinine glucuronide, 3'-hydroxycotinine glucuronide, 3'-hydroxycotinine and caffeine as well as cotinine and nicotine. Cotinine glucuronide and 3'-hydroxycotinine glucuronide were determined indirectly via basic hydrolysis. This involved adding sodium hydroxide to the plasma sample and heating at 70 °C for 30 minutes. The mixture was then extracted using dichloromethane, the organic layer centrifuged and the supernatant analysed.

43

Seaton et al (1993) used a gradient HPLC method with UV detection to determine the concentration of nicotine N-glucuronide, cotinine N-glucuronide and 3'-hydroxycotinine in rat bile. The cotinine N-glucuronide standard was synthesised by another group, nicotine N-glucuronide was synthesised in house and 3'-hydroxycotinine glucuronide was measured indirectly because it had not yet been synthesised. Overall, the glucuronide standards of cotinine and nicotine provided an alternative method for the measurements of such metabolites. It was also suggested that this method could allow a more thorough investigation of the pathways of nicotine metabolism than previously possible using the indirect method, i.e. treating samples with the enzyme β glucuronidase.

1.6.2 Gas chromatography techniques

Curvall et al (1982) described a rapid and sensitive method for the determination of nicotine and cotinine in plasma using capillary column gas chromatography with nitrogen-sensitivity detection using N-methylanabasine and N-ethylnorcotinine as internal standards. The sensitivity and selectivity of the thermionic specific detector for nitrogen-containing compounds that they used allowed a relatively precise determination of small amounts of nicotine and cotinine. The detection limit at a signal to noise ratio of four was estimated to be 5 pg of nicotine and 20 pg of cotinine. The detector response was found to be linear and reproducible down to 5 pg of nicotine and 20 pg of cotinine, which corresponds to a minimal detectable amount of 0.1 ng of nicotine and 0.4 ng of cotinine per mL of plasma. Calibration curves were determined between 5 and 100 ng for nicotine and between 5 and 500 ng for cotinine per mL of plasma using 1 mL and 0.1 mL samples.

Domino et al (1992) looked at two GC methods previously described (Hariharan et al 1991 and Jacob et al 1991), a gas chromatographic-nitrogen specific detector (GC-ND) which had a column sensitivity of 10 pg for both nicotine and cotinine and a capillary gas chromatography-mass spectrometry (GC-MS) method. The capillary GC-MS method could simultaneously analyse both nicotine and cotinine using the corresponding

deuterated compounds as internal standards and had a limit of detection of 1 ng/mL for nicotine and 10 ng/mL for cotinine. Domino et al (1992) reported that they had improved the sensitivity of the capillary GC-MS method to enable 10 pg/mL of nicotine and 50 pg/mL of cotinine to be determined, using non-deuterated internal standards. The mean inter-assay coefficient of variation for nicotine was 5 percent and for cotinine 7 percent. Domino et al (1992) experience of nicotine and cotinine assays was limited to HPLC, GC-ND and GC-MS techniques. They suggested that HPLC is less expensive than the other two methods and is recommended for routine analysis. However, greater sensitivity can be achieved with GC-ND but for results that require high sensitivity and selectivity then they preferred to use GC-MS.

Baskin et al (1998) described a solid phase extraction method for determination of nicotine in serum and urine by isotope dilution GC-MS with selected ion monitoring. The method has a lower limit of determination of approximately 2 µg/mL and is linear to at least 2000 µg/mL. The within-run coefficients of variation were less than 4 percent for both assays over a nicotine concentration of 10-200 µg/mL. The between-run coefficients of variation in serum were 5.4, 5.2, 4.8 and 5.9, respectively, at nicotine concentrations of 10, 15, 25 and 50 µg/mL, and for the urine assays were 5.9, 4.5, 2.7 and 5.2, respectively at nicotine concentrations of 100, 250, 500 and 2000 µg/mL. The absolute recovery of nicotine was 61± 6 percent (mean ± SD) over the range of 10-250 µg/mL.

Jacob et al (1981) described an improved GC method for nicotine and cotinine determination that had been previously described (Langone et al 1973, Falkman et al 1975 and Feyerabend et al 1979). The internal standard used by the previous methods was quinoline but the results obtained in these studies were unable to be reproduced by Jacob et al (1981). They suggested that quinoline was a poor choice as nicotine is considerable more basic (pK_a 8.0) than quinoline (pK_a 4.9) and synthesised a new internal standard N-ethylnornicotine, a structural analog with chemical properties similar to nicotine. They also improved the previously described extraction procedure for extracting cotinine from whole blood or plasma (Beckett 1966 and Hengen and Hengen 1978) by using 1-

butanol instead of dichloromethane, which resulted in less emulsion forming and a cleaner separation of layers.

1.6.3 The König reaction

The colorimetric method (commonly known as the König reaction) used to detect urinary concentrations of nicotine and its metabolites, was first described by Peach et al in 1985 (further details on the König reaction are described in chapter 2). This method involves the addition of sodium acetate buffer, potassium cyanide, chloramine-T and a condensing reagent, barbituric acid, to a urine sample. Peach et al (1985) reported that when the condensing reagent, barbituric acid was used then red chromophores were formed, that were not extractable into ethyl acetate. On changing the condensing reagent to a less polar compound, diethyl thiobarbituric acid (DETB) they found that the chromophores produced were extractable into ethyl acetate. Using measurements taken from urine samples from 103 male smokers and 78 male non-smokers, they found that the false positive rate for the DETB chromophores extracted into ethyl acetate was 3 percent, compared to 12 percent and 6 percent for DETB or barbituric respectively obtained when extraction was not carried out.

Barlow et al (1987) compared the quantitative direct barbituric acid (DBA) with a qualitative DBA method and an established cotinine radioimmunoassay (RIA). They used 128 reported smokers and 383 reported non-smokers. The quantitative DBA method results correlated with the RIA results, (r = 0.85), and coefficient of variation was 6 percent for the quantitative DBA method and 10 percent for the RIA method. Both the qualitative and quantitative DBA methods gave similar limits of detection rates of 91 percent and 93 percent, respectively, which corresponded to a false positive rate of 3 percent.

Barlow et al (1987) had also automated the quantitative DBA method, using a Flow Laboratories micronic tube system. This system increased sample throughput (approximately 170 samples per day), increased the speed at which the absorbance of each sample, within an analytical batch, could be measured (this was necessary since the coloured product is unstable, decaying at a rate of about 10 percent in 5 minutes) and also enabled a reduction in both sample and reagent volume. This method was reported to have an assay linear of at least 0-250 µmol/L cotinine.

Smith et al (1998) reported using the König reaction to measure urinary nicotine metabolites to assess smoking status in 251 diabetic subjects. Measurements were made qualitatively and quantitatively using DETB as the condensing reagent. Qualitative measurements were extracted into ethyl acetate and overall performance achieved a sensitivity of 95 percent and specificity of 100 percent. However, the quantitative results performed less well with a sensitivity of 92 percent and specificity of 97 percent.

The derivatives obtained from the König reaction have also been determined by various HPLC methods. O'Doherty et al (1990) used neat metabolites, 3-pyridyl acetic acid, *trans*-3′-hydroxycotinine, cotinine, nicotine, N^1-acetylnornicotine and demethylcotinine derivatized with either DETB or DMDD. Moore et al (1990) determined nicotine, cotinine and 3′-hydroxycotinine in urine samples derivatized with DETB using a gradient HPLC method. Mariner et al (1992) studied an automated HPLC method using the analytical procedure described by Moore et al (1990) to study nicotine, cotinine and 3′-hydroxycotinine and treatment with β-glucuronidase in urine. Ubbink et al (1993) also used urine samples to separate out cotinine and nicotine but they used barbituric acid as the condensing reagent. Vindatiche et al (2000) optimised the conditions of the König reaction to enable nicotine and its metabolites cotinine, *trans*-3′-hydroxycotinine and pyridylcarbinol to be measured in urine samples derivatized with thiobarbituric acid. Modifications included the addition of sodium hydroxide to the reaction mixture and then the reaction mixture was then centrifuged, the supernatant filtered and analysed but no time was stated to leave the reaction before analysis.

Chapter 2

The König reaction

2.1 Introduction

The König reaction was first described in 1904 (König 1904) and over the years has been used to measure compounds like cyanide, thiocyanate (Aldridge 1944) and derivatives of pyridine such as nicotinic acid (Leifer et al 1950 and Huebner 1951). More recently, this reaction was developed as a simple colorimetric test to measure urinary concentrations of isonicotinic acid and isonicotinylglycine, the major metabolites of the antituberculosis drug isoniazid (Ellard et al 1972). This test involves the sequential addition of sodium acetate buffer, potassium cyanide, chloramine-T and a condensing reagent, barbituric acid to a urine sample. During observations made whilst measuring urinary concentrations of isonicotinic acid and isonicotinylglycine, Peach et al (1985) reported that urine samples containing isonicotinic acid taken from non-smokers gave a characteristic blue colour, compared to urine samples taken from smokers not taking isoniazid, which gave a distinctive orange colour. The colour of the urine samples observed from smokers taking isoniazid was not reported, although it was later reported that the derivatives that formed the blue chromophores were not extractable in ethyl acetate (Ellard 1995). It was therefore suggested, that this orange colour could be due to nicotine and its metabolites.

Peach et al (1985) carried out a series of experiments using the König reaction and reported that this reaction could be used as a novel, simple and inexpensive urine test to determine smoking status. They also improved this reaction by changing the condensing reagent from the previously used condensing reagent, barbituric acid, to 1,3-diethyl-2-thiobarbituric acid (DETB), which forms pink-red chromophores, rather than orange and which are also readily extracted into ethyl acetate. The reason why they

48

wanted to extract the pink chromophores into ethyl acetate was to increase the sensitivity of qualitative or semi quantitative measurements.

The chemistry involved in the König reaction when used in this way is not understood with certainty, although reaction mechanisms have been suggested by several groups (O'Doherty et al 1990 and Rustemeier et al 1993). Both workers proposed that the oxidising agent, chloramine-T first oxidises potassium cyanide to form cyanogen chloride. Once cyanogen chloride is produced, the pyridine ring of the nicotine metabolite is broken, which enables a condensing reagent (for example, barbituric acid or DETB) to react with the nicotine metabolite to form either structure 1 suggested by O'Doherty et al (1990) or structure 2, suggested by Rustemeier et al (1993) (figure 2.1).

It has been assumed that this reaction may be applied to the majority of the nicotine metabolites, and indeed is often referred to as a test for "total nicotine metabolites". However, Rustemeier et al (1993) put forward the suggestion that only nicotine metabolites that contain an intact pyridine ring and also have no other atoms or groups attached to the nitrogen atom will react. Therefore, the majority of nicotine metabolites may be expected to undergo this reaction, with the exception of only a few metabolites such as cotinine-*N*-oxide and the *N*-glucuronides of cotinine and nicotine (figure 2.2).

Potassium cyanide + Chloramine-T ⟶ Cyanogen chloride

Cyanogen chloride +

Nicotine metabolite

Glutacondialdehyde derivative
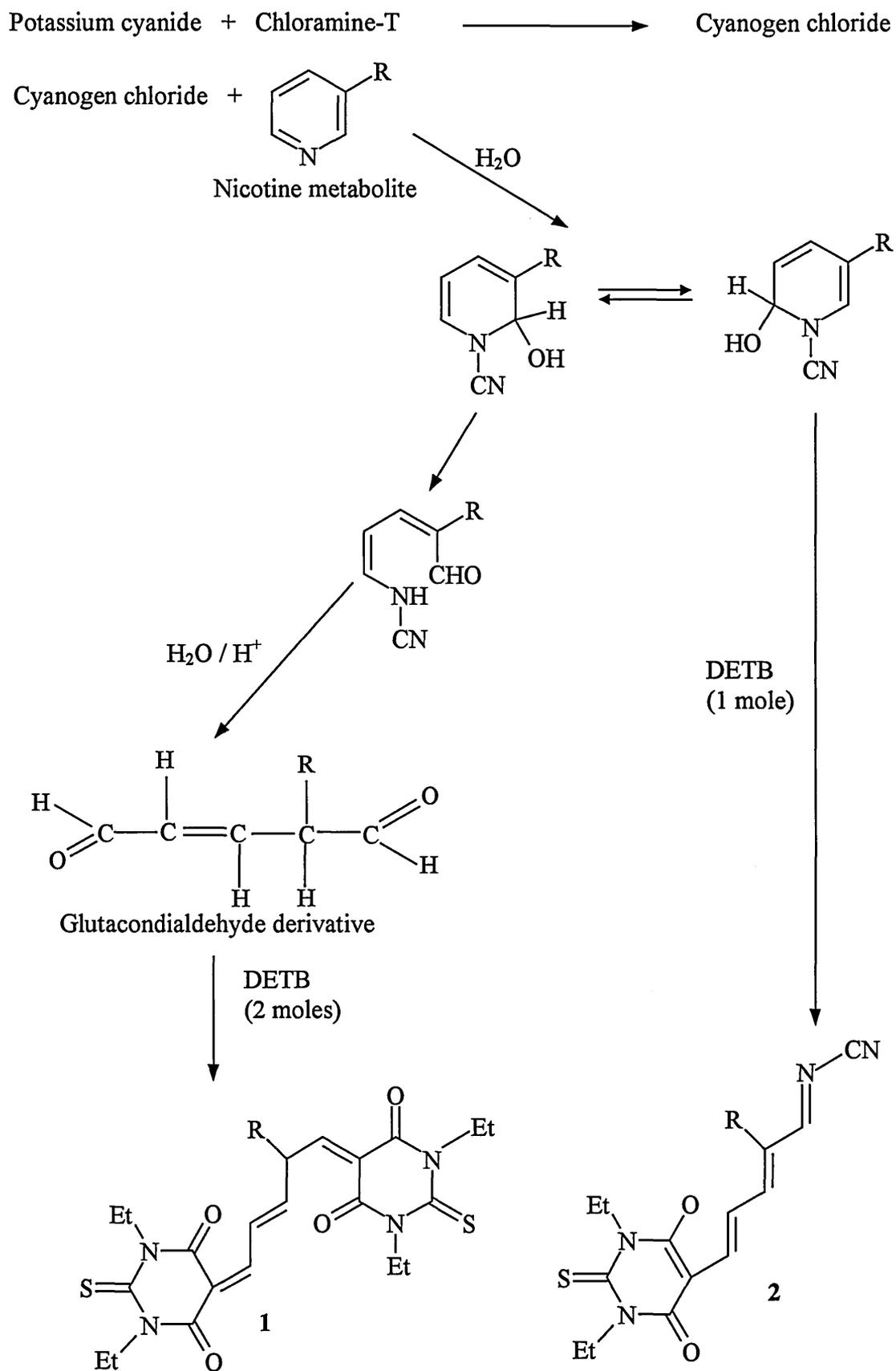
Figure 2.1 Possible mechanisms for DETB derived nicotine metabolites as proposed by O'Doherty et al (1990) (**1**) and Rustemeier et al (1993) (**2**)

50

Nicotine *N*-glucuronide   Cotinine *N*-glucuronide
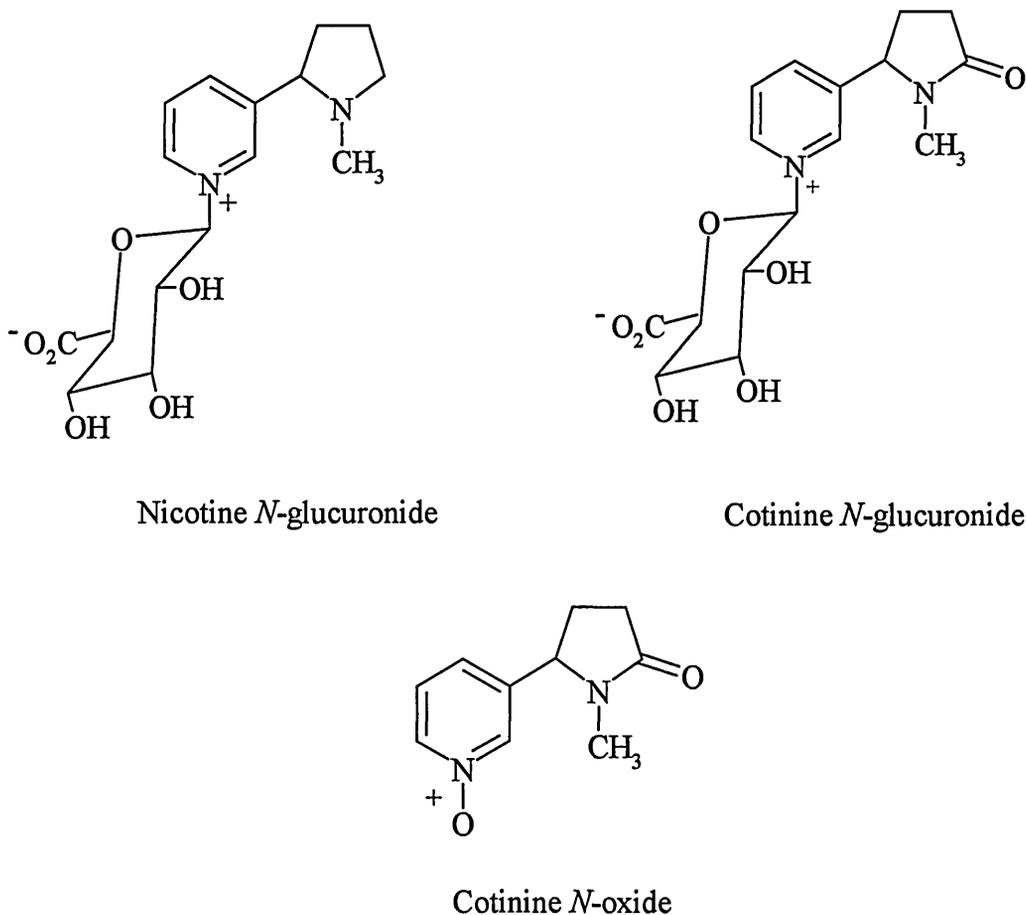
Cotinine *N*-oxide

Figure 2.2 Structures of cotinine-*N*-oxide and the *N*-glucuronides

The König reaction may be used to measure nicotine metabolites present in a biological sample. For purposes of quantification the unknown sample is often standardised against a known concentration of cotinine and the result expressed as "cotinine equivalent". However, there is no real reason why cotinine should be used, except that it has been thought to be the major nicotine metabolite and therefore has become the most commonly used marker for the assessment of exposure to nicotine. It would therefore be as justifiable to express the total nicotine metabolite concentration as nicotine or 3′-hydroxycotinine equivalent concentrations rather than cotinine, although throughout this research cotinine equivalent concentrations have been used. Another reason why cotinine is used to determine nicotine exposure is due to its longer half-life of approximately 17 hours in serum, compared to nicotine which has a half-life of only 2 hours in serum (Wall et al 1988).

The main aim of the work described in this chapter was to extend the study of the König reaction and its relationship to nicotine and its metabolites. Various studies have looked briefly at the relationship between the König reaction and certain nicotine metabolites, such as nicotine, cotinine and 3′-hydroxycotinine (Smith and Cooke 1987, O'Doherty et al 1990 and Rustemeier et al 1993). However, no other work has been reported or extended to include a larger range of possible metabolites.

Detailed investigations of the König reaction were carried out using four condensing reagents (figure 2.3), 1,3-diethyl-2-thiobarbituric acid (DETB), 1,3-dibutyl-2-thiobarbituric acid (DBTB), 2,2-dimethyl-1,3-dioxane-4,6-dione (commercially known as Meldrum's acid) (DMDD) and 2-methyl-2-(2-phenylethyl)-1,3-dioxane-4,6-dione (MPED).

DETB

DBTB

DMDD

MPED
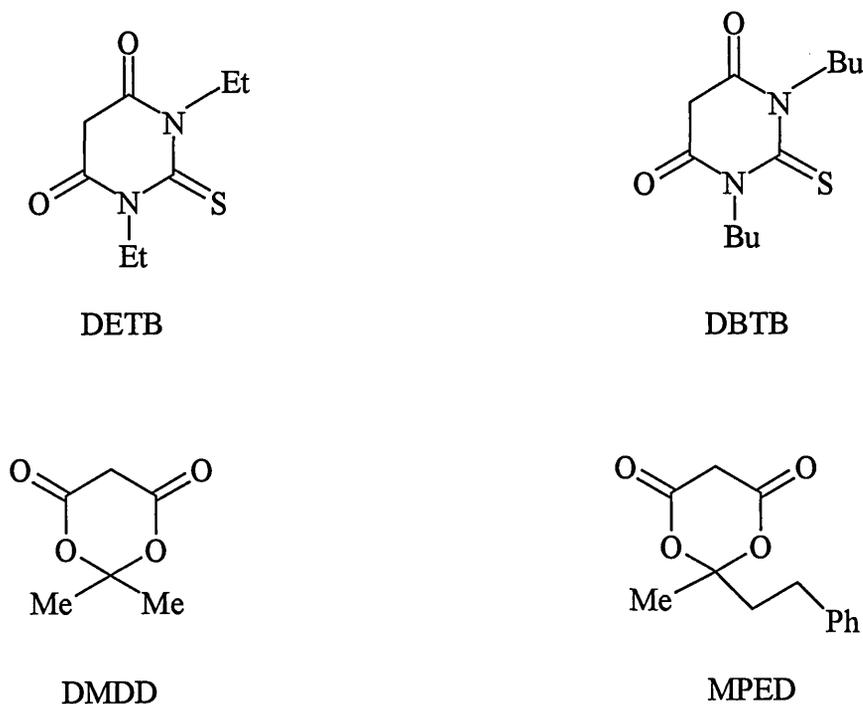
Figure 2.3 Structures of the four condensing reagents

Both DETB and DMDD have been used as condensing reagents in previous studies (Peach et al 1985 and O'Doherty et al 1990). However, when the coloured derivative produced using DETB or DMDD was extracted into ethyl acetate, it was observed that the colour was not fully extracted, although DETB derivatives appeared to be extracted more efficiently than when DMDD was used. Since DETB is less polar than DMDD, it was decided to synthesise the novel more non-polar condensing reagents DBTB and MPED in order to investigate the properties of derivatives obtained from these compounds.

The aim of synthesising these more non-polar condensing reagents was to try to improve the degree of extraction into organic solvent in order to enhance the overall sensitivity of the test. An increase in sensitivity might be expected to make qualitative assessment of smoking status more easily distinguishable between urine samples taken from smokers and non-smokers and therefore, might also reduce the number of samples misclassified, i.e. the number of false positives and false negatives. A result from an individual who is a smoker but is classified as a non-smoker by a diagnostic test is referred to as a false negative result, whereas false positive results refer to a non-smoker who is classified as a smoker by a laboratory test. Furthermore, if the König reaction could be further enhanced so as to sufficiently increase the overall assay sensitivity, allowing very small quantities of nicotine metabolites to be detected, then it might be possible to use this reaction as a simple means for determining the exposure to environmental tobacco smoke or to measure cotinine in samples other than urine, for example, saliva, where the concentration is considerably lower.

The only major nicotine metabolites that were not available for this research project were the glucuronides of cotinine, nicotine and 3′-hydroxycotinine. One established method for the determination of the concentrations of glucuronides in a urine sample is to use the enzyme β-glucuronidase, an enzyme that cleaves the bond joining the carbohydrate residue to the nicotine-derived portion (Bryd et a 1992 and Rustemeier et al 1993). The glucuronide can be either an O-glucuronide (3′-hydroxycotinine) or an N-glucuronide (nicotine and cotinine) (figure 2.4). Once a urine sample has been treated with β-glucuronidase, the sugar residue is no longer associated with the nicotine-derived portion, enabling the metabolite to be measured using the König reaction. However, it is

highly unlikely that the glucuronides, especially those of cotinine and nicotine, will react using the König reaction, and as there was no standard solution available to compare a concentration of a known glucuronide to an unknown urine sample the synthesis of cotinine N-glucuronide was attempted.

Nicotine N-glucuronide

Cotinine N-glucuronide

3'-Hydroxycotinine O-glucuronide
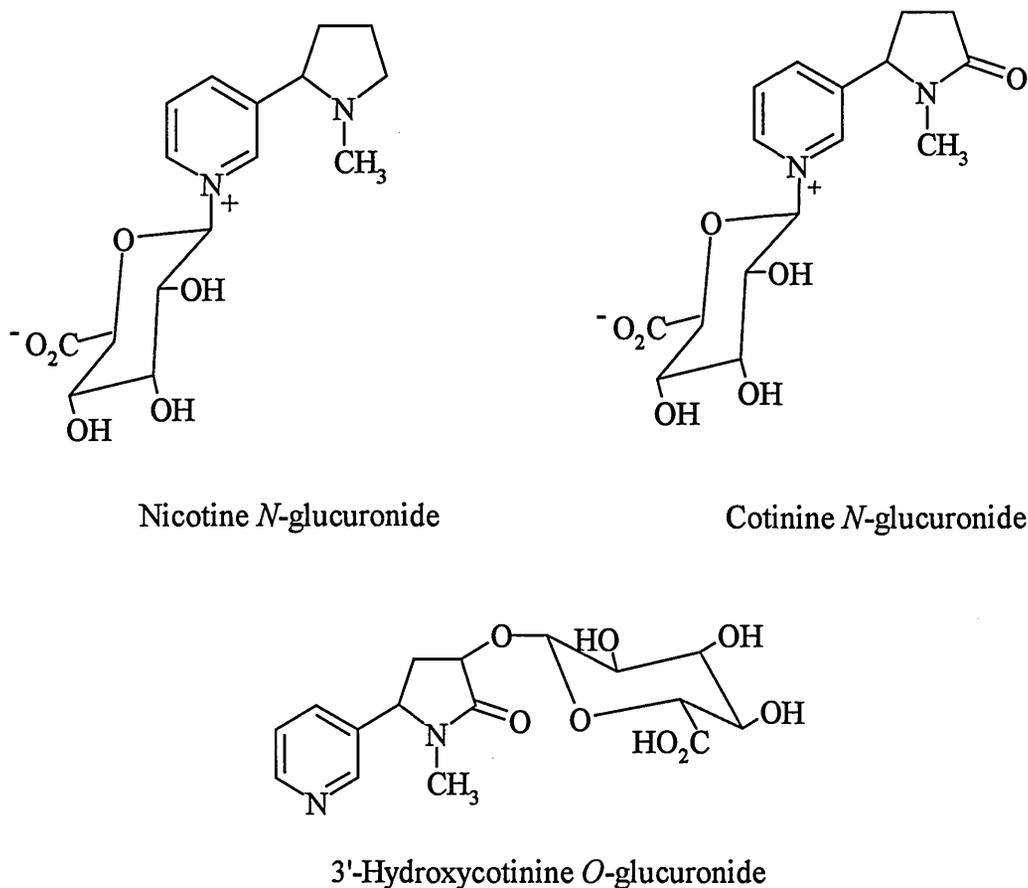
Figure 2.4 Structures of the O-glucuronide and N-glucuronides

2.2 Materials and methods

Potassium cyanide and chloramine-T were purchased from Merck BDH. *N*, *N*-Dibutyl-2-thiourea, 4-phenyl-2-butanone, diethyl malonate and Dowex 50X were purchased from Aldrich (Gillingham, UK). Amberlite XAD-4 was purchased from Fisher Scientific (Loughborough, UK). 1,3-Diethyl-2-thiobarbituric acid, malonic acid, glucuronolactone, 30% hydrogen bromide in acetic acid and β-glucuronidase were all purchased from Sigma (Poole, Dorset. UK). Methanol, ethyl acetate and acetone were all general-purpose reagent grades. Nicotine metabolites nicotine, cotinine and 3-pyridyl acetic acid were purchased from sigma and nornicotine, norcotinine, 5′-hydroxycotinine, 4-3-pyridyl-4-oxo-*N*-methyl butyramide, *trans*-3′-hydroxycotinine, 4-3-pyridyl-4-oxo butyric acid, cotinine-*N*-oxide and nicotine-1′*N*-oxide were a kind gift from Professor Peyton Jacob, USA. Absorbance readings were obtained using a Cecil 9500 Super Aquarius. The NMR spectra were obtained using a Bruker AC 250.

2.2.1 Synthesis of 1,3-dibutyl-2-thiobarbituric acid

1,3-Dibutyl-2-thiobarbituric acid was prepared according to the modified method of Hahn et al (1990). Sodium (6.5 g washed with petroleum spirits and dried) was placed in a round bottom flask containing methanol (75 mL). *N*, *N*-Dibutyl-2-thiourea (10 g, 0.076 mol) and diethylmalonate (17.5 g, 0.1 mole) were added to the flask and the resulting reaction mixture was allowed to reflux for at least 36 hours. After this period water (50 mL) was added to the solution and partly evaporated under pressure to remove any remaining methanol. Any white precipitate (unreacted thiourea) which may have formed during evaporation was removed by filtration under reduced pressure. The solution was further diluted with water (100 mL), chilled on ice and then acidified with concentrated hydrochloric acid to a pH between 1 and 2. The resulting precipitate was collected and dried under reduced pressure producing a light yellow powder 1,3-dibutyl-2-thiobarbituric acid (9.5 g). Melting point 56-60^{0}C. ^{1}H NMR (250MHz; CDCl$_3$) δ ppm 0.94 (6H, t, *J*7.5, CH$_3$x2); 1.35 (4H, m, CH$_2$C̲H̲$_2$CH$_3$); 1.6(4H, m, C̲H̲$_2$CH$_2$CH$_3$); 3.7 (2H, s, O=CC̲H̲$_2$C=O); 4.3 (4H, t, *J*7.5-NC̲H̲$_2$CH$_2$-)

2.2.2 Synthesis of 2-methyl-2- (2-phenylethyl)-1,3-dioxane-4, 6-dione

To a mixture of malonic acid (13.5 g, 0.13 mol), acetic anhydride (16.2 g, 15 mL, 0.16 mol) and concentrated sulphuric acid (0.5 mL) was added 4-phenyl-2-butanone (22.2 g, 0.15 mol). The mixture was stirred at room temperature overnight and poured into water (200 mL) to afford an oil, which crystallised on standing. The solid product was filtered off, dried and recrystallised from ethyl acetate/ petroleum spirits to give colourless crystals (10.3 g, 34%). Melting point 82-84°C. ^{1}H NMR (250MHz; CDCl$_3$) δ ppm 1.79 (3H, s, CH$_3$); 2.26 (2H, m, CH$_2$); 2.80 (2H, m, CH$_2$); 7.28 (5H, m, ArH); ^{13}C NMR (62.5 MHz; CDCl$_3$) δ ppm 26.31, 29.40, 36.58, 42.41, 107.60, 126.81, 128.66, 129.04, 140.19, 163.47.

2.2.3 Attempted synthesis of cotinine-*N*-glucuronide

The attempted synthesis of cotinine *N*-glucuronide was carried out using a modified method as described by Caldwell et al (1992). The procedure involved using a four step synthesis (figure 2.5).

Step 1: Synthesis of methyl tetra-*O*-acetyl-β-D-glucopyranurate **4**.

Glucuronolactone **3** (20 g) was stirred with methanol (155 mL) containing sodium hydroxide (55 mg) for 1 hour at ambient temperature. The methanol was removed under reduced pressure, keeping the bath temperature less than 50 °C. A mixture of acetic anhydride (75.5 mL) and pyridine (55 mL) was then added to the remaining residue. Note that when the acetic anhydride and pyridine solutions were added to the remaining residue, all solutions were pre-cooled on ice to ensure the temperature of the solution did not exceed 40 °C. The solution was then left overnight in the fridge to provide the solid product **4** (7.24 g). Melting point 168-176 °C, literature melting point (Caldwell et al 1992) 177°C.
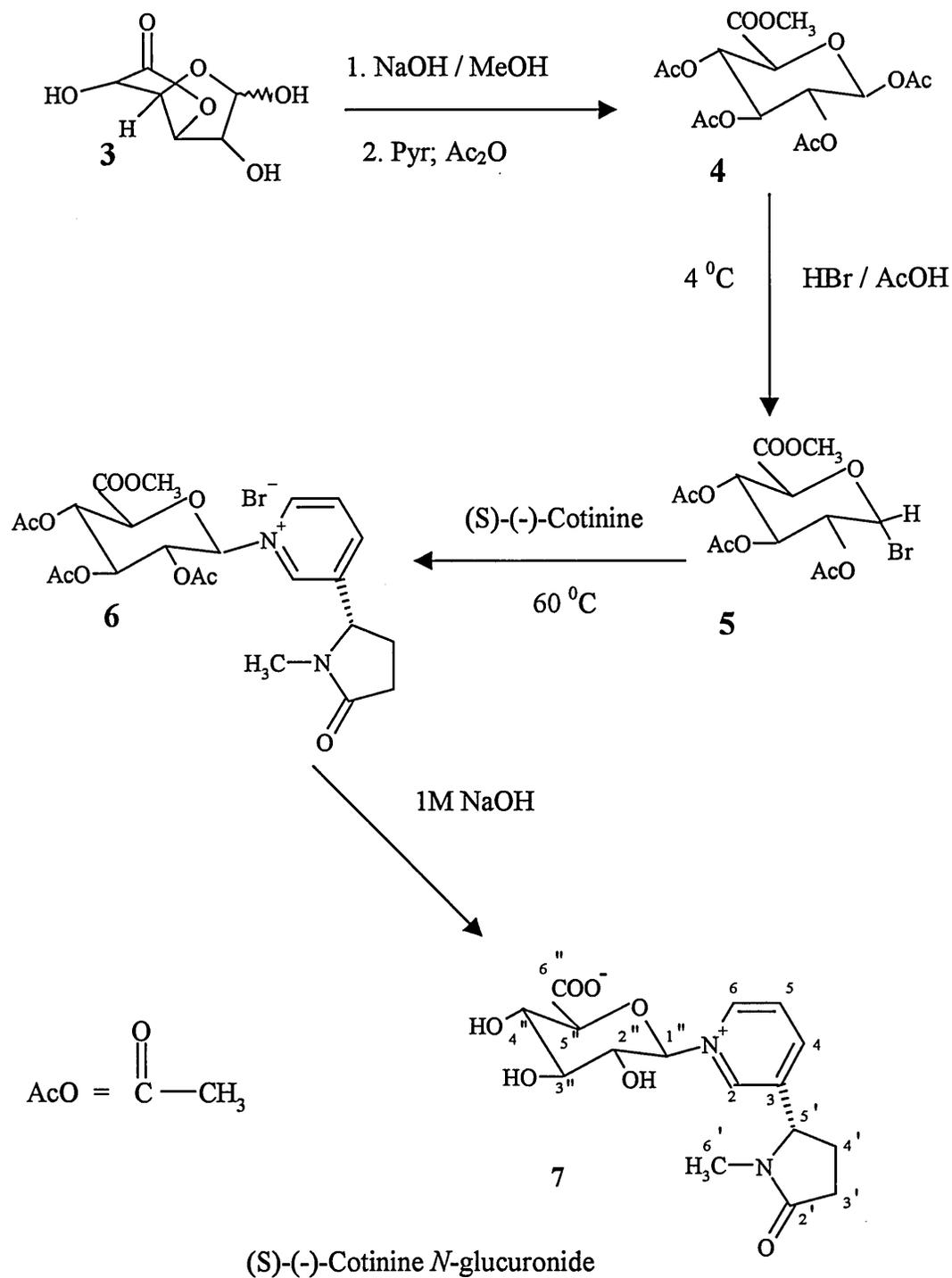
Figure 2.5 Synthesis of *N*-β-D-glucopyranuronosyl-(s)-(-)cotininium inner salt (GA)$^{\alpha}$ (cotinine *N*-glucuronide) (Caldwell et al 1992)

Step 2: Synthesis of methyl 2,3,4-tri-*O*-acetyl-1-bromo-1-deoxy-α-ᴅ-glucopyranurate **5**.

Compound **4** (7.24 g) was added to 30% hydrogen bromide in acetic acid (36 mL) and allowed to stand overnight at 4°C. The resulting residue was dissolved in dichloromethane (15 mL), and the organic solution extracted with saturated sodium bicarbonate solution (15 mL), followed by water (15 mL). The resulting dichloromethane layer was dried with magnesium sulphate and the solvent evaporated. The residue was crystallised from absolute ethanol to afford **5** as a cream-coloured crystalline solid (5.1 g). (Note this product readily decomposes if left at ambient temperature, and therefore it is best stored at –20 °C). Melting point 74-78 °C, literature melting point (Caldwell et al 1992) 102-105°C. TLC ethyl acetate/ petroleum spirits 30:70 R_f 0.80, (TLC plate developed with ceric ammonium sulphate). ^{1}H NMR (250MHz; $CDCl_3$) δ ppm 2.04-2.08 (9H, 3 x s, CH_3COO); 3.75 (3H, s, $COOCH_3$); 4.57 (1H, d, *J*10.2); 4.84 (1H, dd, *J*10.0, 4.0); 5.23 (1H, t, *J*10.0); 5.61 (1H, t, *J*9.7); 6.64 (1H, d, *J*4.1).

Step 3: Synthesis of *N*-(2,3,4-tri-*O*-acetyl-6-methyl-β-ᴅ-glucopyranuronosyl)-(S)-(-)-continium bromide **6**.

Compound **5** (2.37 g) was added to cotinine (0.2 g) and acetonitrile (10 mL) and refluxed under nitrogen for 4 days. The acetonitrile was removed under pressure. The resulting melt was partitioned between water and diethyl ether (5 x 20 mL). The aqueous layer was eluted through an XAD-4 column, followed by two bed volumes of distilled water. The aqueous layer was collected and the water evaporated under reduced pressure. From the NMR, it appeared that **6** had been synthesised, although impure, and therefore it was further purified on a silica column using dichloromethane/methanol 9:1 as the mobile phase. Once purified, the fractions containing **6** were evaporated under reduced pressure, leaving the solid product **6** (148 mg). TLC dichloromethane/methanol 9:1, R_f 0.3. NMR spectrum before purification the following peaks were observed, ^{1}H NMR (250MHz; $CDCl_3$) δ ppm 9.41-9.32 (m), 8.81- 8.78 (d), 8.34-8.29 (t), 6.44 (d), 5.72-5.69 (d), 5.6-5.5 (m), 4.93-4.86 (m), 3.67 (s), 2.29 (s), 2.14 (s), 1.86 (s), 1.78 (s) other peaks were also observed 3.36 (s), 2.50-2.54 (m), 1.23-1.37 (m), 0.8-0.89 (m) which are believed to be solvent residues and 7.68-

7.69 (m), 4.11-4.14(d), 3.9-3.93 (d) are believed to be impurities. NMR spectrum for **6** (Caldwell et al 1992) ^{1}H NMR (DMSO-d_6) δ 9.45-9.32 (2 H, m, pyridinium H-2 and H-6), 8.84-8.80 (1 H, d, pyridinium H-4), 8.40-8.30 (1 H, t, pyridinium H-5), 6.40 (1 H, d, H-1''), 5.80-5.70 (2 H, m, H-2'' and H-5'), 5.58-5.48 (1 H, t, H-4''), 4.83-4.95 (3 H, m, H-3'', H-4' and H-5'), 3.70 (3 H, s, CO$_2$CH$_3$), 2.52 (3 H, s, NCH$_3$), 2.23-2.43 (3 H, m, H-3'a, H-3'b and H-4'b), 2.12-2.20 (1 H, m, H-4'a), 1.8, 1.9, 2.1 (9 H, 3 X s, 3 X OCOCH$_3$).

Step 4: synthesis of *N*-β-D-glucopyranuronosyl-(S)-(-)-cotininium inner salt **7**

Compound **6** (148 mg) was dissolved in sodium hydroxide (1 mL, 1 M), and the solution was left at ambient temperature for 16 hours. The pH of the sample was then adjusted to 7.0 with dilute aqueous acetic acid, and the mixture was applied to a column containing strong cation-exchange resin (Dowex 50X 1, 50 – 100 mesh, 7 x 12 cm). The column was eluted with 4 bed volumes of distilled water, followed by 2 M aqueous ammonia solution. The first UV-absorbing basic fractions were collected and evaporated under reduced pressure but no significant amount of **7** was produced.

2.2.4 The König reaction

The König reaction, based on the method described by Peach et al (1985) was used to determine the total nicotine metabolite concentration in urine samples. The method involved taking a known concentration of an individual nicotine metabolite dissolved in distilled water (1 mL) or a neat urine sample (1 mL) and mixing with sodium acetate buffer (400 μL, 4 M, pH 4.7). This was then followed by potassium cyanide (200 μL, 10% w/v) and chloramine-T (200 μL, 10%w/v). The solution was mixed well, before the addition of a condensing reagent (1 mL, 1% w/v, DBTB and MPED in acetone, DETB and DMDD 50:50 acetone: water). The reaction mixture was again mixed well and allowed to stand for 20 minutes.

2.2.4.1 Qualitative or quantitative measurements of the König reaction

The König reaction can be used qualitatively or quantitatively. Qualitative determinations were made by a simple observation of a pink colour either in the aqueous layer or after extraction in ethyl acetate (1 mL). Quantitative measurements were determined either by measuring the absorbance of the aqueous layer (aqueous results) or of the organic layer after extraction with ethyl acetate (1 mL) (organic results), at the appropriate wavelengths. The spectrophotometer was set to zero using a sample blank, which was analysed following the method above except that chloramine-T was excluded.

An example is given below of the calculation to determine the concentration of a urine sample derivatized with DETB

Absorbance obtained from urine sample = 0.120

Absorbance obtained from cotinine (10 μg/mL) standard solution = 0.644

$$\frac{\text{Absorbance of urine sample}}{\text{Absorbance of standard}} \times \text{concentration of cotinine} \quad \frac{0.120}{0.644} \times 10 = 1.86 \ \mu g/mL$$

2.2.5 Relative molar absorptivity and partition coefficients

The relative molar absorptivity and partition coefficients for individual nicotine metabolites were determined using the König reaction as in 2.2.4. When nicotine metabolites were derivatized with either DETB or DBTB a 5 μg/mL stock solution was used, except for 4-3-pyridyl-4-oxo methyl butyramide and *trans*-3′-hydroxycotinine where a 10 μg/mL stock solution was used and for 4-3-pyridyl-4-oxo butyric acid when a 20 μg/mL solution was used. When metabolites were derivatized with either DMDD or MPED, a 10 μg/mL solution was used for all relative molar absorptivities and

partition coefficients. The absorbance maximum for each nicotine metabolite after being subjected to the König reaction was determined by measuring the absorbance between 400 nm and 700 nm. The absorbance of each nicotine metabolite was then measured, post derivatization, using the aqueous layer (aqueous results), organic layer (organic results) and the aqueous layer post extraction (post extraction aqueous results) at their absorbance maxima using each of the four condensing reagents (figure 2.6). Each nicotine metabolite was measured at least twice.
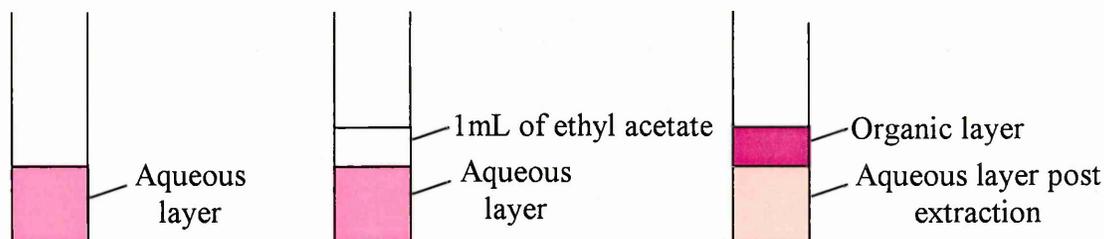
Figure 2.6 Schematic diagram that represents the extracted and unextracted phases when using DETB or DBTB.

The absorbance values for each nicotine metabolite were obtained and appropriate calculations were carried out to determine both the relative molar absorptivity and partition coefficient. The relative molar absorptivity measurements were determined using the aqueous results, whereas the partition coefficients require the organic and post extraction aqueous results.

The relative molar absorptivities were calculated using the Beer-Lambert Law,

$$A = \varepsilon bc$$

Where A represents the absorbance, ε is known as the molar absorptivity and is expressed as litres per mole per centimetre (L mol^{-1} cm^{-1}), b represents the pathlength which is usually expressed in centimeters and c is the concentration of the sample in moles per litre.

Therefore, molar absorptivity, $\varepsilon = \dfrac{A}{bc}$

An example of the calculation of relative molar absorptivity using cotinine derivatized with DETB.

RMM of cotinine = 176, pathlength = 1 cm

Concentration of cotinine used = 5 µg/mL or 0.005 g/L

Molar concentration used:

$$\dfrac{\text{concentration of cotinine}}{\text{RMM}} \qquad \dfrac{0.005}{176} \qquad = 2.84 \times 10^{-5}$$

However, the sample was diluted to 2.8 mL (total volume of reaction mixture)

$$\dfrac{\text{Molar concentration}}{\text{Dilution factor}} \qquad \dfrac{2.84 \times 10^{-5}}{2.8} \qquad = 1.01 \times 10^{-5} \text{ g/L}$$

Average cotinine absorbance = 0.421

$$\varepsilon = \dfrac{A}{bc} \qquad \dfrac{0.421}{1.01 \times 10^{-5} \times 1} \qquad = 4.1 \times 10^{4} \text{ L M}^{-1}\text{cm}^{-1}$$

Partition coefficients were calculated using the following equation:

Partition coefficient, $k = \dfrac{A_{s2}}{A_{s1}}$

Where A_{s2} represents the absorbance obtained from the organic layer (solute 2) and A_{s1} represents the absorbance obtained from the aqueous layer post extraction (solute 1).

An example is given below for the calculation of the partition coefficient using cotinine 5 µg/mL derivatized with DETB

Average absorbance obtained from organic layer = 0.819

Average absorbance obtained from the aqueous layer post extraction = 0.140

$$\dfrac{A_{s2}}{A_{s1}} \qquad \dfrac{0.819}{0.140} \qquad = 6$$

2.2.6 Cotinine calibration curves

The aim of this experiment was to investigate the claim of Smith and Cooke (1987) that the König reaction does not follow the Beer-Lambert Law. Their findings suggested that the sum of the absorbance values obtained for three individual metabolites (nicotine, cotinine and nicotine-1'N-oxide) were not the same as the predicted absorbance value when a mixture of the same three metabolites was used. From these results, they concluded that their results indicated that the König reaction significantly departs from the classical behaviour of the Beer-Lambert Law, which could have implications for the reliability of the method as a quantitative analytical tool.

To determine if the König reaction followed the Beer-Lambert Law, calibration curves were prepared using pure cotinine dissolved in distilled water. The concentration of cotinine used for the calibration curves ranged from 4 µg/mL to 20 µg/mL. Measurements were taken at the optimum wavelength using all four condensing reagents. Each sample was measured in triplicate, and the results presented as the average absorbance.

2.2.7 Optimum reaction time courses

Smith and Cooke (1987) further suggested that the time required for the reaction to reach completion was unique to each of the compounds they investigated (nicotine, cotinine and nicotine-1'N-oxide). In order to investigate this observation further individual reaction time courses were obtained using these three compounds and a further eight nicotine metabolites. Therefore, to determine the optimum time to leave the König reaction to measure the maximum total nicotine metabolite concentration, individual nicotine metabolite time courses were carried out. Once the König reaction (2.2.4) had been initiated with one of the four condensing reagents, the solution was mixed well and transferred into a cuvette. A spectrophotometer was set to record the absorbance values every four minutes, for a total of forty minutes.

2.2.8 Imprecision studies

Imprecision measurements were made using a "pooled" urine sample taken from five confirmed smokers. The pooled urine sample was subdivided into ten aliquots (1 mL) and each aliquot was subjected to the König reaction (2.2.4) using each of the condensing reagents. The concentration of each aliquot was determined by comparison with the absorbance value obtained from a cotinine (10 µg/mL) standard solution and the coefficient of variation calculated.

2.2.9 Determination of the effects of β-glucuronidase

Byrd et al (1992) described a method to quantify the glucuronide concentration of cotinine, nicotine and 3′-hydroxycotinine in urine samples by treating the sample with the enzyme β-glucuronidase. Modifications were made to this method in order to determine the optimal amount of β-glucuronidase required. A urine sample (10 mL) was taken from a self-reported smoker and adjusted to pH 5.0 with 4 M hydrochloric acid (0.5 mL). An aliquot of this sample (1 mL) was taken and a known amount of β-glucuronidase was added. The sample was then incubated at 37^0C for approximately twenty-four hours. To determine the optimum amount of β-glucuronidase, different amounts of the enzyme were prepared between 500 IU and 1500 IU (table 2.1). This range was chosen because Byrd et al (1992) had used 3650 IU of β-glucuronidase per 3 mL or 1216.7 IU per mL.

Calculations to decide the amount of β-glucuronidase required were carried out using the following equation.

$$\frac{\text{Number of IU required per mL}}{\text{IU of stock solution of β-glucuronidase per μL}} = \text{amount required in μL}$$

IU of β-glucuronidase per mL (obtained from label on the β-glucuronidase bottle)

= 89400 IU/mL or 89.4 IU/ μL

For example:
Amount required for 1000 IU $\quad \frac{1000}{89.4} \quad = 11.19 \, μL$

For example:
Number of IU in 13.5 μL $\quad 89.4 \times 13.5 \quad = 1206.9 \, \text{IU/mL of urine}$

IU REQUIRED PER ML OF URINE	μL REQUIRED PER ML OF URINE	ACTUAL μL USED PER ML OF URINE	ACTUAL IU USED PER ML OF URINE
500	5.59	6	536.4
750	8.39	8	715.2
1000	11.19	11	983.4
1200	13.42	13.5	1206.9
1500	16.79	17	1519.8

Table 2.1 Required amount β-glucuronidase used to determine optimal concentration

After a twenty-four hour incubation, the urine samples were subjected to the König reaction (2.2.4) using DBTB as the condensing reagent. Absorbance measurements were carried out in duplicate at 525 nm, using the aqueous layer and the cotinine equivalent concentration of each sample was determined using the absorbance value obtained from a cotinine (10 μg/mL) standard solution.

Further studies using β-glucuronidase were carried out to determine if the cotinine equivalent concentration after treatment with β-glucuronidase increased significantly more in urine samples taken from self-reported smokers, compared to urine samples taken from self-reported non-smokers. Urine samples were taken from five smokers and five non-smokers. Each urine sample (30 mL) was split into three aliquots (10 mL each). The first aliquots (batch one) of urine samples were adjusted to pH 5.0 and 1 mL of urine was then transferred to a second container, where the addition of the enzyme β-glucuronidase (10 μL, 894 IU) occurred. The samples were then placed in a water bath and incubated at 37^0C. The first batch is the only batch of urine samples that had the enzyme added, although a further two batches were used as controls to ensure that the temperature of the water bath or changing the pH of the urine sample had no effect on the total nicotine metabolite concentration. The second aliquots (batch two) of urine samples were placed in the water bath alongside those from batch one. The third aliquots (batch three) of urine samples were adjusted to pH 5.0 and placed in the water bath. After approximately twenty-four hours, all urine samples were removed from the

water bath and the total nicotine metabolite concentrations were measured using DBTB as the condensing reagent. Measurements were carried out at 525nm using the aqueous layer.

2.3 Results and discussion

2.3.1 Relative molar absorptivities and partition coefficients

Relative molar absorptivities for each individual nicotine metabolite post derivatization using either DETB or DBTB are shown in table 2.2. The relative molar absorptivities for nicotine metabolites derivatized using DETB range from 0.55×10^4 L $M^{-1}cm^{-1}$ for 4-3-pyridyl-4-oxo butyric acid to 4.1×10^4 L $M^{-1}cm^{-1}$ for cotinine, whereas nicotine metabolites derivatized using DBTB range from 0.46×10^4 L $M^{-1}cm^{-1}$ for 4-3-pyridyl-4-oxo butyric acid to 5.2×10^4 L $M^{-1}cm^{-1}$ for cotinine. Overall, the relative molar absorptivities were higher when the condensing reagent DBTB was used, compared to DETB, although 4-3-pyridyl-4-oxo butyric acid appeared to be the exception.

Cotinine-N-oxide was the only nicotine metabolite that produced a solution of insufficient colour to measure either the relative molar absorptivity or partition coefficient. This is in accordance with the predictions of Rustemeier et al (1993) who suggested cotinine-N-oxide would be one of the few metabolites that would be unable to undergo the König reaction, due to an oxygen atom being attached to the nitrogen atom situated in the pyridine ring (figure 2.2).

Relative molar absorptivities for nicotine metabolites derivatized with either DMDD or MPED are shown in table 2.3. The relative molar absorptivities obtained using the condensing reagent DMDD, ranged from 0.8×10^4 L $M^{-1}cm^{-1}$ for 3-pyridyl acetic acid to 4.4×10^4 L $M^{-1}cm^{-1}$ for cotinine, compared to nicotine metabolites derivatized using

NICOTINE METABOLITE (CONCENTRATION µg/mL)	DETB MAXIMUM WAVELENGTH (nm)	DETB MOLAR ABSORPTIVITIES x 10^4 L M^{-1} cm^{-1}	DBTB MAXIMUM WAVELENGTH (nm)	DBTB MOLAR ABSORPTIVITIES x 10^4 L M^{-1} cm^{-1}
Cotinine (5)	525	4.1	528	5.2
Cotinine-*N*-oxide	533	No significant absorbance	532	No significant absorbance
5′-hydroxycotinine (5)	531	1.6	533	3.1
Trans-3′-Hydroxycotinine (5)	525	3.1	527	4.4
Nicotine (5)	532	3.3	534	3.5
Nicotine-1′*N*-oxide (5)	528	1.4	531	2.2
Norcotinine (5)	525	2.5	526	3.6
Nornicotine (5)	531	2.6	533	3.3
3-Pyridyl acetic acid (10)	522	1.1	527	1.9
4-3-Pyridyl-4-oxo butyric acid (20)	542	0.55	542	0.46
4-3-Pyridyl-4-oxo-*N*-methyl butyramide (10)	533	1.3	533	2.4

Table 2.2 Relative molar absorptivities for the individual nicotine metabolites derivatized using DETB or DBTB.

68

NICOTINE METABOLITE (CONCENTRATION µg/mL)	DMDD MAXIMUM WAVELENGTH (nm)	DMDD MOLAR ABSORPTIVITY x 10^4 L $M^{-1}cm^{-1}$	MPED MAXIMUM WAVELENGTH (nm)	MPED MOLAR ABSORPTIVITY x 10^4 L M^{-1} cm^{-1}
Cotinine (10)	495	4.4	500	4.0
Cotinine-*N*-oxide	496	No significant absorbance	499	No significant absorbance
5'-hydroxycotinine (10)	496	3.3	497	3.4
Trans-3'-Hydroxycotinine (10)	493	4.1	497	3.2
Nicotine (10)	495	3.1	498	2.5
Nicotine-1'*N*-oxide (10)	492	1.7	494	1.9
Norcotinine (10)	495	3.0	499	3.0
Nornicotine (10)	496	2.7	499	3.2
3-Pyridyl acetic acid (10)	496	0.8	499	1.7
4-3-Pyridyl-4-oxo butyric acid	495	No significant absorbance	505	No significant absorbance
4-3-Pyridyl-4-oxo-*N*-methyl butyramide (10)	494	3.0	498	3.7

Table 2.3 Relative molar absorptivities for individual nicotine metabolites using DMDD or MPED.

MPED which range from to 1.7×10^4 L M^{-1}cm^{-1} for 3-pyridyl acetic acid to 4.0×10^4 L M^{-1}cm^{-1} for cotinine.

The relative molar absorptivities for MPED were not always higher than DMDD and therefore cannot follow the same trends as for the relative molar absorptivities obtained using DETB and DBTB. If MPED had followed the same trend as DBTB, then it would have been expected that MPED, being the more non-polar condensing reagent compared to DMDD, would have had higher relative molar absorptivities.

The nicotine metabolite, 4-3-pyridyl-4-oxo butyric acid produced no significant absorbance when the condensing reagents DMDD or MPED were used to determine the relative molar absorptivity or partition coefficient. One possible reason could be that the relative molar absorptivities for 4-3-pyridyl-4-oxo butyric acid using DETB or DBTB were low and therefore, if 4-3-pyridyl-4-oxo butyric acid does not react as readily with DMDD or MPED then the absorbance value may fall just below the limit of detection.

The maximum wavelength used to determine the absorbance of the derivative also slightly varied depending on the nicotine metabolite and condensing reagent used, although for the majority of nicotine metabolites the maximum wavelength was between 531nm and 533 nm.

Each nicotine metabolite, post derivatization, had its own relative molar absorptivity, which varied depending on the condensing reagent used and similar trends for all four condensing reagents were generally followed. For example cotinine always had the highest relative molar absorptivity and 4-3 pyridyl-4-oxo-butyric acid and cotinine-*N*-oxide had the lowest molar absorptivity. However, when the molar absorptivities for each nicotine metabolite were ranked from the highest to lowest molar absorptivity for each condensing reagent (table 2.4), it was determined that not all nicotine metabolites were ranked in the same order for each condensing reagent.

NICOTINE METABOLITE (CONCENTRATION µg/mL)	DETB	DBTB	DMDD	MPED
Cotinine (10)	1	1	1	1
Cotinine-*N*-oxide	11	11	10	10
5´-hydroxycotinine (10)	6	6	3	3
Trans-3´-Hydroxycotinine (10)	3	2	2	4
Nicotine (10)	2	4	4	7
Nicotine-1´*N*-oxide (10)	7	8	8	8
Norcotinine (10)	5	3	5	6
Nornicotine (10)	4	5	7	5
3-Pyridyl acetic acid (10)	9	9	9	9
4-3-Pyridyl-4-oxo butyric acid	10	10	10	10
4-3-Pyridyl-4-oxo-*N*-methyl butyramide (10)	8	7	5	2

Table 2.4 Nicotine metabolite ranked according to their molar absorptivity.

71

For example 5′-hydroxycotinine was ranked sixth using DETB or DBTB but third when DMDD or MPED were used.

The molar absorptivities using DETB or DMDD have been previously reported (Smith and Cooke 1987 and O'Doherty et al 1990). Smith and Cooke (1987) reported that the three nicotine metabolites cotinine, nicotine and nicotine-1′N-oxide, derivatized with DETB, gave molar absorptivities which ranged from 1.3×10^4 L $M^{-1}cm^{-1}$ for nicotine-1′N-oxide to 3.1×10^4 L $M^{-1}cm^{-1}$ for nicotine. The results obtained in this study agree with the results obtained by Smith and Cooke (1987), except for cotinine which had a higher molar absorptivity than nicotine in this study but was reported to have a molar absorptivity less than nicotine by Smith and Cooke (1987).

One possible reason why the molar absorptivities differ in these two studies is that Smith and Cooke (1987) measured all the derivatized nicotine metabolites at 536 nm, whereas in this current study each nicotine metabolite was measured at the appropriate wavelength. In this study cotinine derivatized with DETB was measured at 525 nm and therefore it may be possible that when the derivative of cotinine was measured by Smith and Cooke (1987) at 536 nm, the reaction was not measured at the maximum absorbance, which would result in a lower molar absorptivity.

O'Doherty et al (1990) also looked at the molar absorptivities for the four nicotine metabolites; cotinine, nicotine, *trans*-3′-hydroxycotinine and 3-pyridyl acetic acid derivatized with either DETB or DMDD. They also concluded that each metabolite had its own molar absorptivity and that the metabolites derivatized with DETB gave a molar absorptivity which ranged from 4.4×10^4 $M^{-1}cm^{-1}$ for 3-pyridyl acetic acid to 7.0×10^4 $M^{-1}cm^{-1}$ for cotinine, whereas the metabolites derivatized with DMDD ranged from 5.7 $\times$ 10^4 $M^{-1}cm^{-1}$ for 3-pyridyl acetic acid to 7.7×10^4 $M^{-1}cm^{-1}$ for *trans*-3′-hydroxycotinine. Overall, the nicotine metabolites derivatized with DMDD had a higher molar absorptivity, compared to the same nicotine metabolites derivatized with DETB.

The DETB results published by Smith and Cooke (1987) are very similar to DETB results obtain in this current study. However, the results obtained by O'Doherty et al (1990) are significantly higher for the nicotine metabolites derivatized with either DETB or DMDD compared to Smith and Cooke (1987) or this current study. A possible reason for the differences in the molar absorptivities is that this current research investigated the relative molar absorptivities; absorbance measurements were obtained using the crude reaction mixture, rather than isolating the derivatized metabolite to give a true molar absorptivity. O'Doherty et al (1990) used a solid phase extraction cartridge (C_{18}) to remove any undesirable matrix components, leaving the purified derivatized nicotine metabolite to be eluted from the column.

The reason why the true molar absorptivities were not studied is that it is very difficult to isolate the derivatized nicotine metabolites in a pure form that can then be accurately weighed to give a known concentration. The relative molar absorptivities will more realistically reflect the situations used in everyday analysis as it would be extremely difficult to purify the nicotine metabolites present in a biological sample such as urine. Also the sources and the relative purities of the nicotine metabolites are unknown and so it was decided to study the relative molar absorptivities of each nicotine metabolite.

The importance of these findings is that the absorbance obtained from an unknown biological sample, for example urine, may be compiled from different contributions of individual nicotine metabolites depending on an individual nicotine metabolic profile. This then has further implications when determining the true concentration of individual nicotine metabolites by analytical techniques like HPLC.

The partition coefficients for nicotine metabolites derivatized with either DETB or DBTB are shown in table 2.5. Overall, the results obtained suggest that nicotine metabolites derivatized DBTB partition slightly more efficiently than nicotine metabolites derivatized with DETB, except for cotinine and nicotine. The partition coefficients for nicotine metabolites derivatized with DETB range from 5 to 10, compared to metabolites derivatized with DBTB which ranged from 4 to 10.

NICOTINE METABOLITE (CONCENTRATION μg/mL)	DETB PARTITION COEFFICIENT	DBTB PARTITION COEFFICIENT
Cotinine (5)	6	4
Nicotine (5)	9	7
Norcotinine (5)	5	10
Nornicotine (5)	9	9
5'-Hydroxycotinine (5)	5	8
4-3-Pyridyl-4-oxo butyric acid (20)	6	5
Trans-3'-hydroxycotinine (5)	5	8
3-Pyridyl acetic acid (10)	5	7
4-3-Pyridyl-4-oxo-*N*-methyl butyramide (10)	5	10
Cotinine-*N*-oxide	No reaction	No reaction
Nicotine-1'*N*-oxide (5)	10	5

Table 2.5 Partition coefficients for nicotine metabolites derivatized with either DETB or DBTB

The partition coefficients for nicotine metabolites derivatized with either DMDD or MPED are shown in table 2.6. Nicotine metabolites derivatized with DMDD ranged from 1 to 4, compared to nicotine metabolites derivatized with MPED, which ranged from 12 to 32.

NICOTINE METABOLITE	DMDD PARTITION COEFFICIENT	MPED PARTITION COEFFICIENT
Cotinine (10)	1	23
Nicotine (10)	3	12
Norcotinine (10)	1	19
Nornicotine (10)	3	32
5'-hydroxycotinine (10)	2	22
4-3-Pyridyl-4-oxo butyric acid	No reaction	No reaction
Trans-3'-hydroxycotinine (10)	1	17
3-Pyridyl acetic acid (10)	1	18
4-3-Pyridyl-4-oxo-*N*-methyl butyramide (10)	2	23
Cotinine-*N*-oxide	No reaction	No reaction
Nicotine-1'*N*-oxide (10)	4	32

Table 2.6 Partition coefficients for nicotine metabolites derivatized with either DMDD or MPED

The results show that when the more non-polar condensing reagent, MPED, was used, there was a significant increase in the relative partitioning coefficients compared to the other condensing reagents. The increase in the relative partition coefficient suggests that MPED has the potential to increase the sensitivity of the total nicotine metabolite concentration and therefore may prove to be a more suitable condensing reagent when carrying out analytical methods such as HPLC.

2.3.2 Cotinine calibration curves

Calibration curves for the absorbance produced by cotinine are shown in figures 2.7 to 2.10. The results indicate that cotinine derivatized with any of the four condensing reagents overall produces a curve, rather than a continuous straight line over the concentration range chosen. However when cotinine is derivatized with either DETB, DMDD or MPED a straight line is produced between 0-8 µg/mL (0-0.045 µmol/L), which indicates that the König reaction does follow the Beer-Lambert law, i.e. that the absorbance is proportional to concentration between these two values. For cotinine derivatized with DBTB a straight line is produced between 4-12 µg/mL (0.023-0.068 µmol/L), suggesting that the Beer-Lambert law is obeyed between these two values.

However, Pickert et al (1993) verified the linearity of the König reaction by using a cotinine equivalent urine sample by dissolving cotinine (25 mg) in a non-smoker urine sample. They produced a calibration curve from 2.5 to 250 mg/L and found that the reaction was linear up to 100 mg/L. One possible reason why these results differ from the current study is that Pickert et al (1993) determined the linearity of the reaction using a urine sample spiked with cotinine, whereas the current study used pure cotinine.
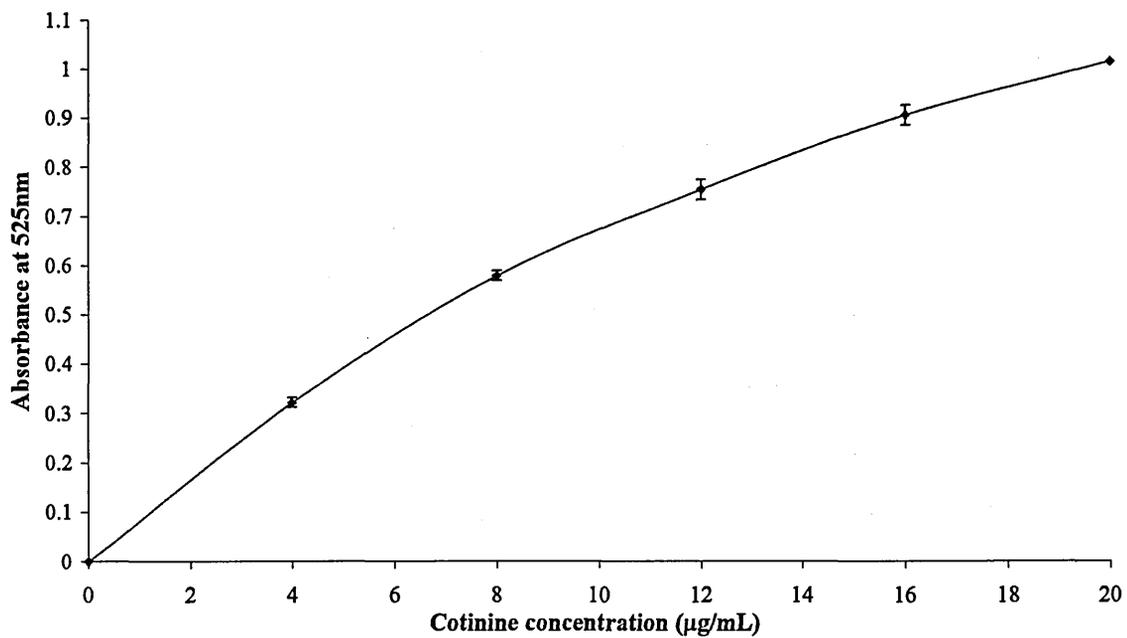
Figure 2.7 A standard curve for the absorbance produced by cotinine using DETB
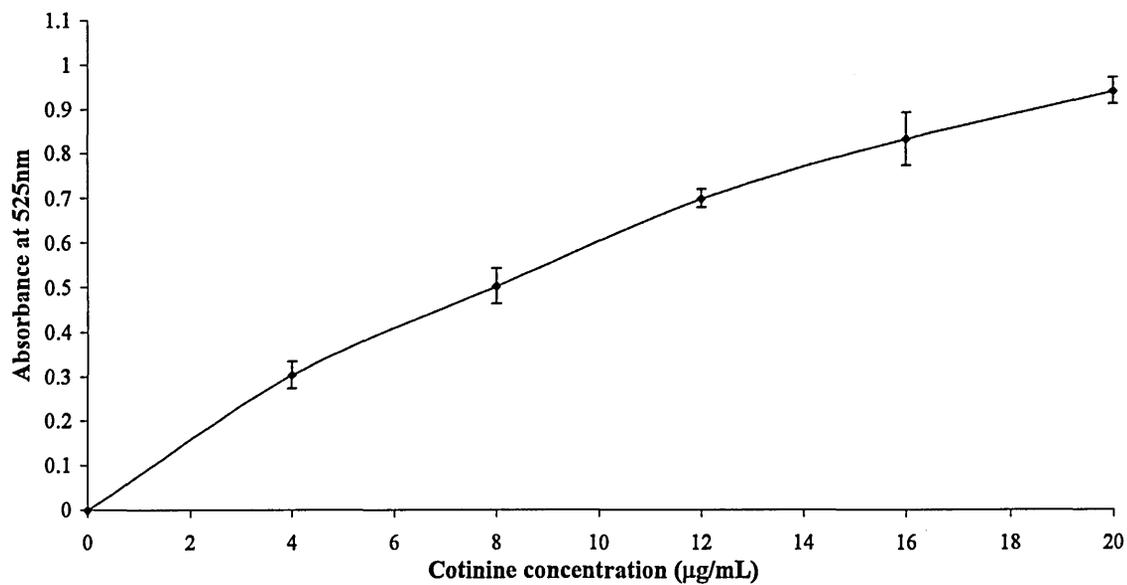
Figure 2.8 A standard curve for the absorbance produced by cotinine using DBTB
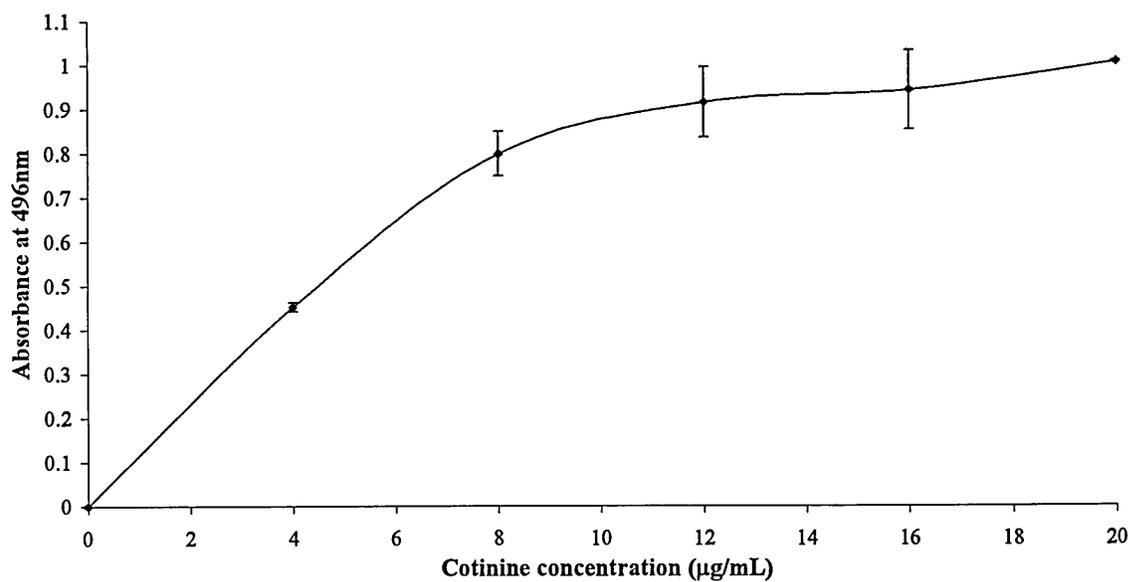
Figure 2.9 A standard curve for the absorbance produced by cotinine derivatized with DMDD
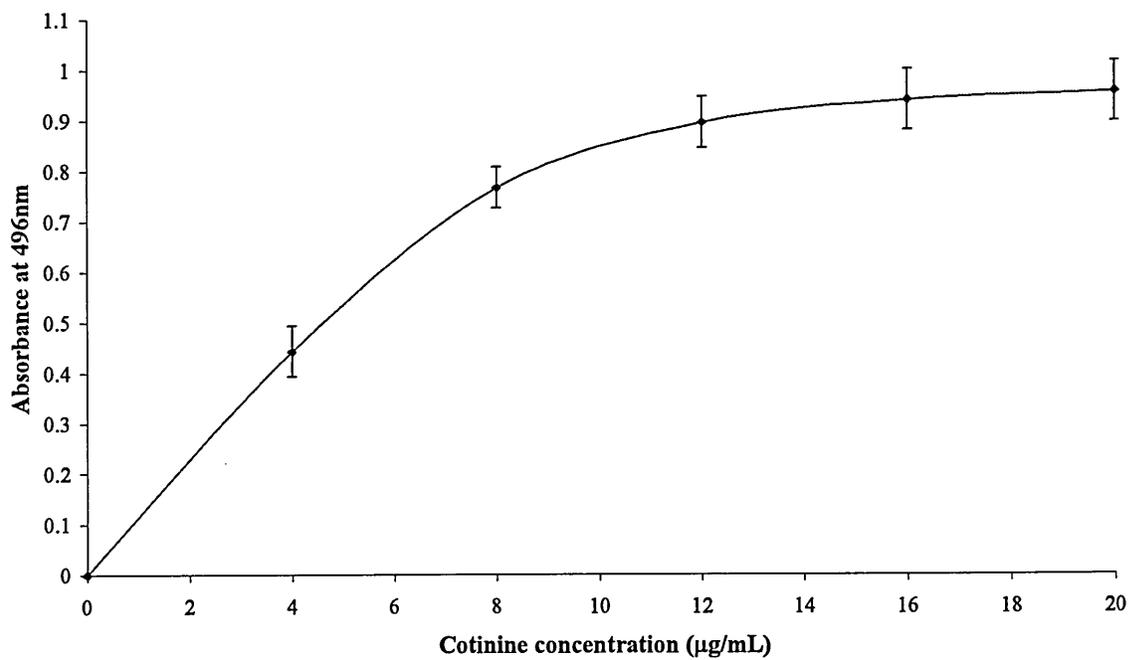
Figure 2.10 A standard curve for the absorbance produced by cotinine derivatized with MPED

77

Smith and Cooke (1987) reported that when they measured the absorbance of a mixture of cotinine, nicotine and nicotine1'N-oxide, the absorbance values obtained were lower than anticipated compared to the total sum of the absorbance values for each individual metabolite. They also suggested that this "indicated that the König reaction had grave implications for the reliability of the method as a quantitative analytical tool". However, a likely reason why Smith and Cooke (1987) observed a lower absorbance value for a mixture of metabolites, compared to the same metabolites measured individually, is that although the mixture contains the same concentration of individual metabolites, overall the mixture has a higher total nicotine metabolite concentration. Therefore, the mixture will contain more molecules, and the more molecules a solution contains then the greater the chance that certain molecules will form weak interactions with each other. If a molecule is interacting with another molecule then it is unable to be excited by the light energy, and this can than produce a lower absorbance value compared to when the same number of molecules are measured individually and their absorbance values grouped together.

2.3.3 Optimum reaction time course

The time for the reaction to reach completion for each nicotine metabolite derivatized with each of the condensing reagents is shown in figures 2.11 to 2.14. The results observed by Smith and Cooke (1987) suggest that nicotine and its metabolites, cotinine and nicotine-1'N-oxide derivatized with DETB had their own time course. Nicotine took 90 minutes to reach its maximum absorbance before the absorbance declined, whereas for cotinine the colour developed rapidly and then remained stable for at least 2 hours, while the colour for nicotine-1'N-oxide developed rapidly, reaching a maximum absorbance within 2 minutes but sharply declined and after 20 minutes there was approximately a 30 percent loss of absorbance. As a result of the different time courses obtained, Smith and Cooke (1987) adopted 20 minutes as a standard waiting period due to operator convenience, although they could have chosen 2 minutes for nicotine-1'N-oxide or 90 minutes for nicotine.
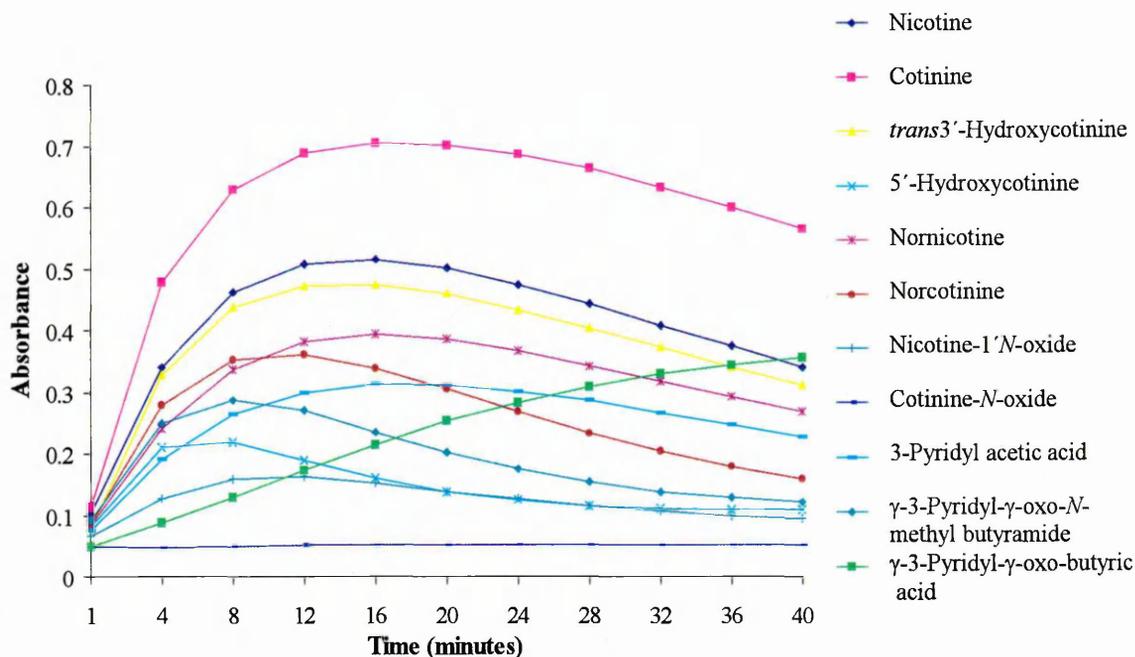
Figure 2.11 Reaction time course for nicotine metabolites derivatized with DETB
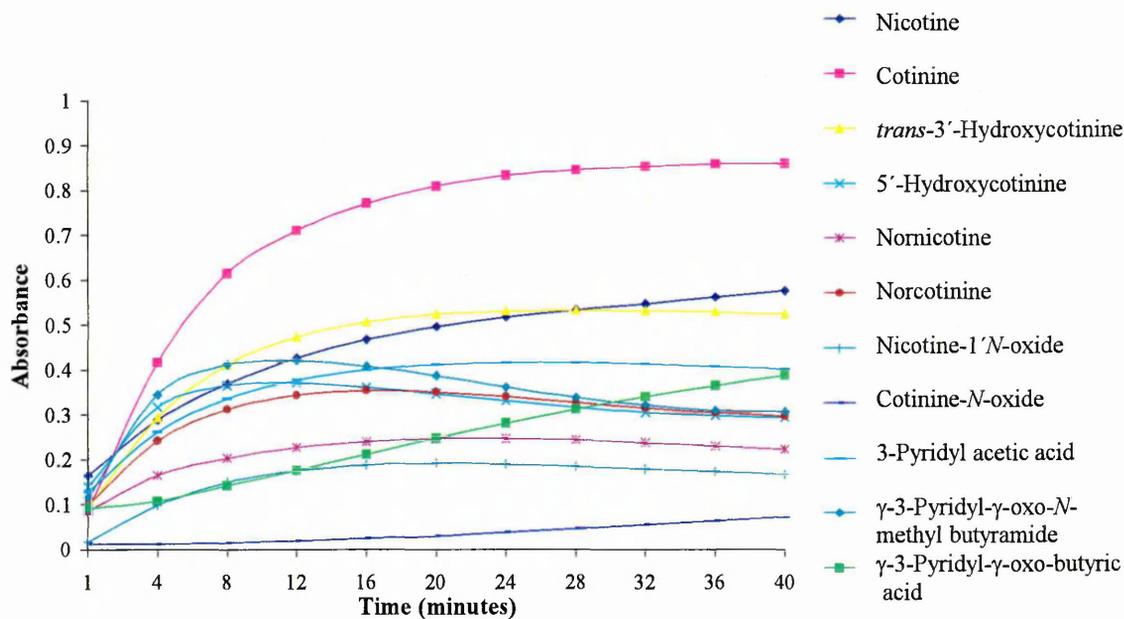
Figure 2.12 Reaction time course for nicotine metabolites derivatized with DBTB
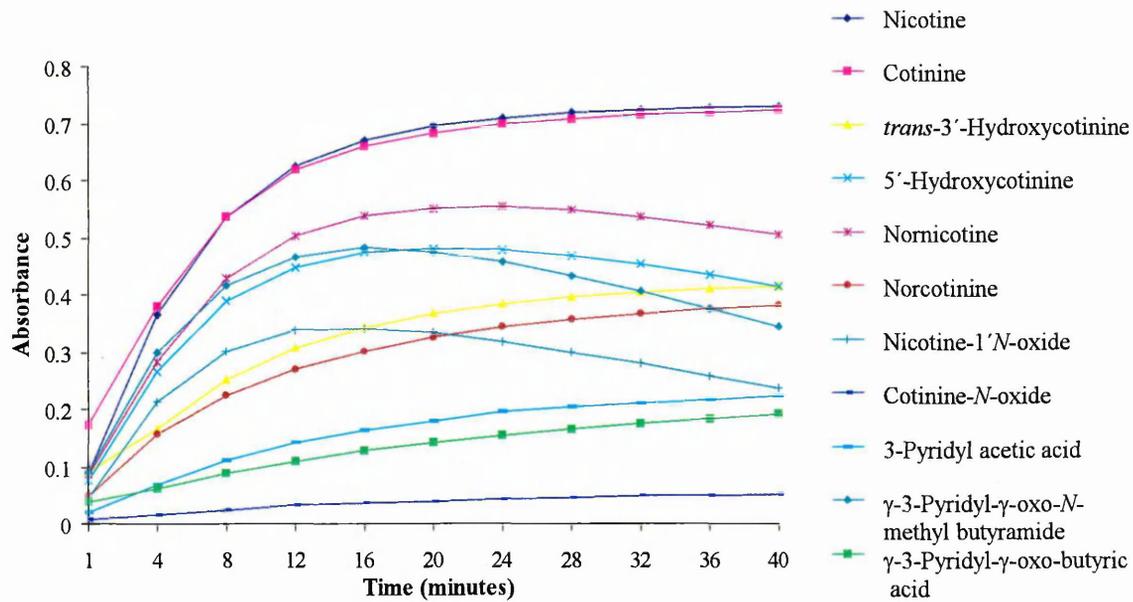
Figure 2.13 Reaction time course for nicotine metabolites derivatized with DMDD
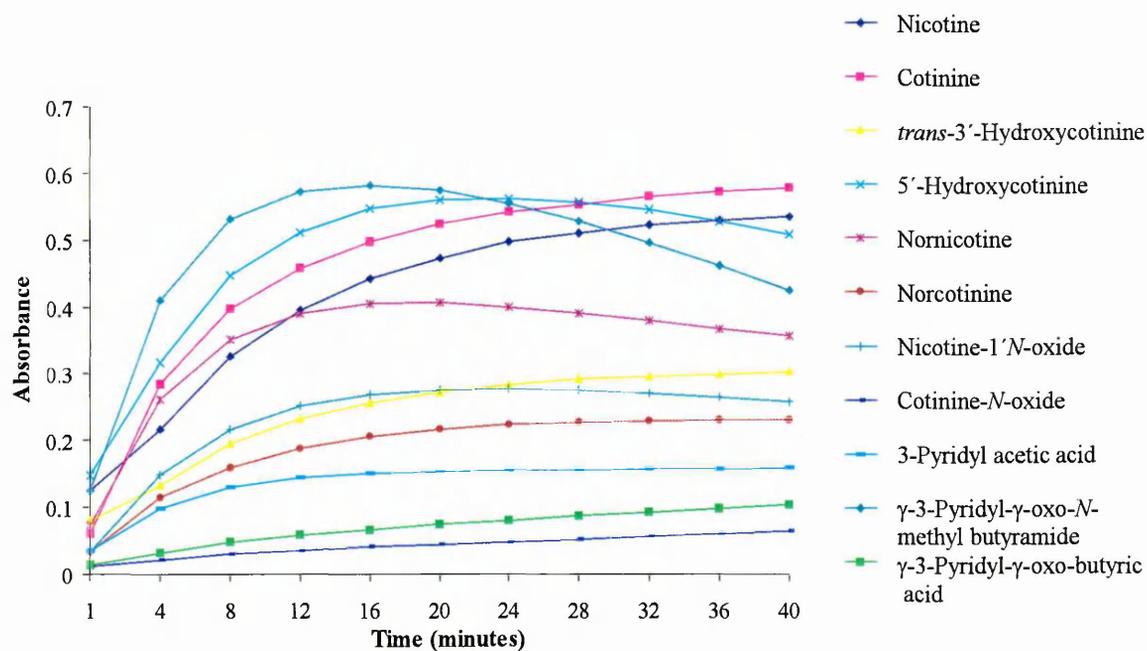
Figure 2.14 Reaction time course for nicotine metabolites derivatized with MPED

The results in this study confirmed that each nicotine metabolite has its own reaction time course and that each nicotine metabolite reacts slightly differently. Some nicotine metabolites reach their absorbance maximum in the first ten to twenty minutes and then start to decline, whereas with other nicotine metabolites absorbance steadily increases over the entire forty minutes of the experiment. However, the time courses for cotinine, nicotine and nicotine-1'N-oxide from this study were not the same shape as reported by Smith and Cooke (1987). The reason for this may be that this current study observed the absorbance using the aqueous layer, whereas Smith and Cooke (1987) determined the absorbance values once extracted into ethyl acetate. Nonetheless, it can be broadly concluded by both studies that twenty minutes is the most appropriate reaction time.

2.3.4 Imprecision studies

Imprecision studies were carried out using a "pooled" smoker's urine sample. The within batch imprecision results obtained for DETB was 5.84 percent, DBTB 5.95 percent, DMDD 8.63 percent and MPED 9.92 percent. The results indicate that all condensing reagents used had an acceptable imprecision value but when derivatized with either DETB or DBTB imprecision was slightly better than with DMDD or MPED. A major contributor to imprecision is the use of multiple steps and therefore one way of improving the overall precision of the reaction would be to reduce the number of steps involved or to automate the König reaction.

Various studies have used the König reaction to measure nicotine and its metabolites (Peach et al 1985, Smith and Cooke 1987, Smith et al 1989, O'Doherty et al 1990, Phillipou et al 1994 and Smith et al 1998) but only two studies that had automated this reaction published imprecision data (Puhakainen et al 1987 and Pickert et al 1993). Puhakainen et al (1987) reported an imprecision of 6.8 percent for 13.6 μmol/L urine pool and 3.4 percent for 39.2 μmol/L using a continuous flow auto-analyser with barbituric acid as the condensing reagent. Pickert et al (1993) reported that their automated König reaction using DETB and a Cobas-Fara II analyser had a within assay imprecision of 4.3 percent for 41.6 mg/ and 5.2 percent for 2.8 mg/L cotinine equivalent in urine and a between

assay imprecision of 8.6 percent for 39.3 mg/L and 16.4 percent for 1.9 mg/L cotinine equivalent in urine.

2.3.5 Determination of the effects of β-glucuronidase

The synthesis of cotinine N-glucuronide was attempted, although no significant yield of 7 (cotinine N-glucuronide) was produced, despite several attempts with various modifications. After Caldwell et al (1992) were consulted about their publication on the synthesis of cotinine N-glucuronide, it become apparent there was some ambiguity about the method they had actually used in the final step of the synthesis, and this was confirmed later after a publication was found describing a modified procedure by the same group (Crooks et al 1992). Due to the time already spent investigating the various steps and waiting for correspondence from Caldwell et al , it was decided not to carry on with the synthesis of cotinine N-glucuronide.

The enzyme β-glucuronidase was used to determine the contributions that the glucuronides make to the total nicotine metabolite concentration in urine samples taken from self-reported smokers and non-smokers. The optimum amount of β-glucuronidase to use was determined from the standard curve to be 900 IU or 10μL (figure 2.15).
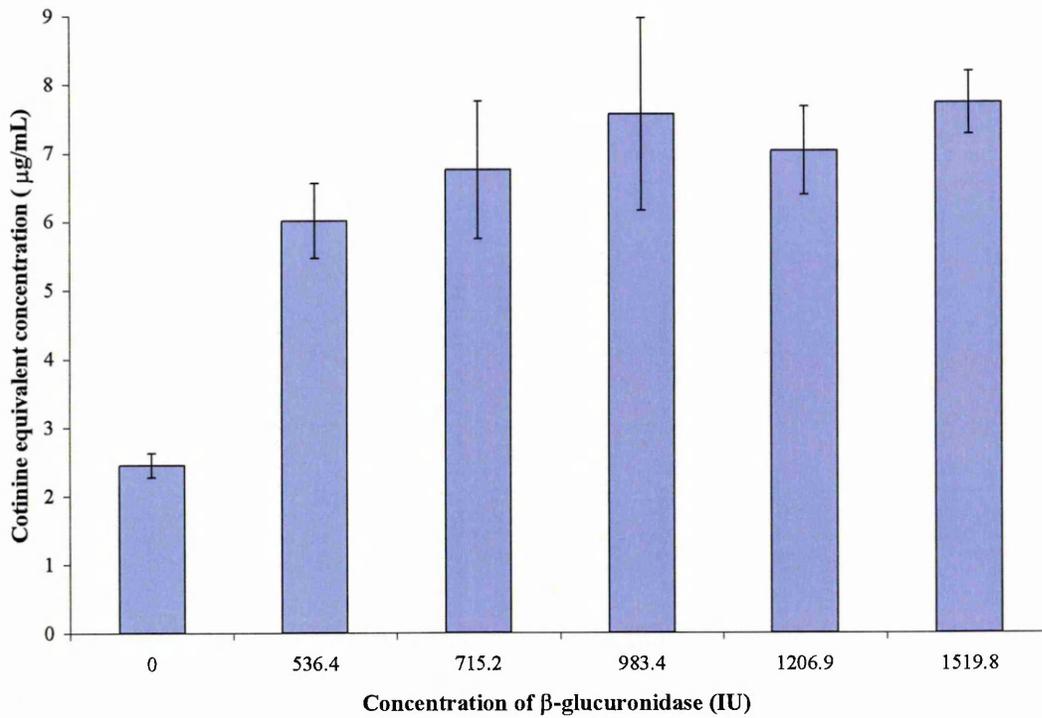
Figure 2.15 Determining the optimum concentration of β-glucuronidase
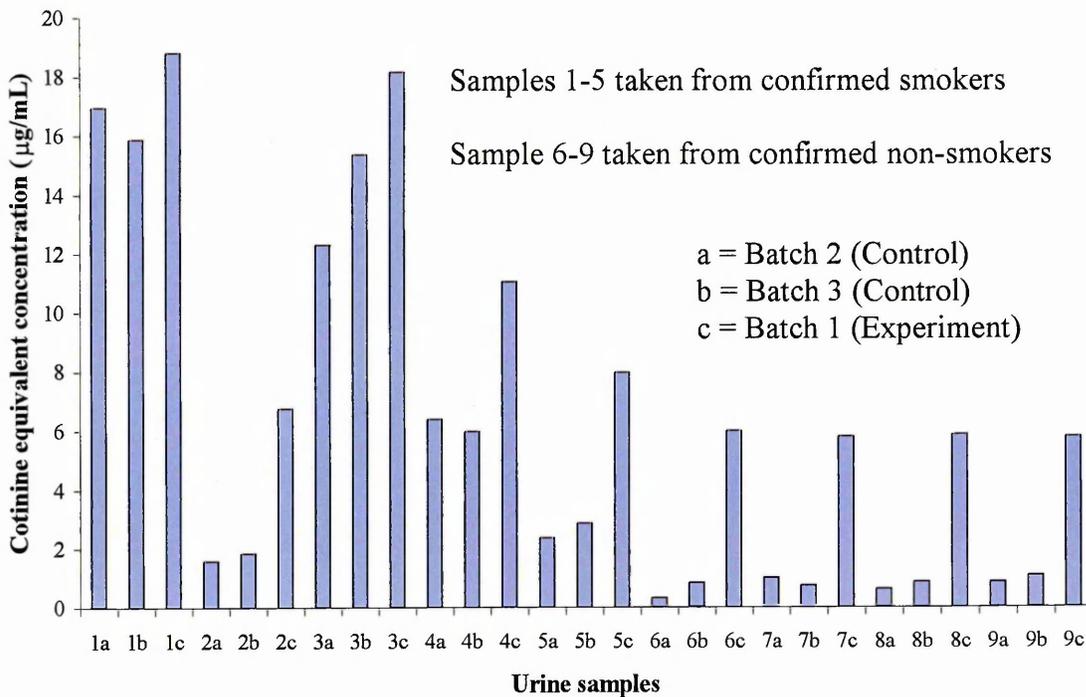
Figure 2.16 Comparing the effects of β-glucuronidase on urine samples taken from confirmed smokers and non-smokers

83

The absorbance measurements obtained from the urine samples from self-reported smokers and non-smokers were converted to cotinine equivalent concentrations. The results from the control samples showed that keeping the urine samples at 37°C or changing the pH to 5.0 had no effect on the total nicotine metabolite concentration (figure 2.16). The average nicotine metabolite concentration increase for the urine samples taken from smokers and treated with β-glucuronidase was 36.7 percent, compared to urine samples taken from non-smokers, which on average had an increase of 90.3 percent. The increase in the total nicotine metabolite concentration in the urine taken from non-smokers is unlikely to be solely due to the glucuronides, especially as the glucuronides of nicotine, cotinine and *trans*-3′-hydroxycotinine constitute on average 29 percent of the urinary metabolites (Byrd et al 1992). Therefore as the expected concentrations for the treated non-smoker urine samples should be less than observed, then the increase must be due to another unknown factor interfering with the reaction. Due to the time available, no further investigations were carried out to determine why a large increase in the concentration of nicotine metabolites was observed in non-smoker urine samples after treatment with β-glucuronidase.

Chapter 3

Urine test for the assessment of smoking status

3.1 Introduction

Smoking is the major preventable cause of mortality and morbidity throughout the western world, and recent evidence has suggested that about half of all regular smokers will die from their smoking habit (Doll et al 1994). For many years questionnaires were used to derive information regarding an individual's smoking habit (Cope et al 1996 and Kharrazi et al 1999). However, often the information an individual gave about their tobacco consumption (i.e. if a particular individual classes themselves as a smoker or a non-smoker) was found to be incorrect compared to laboratory results. The inconsistency in the information obtained may be largely due to the attitude which society has towards smokers, (for example a greater understanding of the risks associated with smoking and passive smoking) and consequently smokers are likely to underestimate their consumption of tobacco or even deny their smoking addiction (Cope et al 1996).

Questionnaires which ask about the frequency and the number of cigarettes smoked or details of smoking habits (i.e. they presume that everyone is a smoker), rather than asking yes/no questions about whether an individual is a smoker or not, were found to be more honestly answered (Kharrazi et al 1999). Questions that require a yes/no answer may not be as honestly answered because it may be confusing to some individuals whether to class themselves as a smoker or not, especially if their smoking habits have changed recently or if they have an irregular smoking habit and therefore prefer to class themselves as a "social smoker". A change in smoking behaviour can occur for a variety of reasons, although it has been documented that smoking behaviour can alter during pregnancy (Fingerhut et al 1990 and Dempsey et al 1998), with some women cutting down on the number of cigarettes smoked or even quitting altogether during pregnancy (Kharrazi et

al 1999). It is important, therefore, to be able to monitor an individual attending a particular health clinic, such as a smoking cessation clinic or an antenatal clinic, using a simple, reliable method to differentiate smokers from non-smokers which can also be used to assess an individual's daily intake of tobacco related compounds.

Using the König reaction Smith et al (1998) reported a clinical sensitivity of 100 percent and specificity of 97 percent compared to self-reported smoking status, and a clinical sensitivity of 95 percent and specificity of 97 percent, compared to a radioimmunoassay (RIA) method (Knight et al 1985), which they used as a gold standard. Clinical sensitivity refers, in this case, to the proportion of individuals who are confirmed to be smokers by RIA (the "gold standard" test) and are also confirmed to be smokers by another particular test (the König reaction).

To distinguish a non-smoker from a smoker can be extremely difficult. This may be as result of an irregular smoking habit ("social smoker"), or the individual not actively smoking but being exposed to high levels of ETS through family or work environments. Therefore it is difficult to correctly identify "social smokers" and those individuals exposed to high levels of ETS using a laboratory test. Thus these individuals may be classified as either non-smokers or smokers, depending on when they last smoked or were exposed to ETS.

The cut off point for a laboratory test in order to distinguish a smoker from a non-smoker can vary depending upon the requirements for which the test is being made. Consequently, the cut off point for a laboratory test required by an antenatal clinic may be different to the cut off point required by an insurance company. The cut off point for an antenatal clinic may be set at a lower point, which would increase the clinical sensitivity rate but decrease the specificity rate. This would mean that all smokers would be detected with a few non-smokers being misclassified, but the benefits of providing pregnant women with all the facts about the effects of smoking and the risks to their unborn child, outweighs the misclassification of a few non-smokers. However, for an insurance company it may be more suitable to have a higher cut off point

compared to the antenatal clinic. This would mean a reduction in clinical sensitivity but an increase in specificity, which would ensure that all non-smokers would be classified correctly but a few smokers would be misclassified. Insurance companies would not want to misclassify any non-smokers for legal reasons, as misclassified non-smokers might choose to sue if they were not offered discounted insurance premiums due to a mistake in a laboratory test distinguishing a smoker from a non-smoker.

The aim of the work in this chapter was to investigate the differences in clinical sensitivity and specificity for both qualitative and quantitative measurements using the condensing reagents, DETB and DBTB. The condensing reagents DETB and DBTB were chosen because they form pink-red chromophores, rather than DMDD or MPED which form orange chromophores. When using DMDD or MPED for qualitative results, it may be difficult to distinguish a urine sample pre-derivatization from a urine sample post-derivatization because of the background colour. However, all four condensing reagents could be used for quantitative analyses, providing appropriate blanks were prepared.

3.2 Subjects

During a previous study, urine samples were collected from 251 patients attending the diabetic clinic at Ealing Hospital (Smith et al 1998). A specific cotinine radioimmunoassay method (Knight et al 1985), indicated that this population consisted of 130 non-smokers and 120 smokers with 1 borderline result. The current study utilised aliquots of 224 urine samples (106 RIA determined smokers and 118 RIA determined non-smokers) remaining from this study.

The urine samples were subjected to both qualitative and quantitative measurements. Quantitative measurements were made before (aqueous results) and after extraction with ethyl acetate (organic results), although measurements made using the organic layer were carried out on only 95 of the samples from RIA determined smokers and 115

samples from RIA determined non-smokers. All samples were collected in accordance with the ethical code at Ealing Hospital. All samples were also deep-frozen until analysis and all analyses were carried out "blind" without reference to self reported smoking status or the results of the RIA.

3.3 Methods

3.3.1 Cotinine radioimmunoassay

For the purpose of this study, an RIA method described by Knight et al (1985) was used as the gold standard. A gold standard is a well-documented method that can reliably distinguish between two categories, for example an individual that is exposed to high levels of nicotine (i.e. a smoker) and an individual who is exposed to low levels of nicotine (i.e. a non-smoker). Aliquots ($10 \mu L$) of the urine samples or cotinine calibrators ($0.14 - 4.26 \mu mol/L$) were incubated overnight with cotinine antibody and ^{125}I cotinine. Bound and free fractions were separated using a polyethylene glycol assisted second antibody technique. The radioactivity in the bound fraction was counted and cotinine concentrations were estimated using a 4-parameter logistic curve-fitting program. Within and between batch imprecision were 5.1 percent and 7 percent respectively. The cut off point was determine to be 1.14 $\mu mol/L$, which was determined by a simple histogram.

3.3.2 The König reaction

The total nicotine metabolite concentration was determined both qualitatively and quantitatively. This involved adding 1 mL of individual nicotine metabolite, urine, or cotinine solution to sodium acetate buffer (400 µL, 4 M, pH 4.7), followed by potassium cyanide (200 µL, 10% w/v) and chloramine-T (200 µL, 10% w/v). The solution was mixed well before the addition of the condensing reagent (1 mL, 1% w/v, DETB water: acetone 50:50 or DBTB in acetone). The reaction mixture was again mixed well and allowed to stand for 20 minutes. Absorbance measurements were made both before and after extraction with ethyl acetate (1 mL).

Qualitative assessment was made by simple observation of the pink colour in the ethyl acetate layer. Absorbance measurements of the aqueous and organic layers were made at 525 nm and 533 nm, respectively. A stock solution of cotinine (5 µg/mL) was used to determine the equivalent nicotine metabolite concentration. In addition a sample blank was used from both self reported smokers and non-smokers, and was analysed following the basic method, except chloramine-T was omitted.

3.3.3 Statistical analysis

The optimum cut off point for each of the condensing reagents was determined by a Receiver Operating Characteristics (ROC) curve. An ROC curve is a simple graph that depicts the pairs of true positive rates and false positive rates that correspond to each possible cut off point for a particular diagnostic test result (Sackett et al 1991). The cut off point is then determined by plotting 1-specificity (false positive rate) against sensitivity (true positive rate).

GOLD STANDARD

		Positive	Negative
TEST Positive		TP	FP
Negative		FN	TN

$$\textbf{SENSITIVITY} = \frac{TP}{TP + FN}$$ TP = True Positive
FN = False negative

$$\textbf{SPECIFICITY} = \frac{TN}{TN + FP}$$ TN = True Negative
FP = False Positive

An overall test efficiency may also be calculated as:

$$\textbf{EFFICIENCY} = \frac{\text{True negative + true positive}}{\text{Total patients tested}}$$

Efficiency is the proportion of patients correctly categorised by the test, in this instance the number of patients correctly categorised as smokers or non-smokers using the König reaction either quantitatively or qualitatively (Jones and Payne 1997).

Correlation studies amongst the RIA determined smokers, were carried out using a Spearman Rank analysis.

3.4 Results

The urinary cotinine concentrations determined by cotinine RIA ranged from 0.06 μmol/L to 0.97 μmol/L with a median of 0.1 μmol/L for the non-smokers, compared to the urine samples taken from smokers, which ranged from 1.48 μmol/L to 76.5 μmol/L with a median of 19.3 μmol/L (Table 3.1).

The urinary cotinine equivalent concentrations determined using DETB (aqueous results) ranged from <0.1 μmol/L to 12.9 μmol/L with a median of 1.25 μmol/L for the non-smokers and 4.3 μmol/L to 282 μmol/L, with a median of 45 μmol/L for the smokers. DBTB (aqueous results) cotinine equivalent concentrations ranged from <0.1 μmol/L to 9.8 μmol/L, with a median of 4.29 μmol/L for the non-smoker, compared to 5.2 μmol/L to 324 μmol/L with a median of 30.3 μmol/L for the smokers (Table 3.1). Urinary cotinine equivalent results for DBTB (organic results) ranged from <0.1μmol/L to 12.6 μmol/L with a median of 0.91 μmol/L for the non-smokers, and 2.6 μmol/L to 212 μmol/L with a median of 23.8 μmol/L for the smoker (Table 3.1).

	NON-SMOKERS		SMOKERS	
	Median (μmol/L)	Range (μmol/L)	Median (μmol/L)	Range (μmol/L)
RIA	0.1	0.06 – 0.97	19.3	1.48 – 76.5
DETB (aqueous results)	1.25	<0.1 – 12.9	45	4.3 – 282
DBTB (aqueous results)	4.29	<0.1 – 9.8	30.3	5.2 – 324
DBTB (organic results)	0.91	<0.1 – 12.6	23.8	2.6 - 212

Table 3.1 A summary of the cotinine equivalent concentrations

The cut off point for the quantitative results were determined by an ROC curve and are summarised in table 3.2. The cut off point for DETB (aqueous results) was determined to be 6 μmol/L, giving a clinical sensitivity of 98 percent and specificity of 97 percent (figures 3.1 and 3.2). The cut off point for DBTB (aqueous results) was determined to be 8.5 μmol/L, giving a clinical sensitivity and specificity of 95 percent (figure 3.3 and 3.4), compared to the cut off point for DBTB (organic results) of 4 μmol/L which had a clinical sensitivity and specificity of 96 percent (figure 3.5 and 3.6).
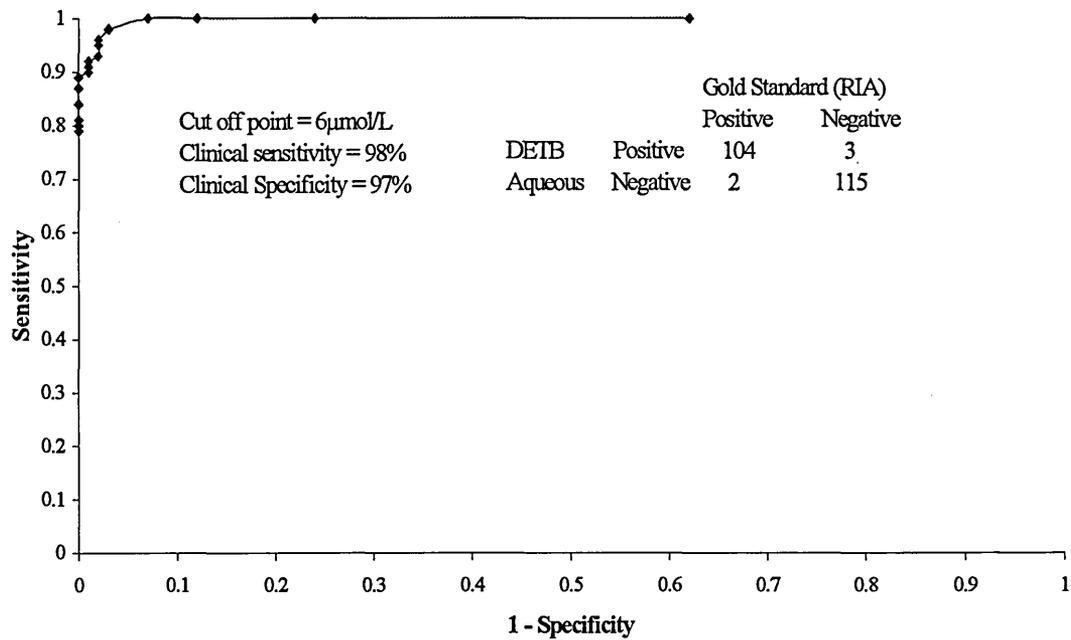
Figure 3.1 ROC curve for DETB aqueous results
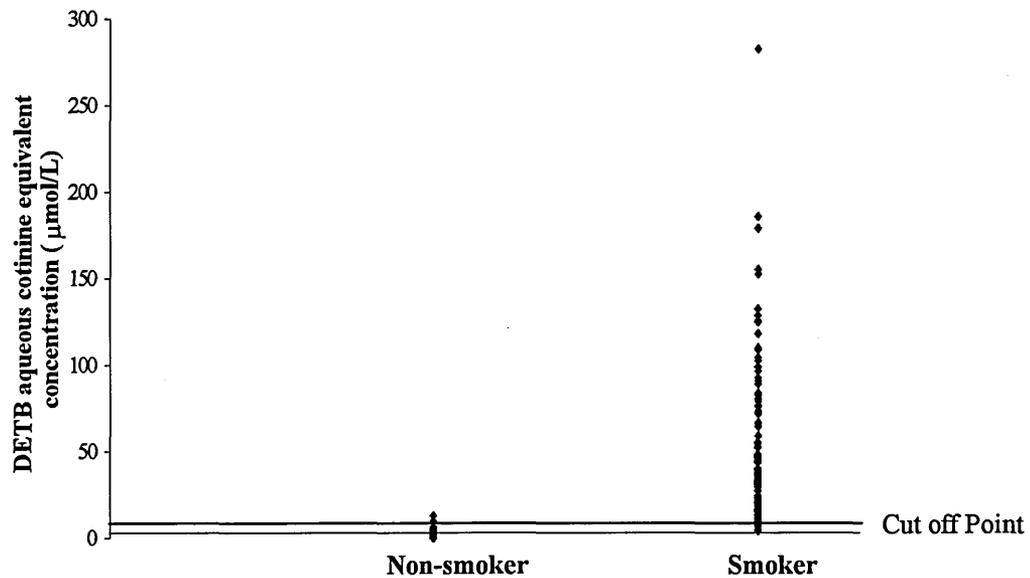
Figure 3.2 Distribution of cotinine equivalent concentrations in relation to smoking status as determined by DETB aqueous results

Cut off point = 8.5μmol/L
Clinical sensitivity = 95%
Clinical Specificity = 95%

Gold Standard (RIA)

		Positive	Negative
DBTB	Positive	101	6
Aqueous	Negative	5	112
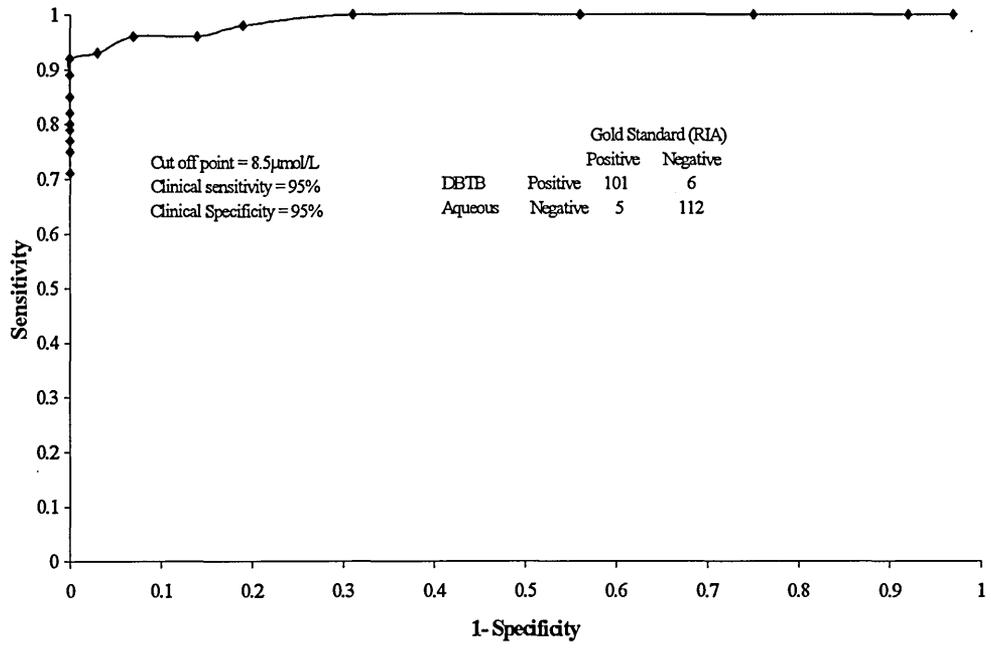
Figure 3.3 ROC curve for DETB aqueous results
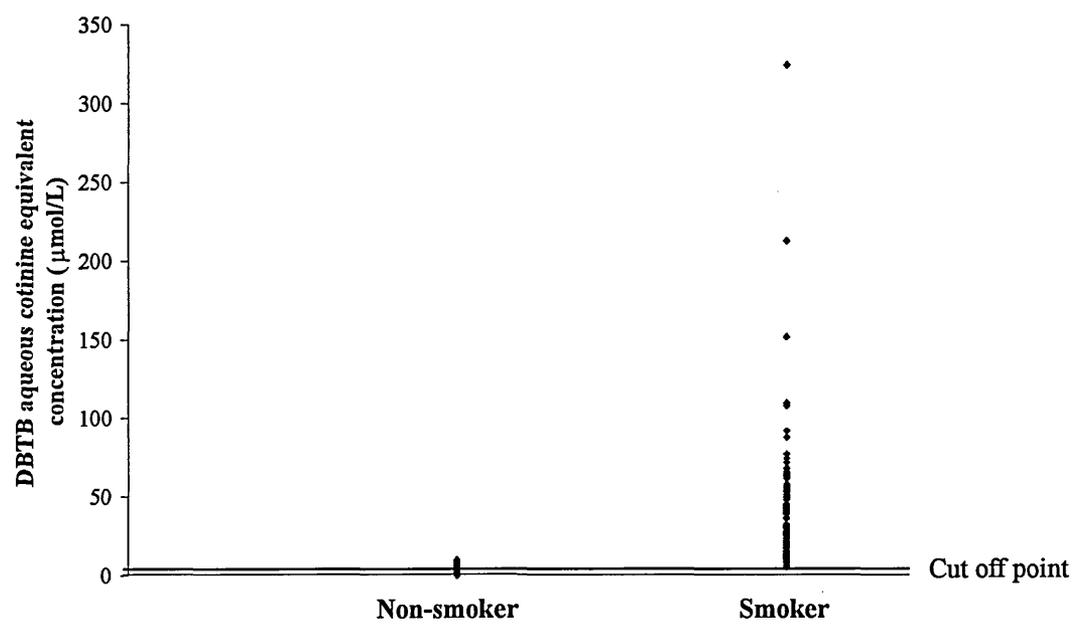
Figure 3.4 Distribution of cotinine equivalent concentrations in relation to smoking status as determined by DBTB aqueous results

94
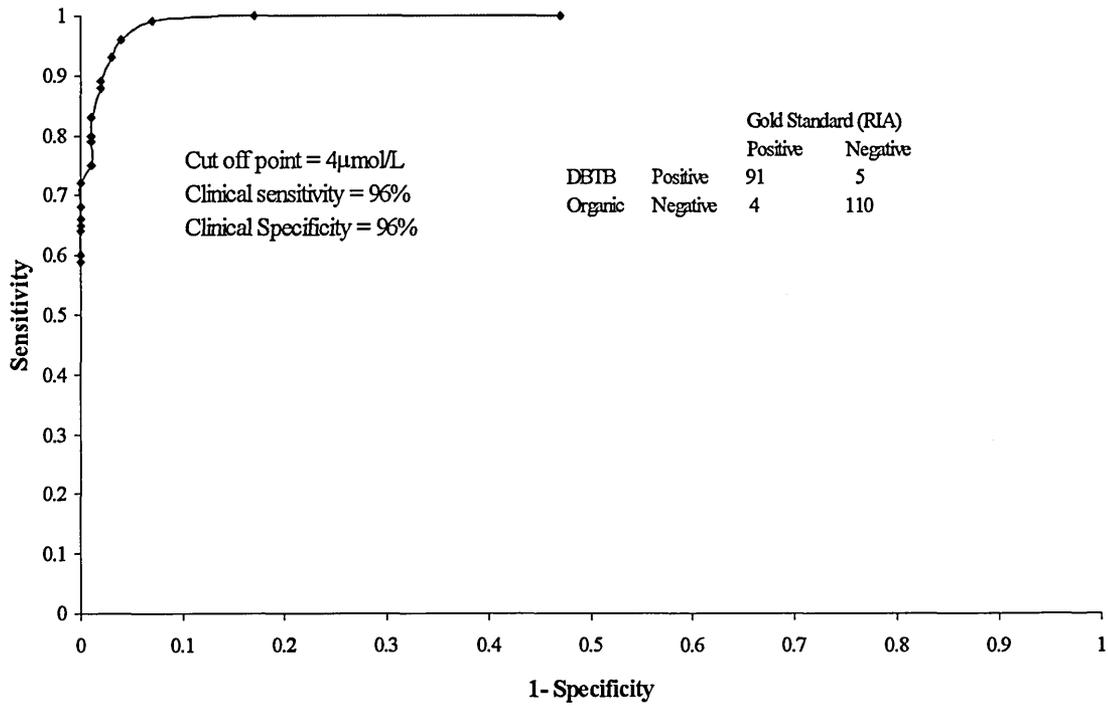
Figure 3.5 ROC curve for DBTB organic results

Cut off point = 4μmol/L
Clinical sensitivity = 96%
Clinical Specificity = 96%

		Gold Standard (RIA)	
		Positive	Negative
DBTB	Positive	91	5
Organic	Negative	4	110
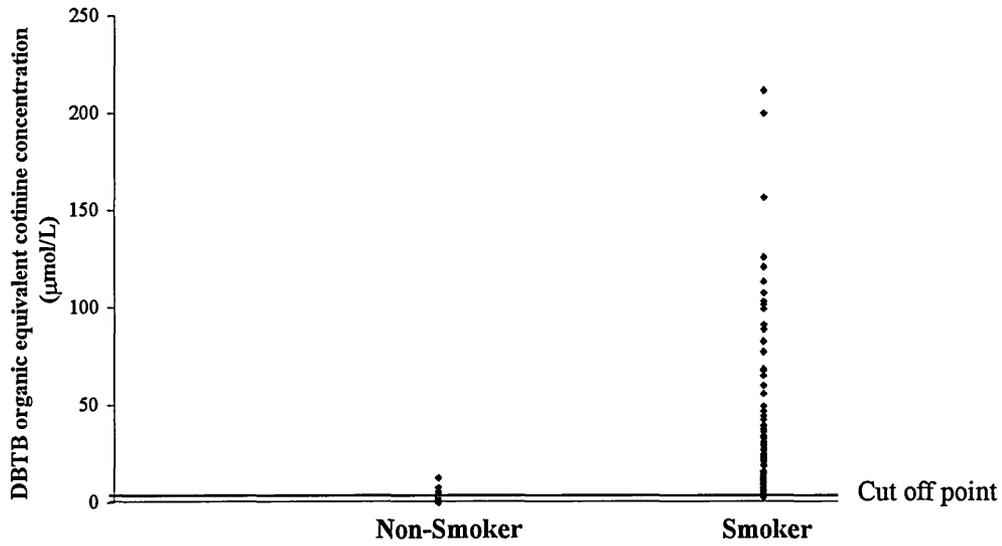
Figure 3.6 Distribution of cotinine equivalent concentrations in relation to smoking status as determined by DBTB organic results

95

	SENSITIVITY	SPECIFICITY
Qualitative (organic results)		
DETB	94%	100%
DBTB	100%	99%
Quantitative (aqueous results)		
DETB	98%	97%
DBTB	95%	95%
Quantitative (organic results)		
DBTB	96%	96%

Table 3.2 A summary of the clinical sensitivity and specificity obtained for qualitative and quantitative results

Qualitative measurements using DETB (organic results), gave a clinical sensitivity of 94 percent and specificity of 100 percent compared to DBTB (organic results) which had a clinical sensitivity of 100 percent and specificity of 99 percent. There was an overall improvement in efficiency from 97 percent using DETB as the condensing reagent to 99.5 percent using DBTB.

Correlation studies amongst the RIA determined smokers, were carried out using a Spearman Rank analysis. The findings were as follows: RIA vs DETB (aqueous results) r_s = 0.890; n =106; p<0.01 (Figure 3.7), RIA vs DBTB (aqueous results) r_s = 0.790; n =106; p<0.01 (Figure 3.8); RIA vs DBTB (organic results) r_s = 0.84; n = 95; p<0.01 (figure 3.9) and DETB (aqueous results) vs DBTB (aqueous results) r_s = 0.871; n = 106; p<0.01 (Figure 3.10).
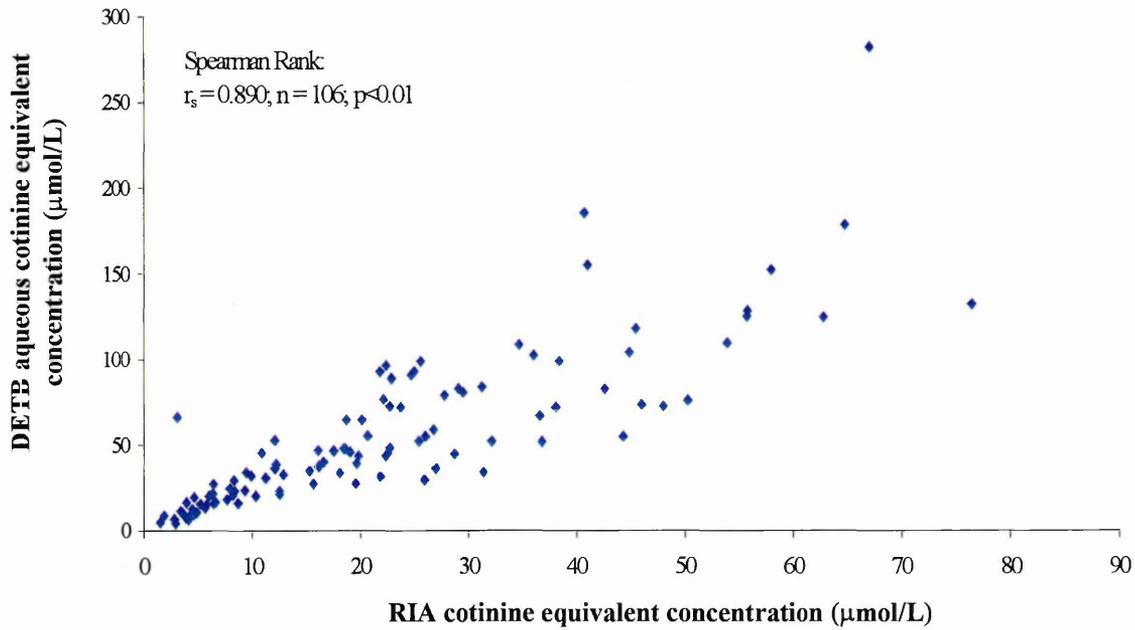
Figure 3.7 A correlation study between RIA determined smokers and DETB aqueous results
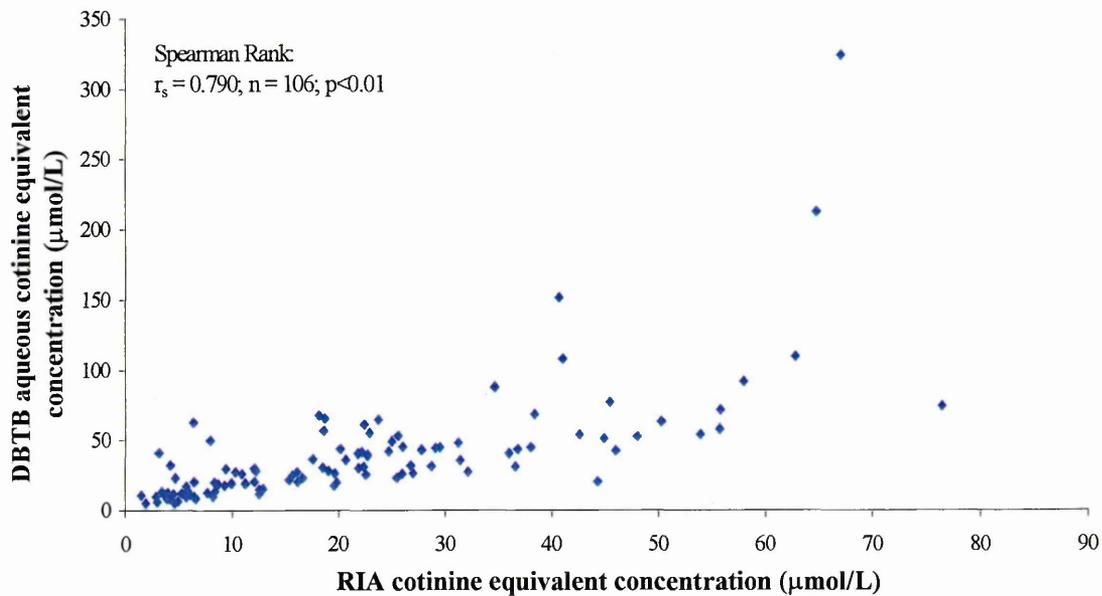
Figure 3.8 A correlation study between cotinine RIA determined smokers and DBTB aqueous results
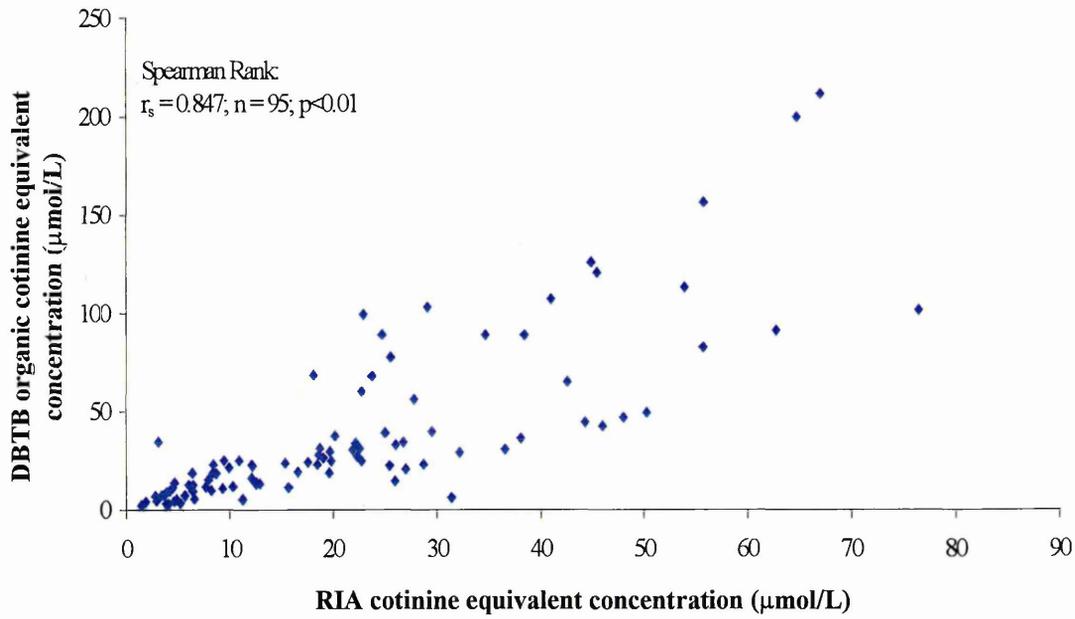
Figure 3.9 A correlation study between cotinine RIA determined smokers and DBTB organic results
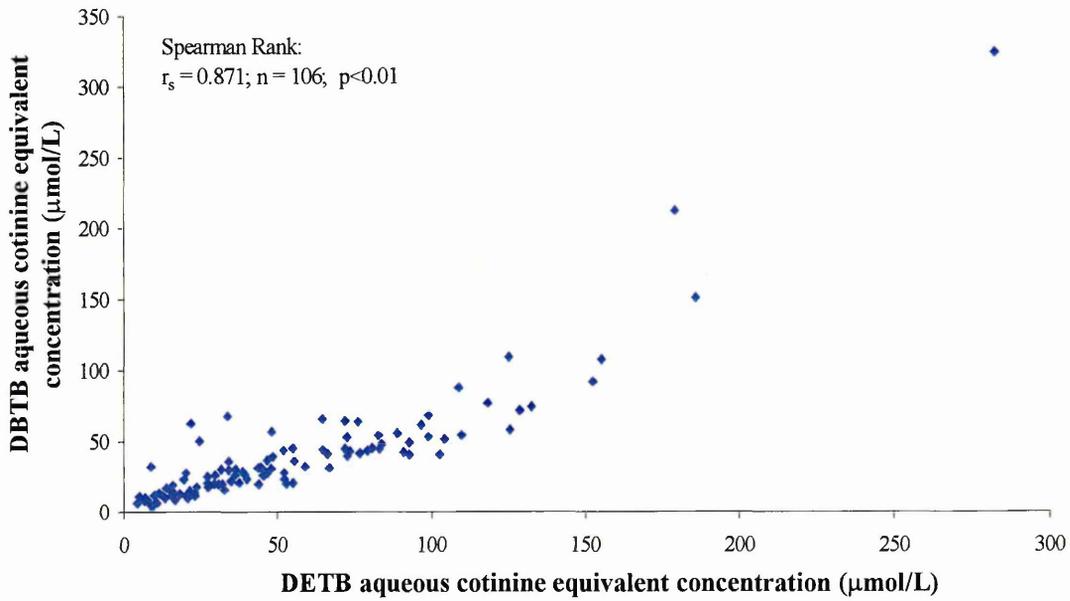
Figure 3.10 A correlation study between DETB smoker aqueous results and DBTB aqueous results

3.5 Discussion

The modified König reaction is a simple test that can be used to determine an individual's smoking status. When used qualitatively, DBTB appeared to be the more efficient condensing reagent with a clinical sensitivity of 100 percent and specificity of 99 percent, compared to DETB, which had a clinical sensitivity of 94 percent and specificity of 100 percent. The overall efficiency was determined to be 97 percent using DETB, which increased to 99.5 percent using DBTB. The difference in the clinical sensitivity and specificity between DETB and DBTB could be due to the improvement in partitioning between the aqueous layer and organic layer. Therefore DBTB has the advantage of being able to distinguish between borderline urine samples (i.e. smoker or non-smoker) which were misclassified by DETB.

Quantitative results suggested that the optimum clinical sensitivity and specificity was achieved using DETB as the condensing reagent (clinical sensitivity of 98 percent and specificity of 97 percent), which implies that, when used quantitatively, measurements obtained using DETB were equal to those with DBTB. The clinical sensitivity and specificity for DBTB results (aqueous results and organic results) were only 3 percent and 2 percent respectively less than DETB results. However, the number of urine samples obtained with a urinary cotinine equivalent concentration around the cut off point was very few and the difference in sensitivity and specificity may be due to one or two samples being misclassified as a smoker or non-smoker, especially as the distinction between a smoker and non-smoker is notoriously difficult to make, since no individual can be said to be totally unexposed to tobacco smoke. Since the numbers around the cut-off point are small, it is likely that these results are not significant.

The correlation studies for the results of the König reaction compared to the results obtained by the "gold standard" (RIA) all gave an $r_s > 0.790$. This confirms that the König reaction and the RIA method are producing similar results, although overall the König reaction produced higher cotinine equivalent values, compared to the RIA method, but this is as expected as the RIA method only measures cotinine and the

König reaction measures the total nicotine metabolite concentration. The correlation studies also showed that there are one or two samples that appear to have a significantly higher cotinine equivalent concentration compared to the cotinine concentration determined by the RIA method. These results may imply that these individuals have a different nicotine metabolic profile and that certain metabolites present in their urine are being measured by the König reaction but not by RIA. One possibility is that these individuals are either unable to metabolise nicotine or metabolise nicotine poorly using phase one metabolism due to a genetic variability of the cytochrome P450 enzyme, CYP2A6. Individuals who carry the null or inactive alleles (CYP2A6*2 and CYP2A6*3) for this enzyme, have an impaired nicotine metabolism and are significantly protected against becoming a tobacco dependent smoker (Pianezza et al 1998). Recent work has also suggested that nicotine is metabolised differently in the pregnant and the non-pregnant (Dempsey et al 1998) and that there are also inter-racial differences in nicotine metabolism (Benowitz et al 1999).

The one or two samples that appear to have a significantly higher cotinine equivalent concentration determined by the König reaction compared to the cotinine concentration determined by RIA may be due to these individuals having an altered metabolic profile and thus a greater concentration of certain nicotine metabolites may be detected; these may include nicotine-1′N-oxide, 3′-hydroxycotinine and 3′-hydroxycotinine glucuronide. These individuals may also have an increase in the concentration of cotinine and nicotine glucuronide, but the König reaction is unlikely to be able measure these particular metabolites directly, due to carbohydrate residue being attached to the nitrogen atom on the pyridine ring (figure 2.2). Therefore to determine the concentration of these metabolites the urine has to be pre-treated with the enzyme β-glucuronidase, which is able to cleave the nicotine-derived portion from the carbohydrate residue, thus allowing the nicotine-derived portion to be measured in the usual way. The increase in the cotinine equivalent concentration between the untreated and treated urine samples is proportional to the amount of cotinine and nicotine glucuronide present in the urine sample.

Chapter 4

Structural determination of the derivatives of nicotine and cotinine produced from the König reaction

4.1 Introduction

The König reaction is known to enable nicotine metabolites to react with a condensing reagent, such as DETB, to form coloured chromophores. The chemistry involved in this reaction and the final derivatized structures are not known for certain, although suggestions have been made (O'Doherty et al 1990 and Rustemeier et al 1993) (figure 4.1). O'Doherty et al (1990) put forward a structure for nicotine and its metabolites derivatized with either DETB or DMDD. However, there appeared to be no evidence given as to why they had suggested these structures. Rustemeier et al (1993) isolated nicotine derivatized with DETB and subjected the isolated compound to various analytical procedures, before they decided on a final structure. The analytical procedures they used included mass spectrometry (MS), infrared spectroscopy (IR) and nuclear magnetic resonance spectroscopy (NMR).
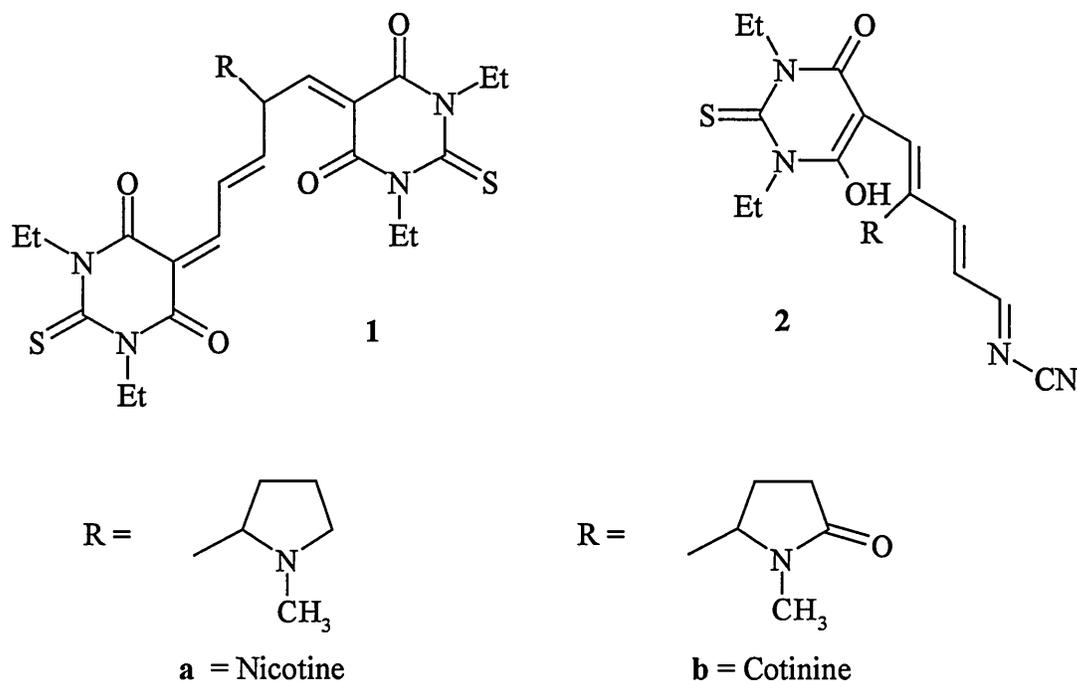
Figure 4.1: Molecular structures of DETB derived nicotine metabolites as proposed by O'Doherty et al (1990) (1) and Rustemeier et al (1993) (2).

The main aim of the work in this chapter was to confirm, or otherwise, the structure of the derivative formed from nicotine and DETB, using the method described by Rustemeier et al (1993). The analysis would be carried out using mass spectrometry, IR and NMR. Mass spectrometry was then to be used to extend this work by determining the relative molecular mass (RMM) of the derivatives formed from nicotine and cotinine using the other condensing reagents.

HPLC was to be used as an analytical procedure to ensure the retention time for the nicotine derivative obtained using the "preparative" conditions described in 4.2.1, was the same as that for nicotine derivatized using the "analytical" conditions described in 4.2.2.

4.2 Materials and methods

The mass spectrometer used was a VG quattro with a Harvard apparatus pump II for direct injection. Infrared spectroscopy was carried out with an ATI Mattson Genesis FTIR. NMR spectra were obtained in $CDCl_3$ solution using a Bruker AC 250. The HPLC system consisted of a Milton Roy CM 4000 multiple solvent delivery system pump, an LKB Biochrom ultraspec II spectrophotometer and a Kipp and Zonen BD9 two channel chart recorder. A 100 µL loop and a C_{18} column were also used and the flow rate was always set to 1 mL/min. Solutions were centrifuged using a Sorvall TC 6 bench top centrifuge. Absorbance measurements were obtained using a Cecil 9500 Super Aquarius. Solid phase extraction was carried out using a Bond Elute C_8 column.

4.2.1 Isolation of nicotine derivative

The structure of the derivative of nicotine derivatized with DETB was determined using a modification of the method described by Rustemeier et al (1993). A mixture of nicotine (40 mL, 0.01 M), sodium acetate buffer (16 mL, 4 M, pH 4.7), potassium cyanide (8 mL, 0.8 g, 1.5 M), chloramine-T (8 mL, 0.8 g, 0.8 M) and DETB (20 mL, 0.4 g, 0.1 M, water: acetone, 50:50 v/v) was allowed to react overnight in the dark. The solution was centrifuged for 10 minutes at approximately 3,000 rpm and the supernatant removed. The precipitate was washed with water and resuspended in distilled ethyl acetate (10 mL). The solution was dried with magnesium sulphate and the solvent removed under reduced pressure. The compound was further purified using a silica column (20 cm x 4 cm) and a gradient mobile phase, ethyl acetate (100 mL), ethyl acetate: 2 % methanol (200 mL), followed by ethyl acetate: 4 % methanol (500 mL). The pink fractions (nicotine derivative) were collected and checked by TLC (ethyl acetate: 4 % methanol, R_f 0.11) and the appropriate fractions pooled together. The solvent was removed under reduced pressure, resulting in a dark red solid (7.8 mg). The isolated fraction (purified nicotine derivative) was further analysed using IR, NMR, mass spectrometry and HPLC.

4.2.2 Analysis by HPLC

HPLC was used to compare the retention time for the isolated DETB nicotine derivative to that of the nicotine derivatized using the "analytical" conditions described below. A small amount of the isolated nicotine derivative was dissolved in methanol to form a concentrated pink solution. The solution was then subjected to chromatography with UV detection at 530 nm using a mobile phase of acetonitrile: heptane sulphonic acid (0.005 M) 45:55. This mobile phase was a modification of mobile phases described by Moore et al (1990) and Ubbink et al (1993).

A 10 μg/mL solution of nicotine (1 mL) was added to sodium acetate buffer (400 μL, 4 M pH 4.7), potassium cyanide (200 μL, 1 % w/v) and chloramine-T (200 μL, 1 % w/v). The solution was mixed well before the addition of the condensing reagent, DETB (1 mL, 1 % w/v 50:50 acetone: water). After 20 minutes the solution was placed on a solid phase extraction (SPE) column, pre conditioned using methanol (2 mL) followed by distilled water (2 mL). The column was washed with distilled water (2 mL) which was used to remove any unreacted potassium cyanide and chloramine-T leaving the nicotine derivative attached to the column. The nicotine derivative was then eluted from the column with methanol (1 mL) and analysed by HPLC using the same conditions as described above.

4.2.3 Analysis by mass spectroscopy

A small amount of the isolated nicotine derivative was resuspended in methanol (2 mL) to form a dark pink solution and was directly injected in to the mass spectrometer set to detect negative ions. Further studies were carried out using the "analytical" conditions as described in 4.2.2 using nicotine or cotinine (50 μg/mL) derivatized with the other condensing reagents, although DBTB and MPED will only dissolve in 100 percent acetone. After twenty minutes, distilled water (2 mL) was added to each sample before the solution was transferred to an SPE column, which had been pre-conditioned with

methanol (2 mL), followed by distilled water (2 mL). It was observed that the addition of water to the sample pre SPE enabled a greater amount of the derivative to remain on the SPE column during the wash stage. The column was then washed with distilled water (2 mL) and the derivative eluted from the column with methanol (2 mL). Each sample was then stored until analysis in a container wrapped in aluminium foil, as the product is light sensitive. This was observed when a sample was left on the bench in direct sunlight for one hour, after which time the colour of the solution had begun to fade.

4.3 Results and discussion

4.3.1 Structural determination of the nicotine DETB derivative

The purified nicotine derivative was subjected to various analytical procedures to confirm if either structure **1a** or **2a** were correct.

The results from the infrared spectroscopy (figure 4.2) showed that there was a strong peak at 2176 cm^{-1}. In this part of the infrared spectrum, very few bonds absorb with the exception of CN triple bonds. Therefore, the peak at 2176 cm^{-1} suggests that the isolated nicotine derivative contains a CN triple bond. The structure **2a**, suggested by Rustemeier et al (1993) contains a CN triple bond, whereas in structure **1a** no such bonds are present.
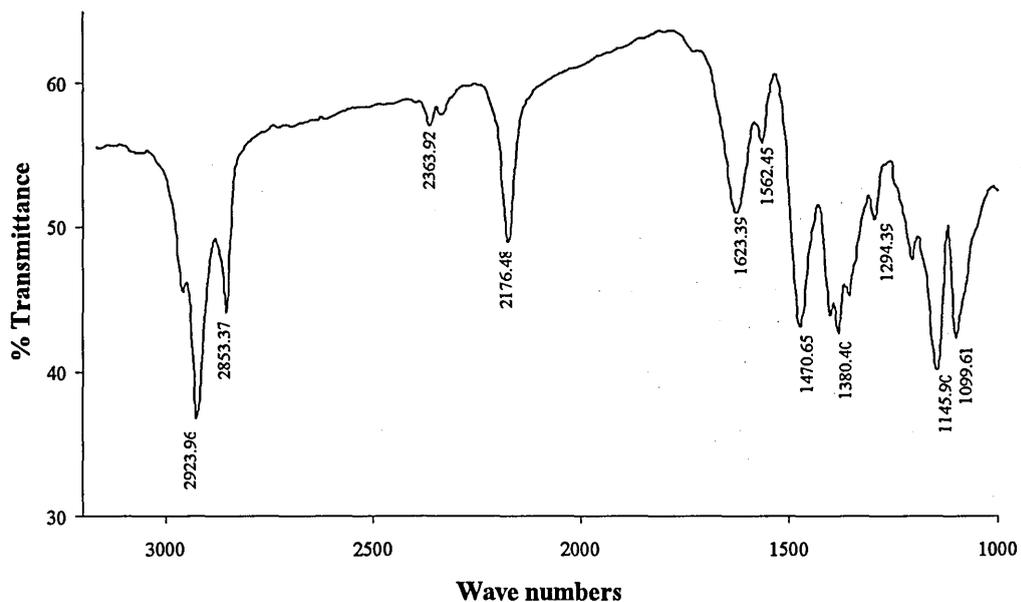
Figure 4.2 Infrared spectrum for isolated nicotine derivative.

Based on the chemical reactions involved in the derivatization of nicotine and its metabolites, using the König reaction (figure 2.1), Rustemeier et al (1993) positioning of the R group may not be the only structure able to form from this reaction. Their suggestion of structure **2** (figure 4.3) would result if the OH group from water attached to the 6-position of the nicotine metabolite. The alternative structure **8** would result if the OH group were attached to the 2-position of the nicotine metabolite.
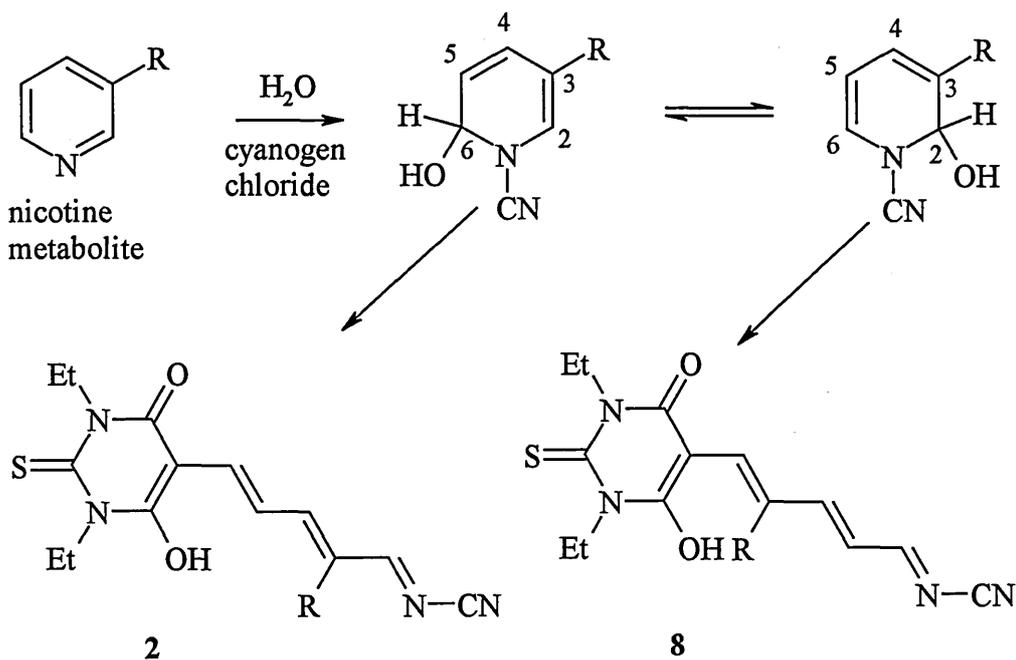
Figure 4.3 Molecular structures of DETB derived nicotine metabolites as proposed by Rustemeier et al (1993) (**2**) and an alternative structure (**8**).

However, it would be extremely difficult to differentiate between these two products using mass spectrometry, IR or NMR. An attempt was made to grow crystals for x-ray analysis, since this technique would have given a definite answer. A small amount of the isolated nicotine derivative was dissolved in ethyl acetate and various mixtures of ethyl acetate and petrol and the solvent allowed to slowly evaporate off, but no suitable crystals were formed, only amorphous powders.

The NMR spectrum of the isolated nicotine derivative is shown in figure 4.4. The relative integration (1:1) of the N-CH₂ peaks of the N-ethyl group(s) at 4.6 δ to that for the alkene protons in the 7.3 - 8.2 δ region indicate that there is only one DETB unit in the compound, as in structure **2a**, whereas structure **1a** would require two such units. This further confirms that the structure suggested by O'Doherty et al (1990) was not correct. The NMR results published by Rustemeier et al (1993) are very vague and no NMR spectrum was given to compare with the results obtained in this study. However, they did comment on the difference between the spectrum of nicotine and their reaction

107
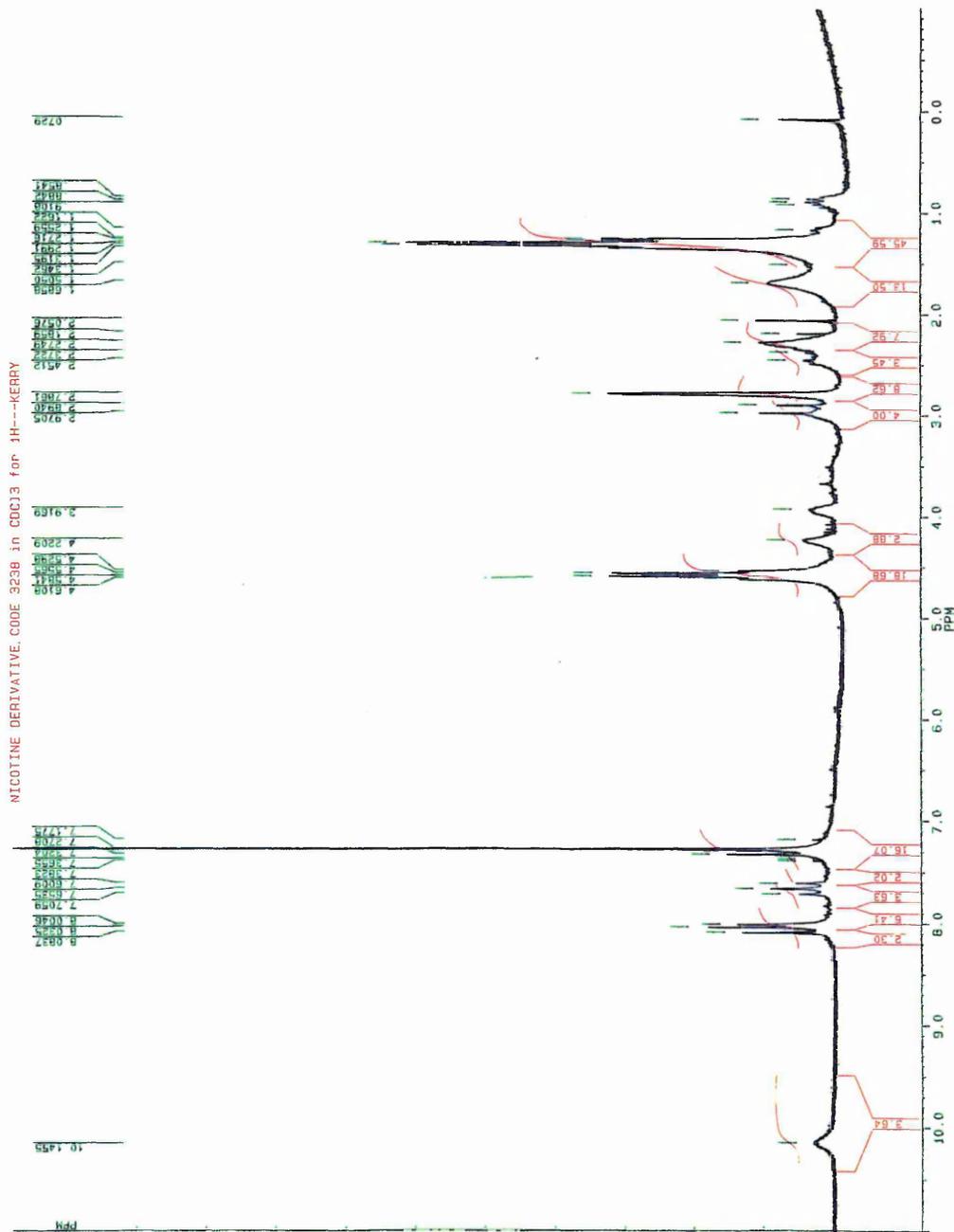
Figure 4.4 NMR spectrum of the isolated nicotine derivative

product. They suggested that the changes for the protons of the double bond region (δ between 7.2 and 8.2), where the coupling constants increased to ca.17 Hz, could be explained by the opening of the pyridine ring leading to an alkene with a trans configuration as in structure **2**. Of course the same would be true for structure **8**.

The results for the HPLC suggest that nicotine derivatized using the "analytical" conditions had the same retention time as the isolated nicotine derivative (9.0 minutes). The HPLC traces for the "analytical" conditions (i.e. the total reaction mixture after SPE) appeared to have one peak up to a retention time of 15 minutes. If any other compounds (for example structure **1a**) were present with a retention time greater than 15 minutes, then they would have been detected in later runs. No such peaks were seen so it can be assumed that no other compounds were present.

4.3.2 Structural determination of derivatives of nicotine and cotinine by mass spectrometry

Mass spectrometry was used by Rustemeier et al (1993) to confirm the RMM of the isolated DETB nicotine derivative. In this current study the predicted and observed molecular ions for either nicotine or cotinine derivatized with the various condensing reagents are shown in table 4.1. The reason for the observed peak being M-1 was a result of the mass spectrometer set to negative ion mode. This results in the observed ions having a RMM of one less than that expected.

NICOTINE METABOLITE	CONDENSING REAGENT	PREDICTED RMM FOR RUSTEMEIER'S STRUCTURE	PREDICTED RMM FOR O'DOHERTY'S STRUCTURE	OBSERVED PEAK (M-1)
Nicotine	DETB	387	543	386
Cotinine	DETB	401	557	400
Nicotine	DBTB	443	599	442
Cotinine	DBTB	457	613	456
Nicotine	DMDD	331	487	330
Cotinine	DMDD	345	501	345
Nicotine	MPED	421	577	420
Cotinine	MPED	435	591	434

Table 4.1 Predicted and observed molecular ions for the derivatives of nicotine and cotinine.

Molecular ions for the derivatives of nicotine and cotinine were determined by observing a range of masses from 50 to 750. The results indicate that structures **2a** and **2b** (figures 4.5- 4.6) (or presumably **8a** or **8b**) were present but no ions were present to confirm either structure **1a** or **1b**.

Overall, the results from the infrared spectroscopy, NMR and mass spectrometry found no evidence for structure **1**. This is supported by a CN triple bond present on the infra red spectrum, only one DETB unit present on the NMR spectrum and correctly identified molecular ions by mass spectrometry. Therefore, these results confirm that the isolated DETB nicotine derivative was the same as structure **2** or **8**. However, the yield for the isolated nicotine derivative was only 5 percent so it may be possible that a compound with the structure **1** was formed, but was not isolated using the "preparative" conditions described in 4.2.1.
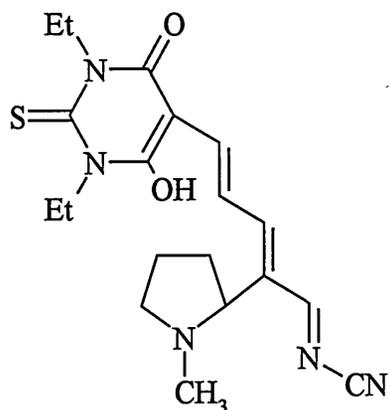
Nicotine derivatized with DETB
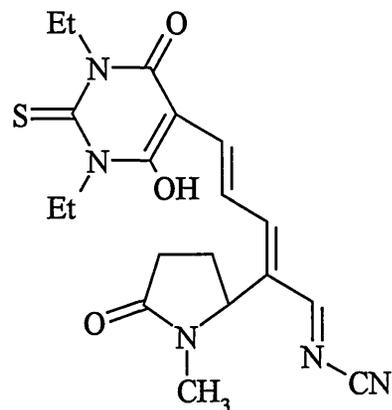
Cotinine derivatized with DETB
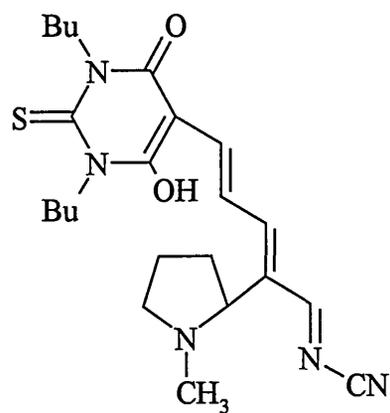
Nicotine derivatized with DBTB
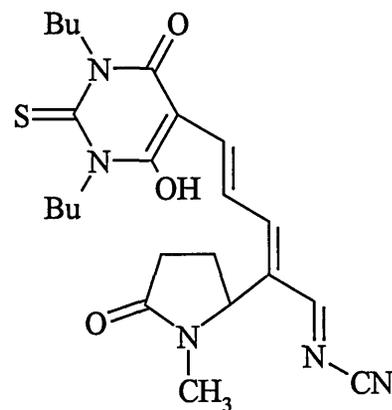
Cotinine derivatized with DBTB

Figure 4.5 Predicted final structures of nicotine or cotinine derivatized using DETB or DBTB

111
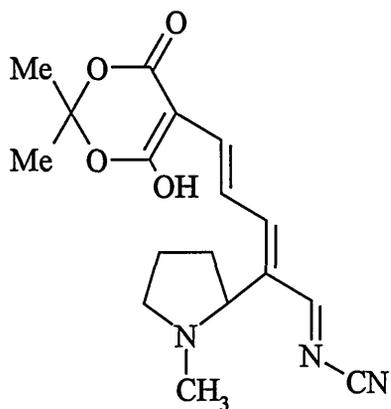
Nicotine derivatized with DMDD
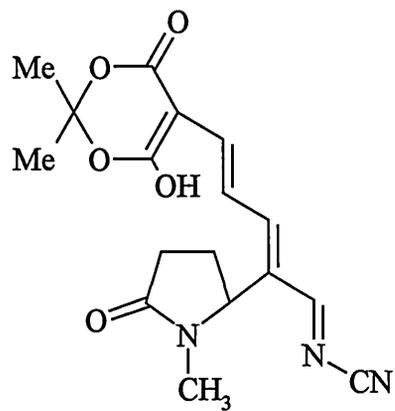
Cotinine derivatized with DMDD
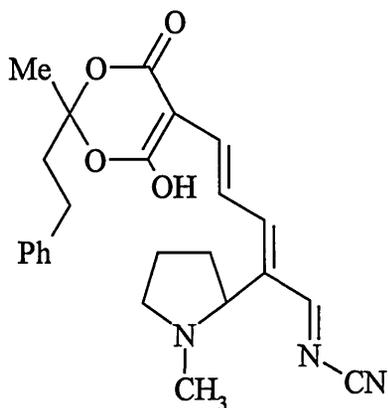
Nicotine derivatized with MPED
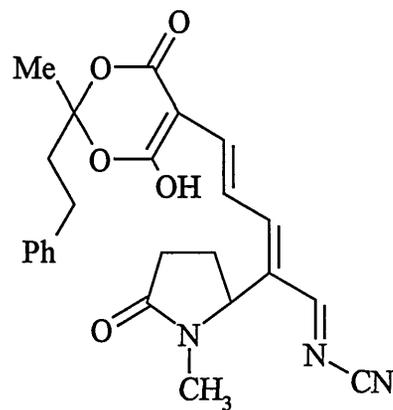
Cotinine derivatized with MPED

Figure 4.6 Predicted final structures of nicotine or cotinine derivatized using DMDD or MPED

112

4.4 Solvatochromism

During the "preparative" reactions (4.2.1), it was observed that the maximum absorbance wavelength for the isolated nicotine derivative altered depending on the solvent used (e.g. water or ethyl acetate). A possible reason for this change is that the derivative is able to exhibit solvatochromism. Solvatochromism refers to a compound whose absorbance maximum wavelength will depend on the polarity of its surroundings.

The pyridinium N-phenoxide betaine dye **9** (figure 4.7a) is a compound showing negative solvatochromism. This dye is known to exhibit one of the largest solvatochromic shifts ever observed (Reichardt 1992). The absorption bandwidth is shifted by 375 nm from a maximum wavelength of 453 nm when in water to 810 nm in diphenyl ether. When **9** is placed in a polar solvent like methanol the highly dipolar ground state is more stable compared to the less dipolar excited state and this results in a greater amount of energy being required to excite an electron from the ground state to excited state, which is observed as a lower wavelength (515 nm) absorption. However, when **9** is placed in a non-polar solvent, such as ethyl acetate, the ground state is made less stable and the excited state more stable and therefore the energy required to excite an electron is less, which results in the maximum wavelength shifting to a longer wavelength (769 nm) (figure 4.7b).
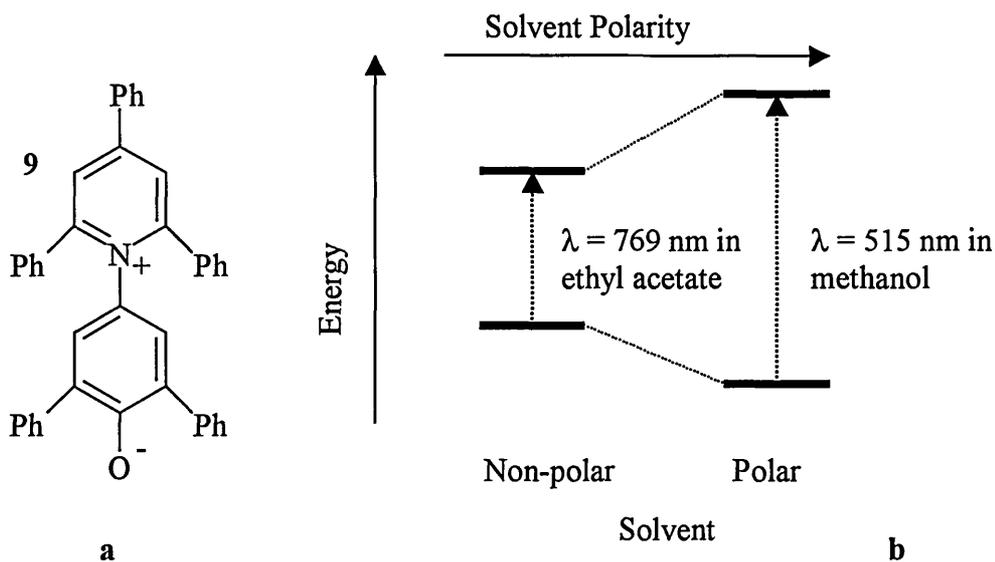
Figure 4.7 a Molecular structure of pyridinium *N*-phenoxide betaine dye **9**
       b Diagrammatic representation of the energy states of **9** in different solvents

The "preparative" conditions described in 4.2.1 were slightly modified to determine if nicotine derivatized with the other condensing reagents formed solvatochromic compounds. A mixture of nicotine (20 mL, 0.01 M), sodium acetate buffer (8 mL, 4M, pH 4.7), potassium cyanide (4 mL, 0.4 g, 1.5M), chloramine-T (4 mL, 0.4g, 0.8 M) and a condensing reagent (10 mL, 0.4 g, DETB and DMDD water: acetone, 50:50 v/v, DBTB and MPED 100 % acetone) was allowed to react overnight. The solution was centrifuged for 10 minutes at approximately 3,000 rpm and the supernatant removed. A small amount of the precipitate was then dissolved in various solvents (water, ethyl acetate: methanol and ethyl acetate) and the maximum wavelength determined by scanning between 250-900 nm.

The results for the wavelengths obtained for all the derivatives in various solvents are shown in table 4.2. The results suggest that, when nicotine is derivatized with any of the condensing reagents the derivative formed is able to exhibit negative solvatochromism, that is the maximum wavelength shifts to a longer wavelength as the surrounding solvent changes from a polar (water) to a less polar (ethyl acetate) solvent.

CONDENSING REAGENT	MAXIMUM WAVELENGTH IN WATER	MAXIMUM WAVELENGTH IN A MIXTURE OF ETHYL ACETATE : METHANOL (1:1)	MAXIMUM WAVELENGTH IN ETHYL ACETATE
DETB	531	549	610
DBTB	518	557	564
DMDD	505	509	518
MPED	497	550	580
Purified DETB nicotine	538 (in methanol)	564	550

Table 4.2 Maximum wavelengths obtained when using different solvents.

Generally the results follow the expected pattern: when the derivatives were placed in water the absorbance at the shorter wavelength was observed, in ethyl acetate the absorbance at a longer wavelength was observed and when the derivatives were placed in a mixture of ethyl acetate and methanol the absorption was in between the previous two wavelengths. However, for the purified DETB nicotine derivative the observed wavelengths did not follow this expected pattern. When the purified nicotine derivative was placed in methanol the maximum wavelength observed was slightly higher than that of the DETB derivative, which is what would have been expected as methanol is less polar than water. When the purified nicotine derivative was placed in a mixture of ethyl acetate and methanol the absorbance shifted from 538nm to 564nm as expected. However, on comparing the wavelengths with the other DETB derivative the wavelengths were similar but the purified nicotine derivative wavelength was slightly longer, 564 nm compared to 549 nm. This can be explained as the ratio of ethyl acetate and methanol were not measured exactly, so if the purified nicotine derivative was added to a mixture containing slightly more ethyl acetate than the mixture used for the DETB derivative then the observed wavelength for the purified nicotine derivative would be expected to be longer. The reason for the wavelength for the purified nicotine derivative when placed in ethyl acetate not being the same as that observed for the DETB derivative is unknown. It is possible that the solvatochromic effects for the

"analytical" samples are a result of additive effects from a number of compounds in the mixture. It may also be that this is an odd result due to contamination of the derivative or solvent. Ideally, this part of the experiment should have been repeated but time did not allow for this to be done.

Overall the results suggest that the derivatives of nicotine do exhibit negative solvatochromism, although when comparing the absorption shifts to 9 the absorption shifts for the derivatives are very small, and therefore it would be highly unlikely that they could be used as solvatochromic compounds. However, from this observation, it is important when reporting the maximum wavelength for all these derivatives and possibly derivatives of other nicotine metabolites, that the solvent system used to determine a spectrum is also reported.

Chapter 5

The effect of surfactants on the König reaction

5.1 Introduction

The König reaction is a simple analytical technique that can be used to measure nicotine and its metabolites in a urine sample, and in recent years this reaction has been incorporated into two different near patient tests to determine an individual's smoking status (Cope et al 1996 and Eswara et al 1996). Cope et al (1996) published a simple near patient urine test they referred to as the Smoking Test Device, although it is now referred to as SmokeScreen (http://www.bham.ac.uk) to distinguish smokers from non-smokers. This device consists of two parts; an adapted fixed-volume 2 mL syringe and cap, and a barrel and lower reaction chamber with a membrane seal. All the dried reagents (potassium cyanide, DETB, citric acid and sodium citrate) are contained in the reaction chamber, except chloramine-T, which is isolated for stability reasons in a washer-like annulus, with a space between two tightly fitting plastic discs, which forms a seal on top of the reaction chamber (figure 5.1).

The fixed-volume syringe can then be used to measure 2 mL of unprepared urine, which is transferred into the barrel, forming a permanent seal between the cap and the barrel. As the syringe passes through the barrel, the tip breaks the seal, causing the chloramine-T to be dispensed into the reaction chamber below. The urine is then injected onto the reagents, which dissolve, starting the reaction. As the device contains DETB as the condensing reagent, pink chromophores will form in a concentration dependent manner, if nicotine and its metabolites are present. After 20 minutes, the colour change of the urine sample can be observed to give either a qualitative or semi-quantitative assessment of an individual's smoking status or measured in a simple spectrophotometer to give the concentration of nicotine and its metabolites with reference to a cotinine standard.
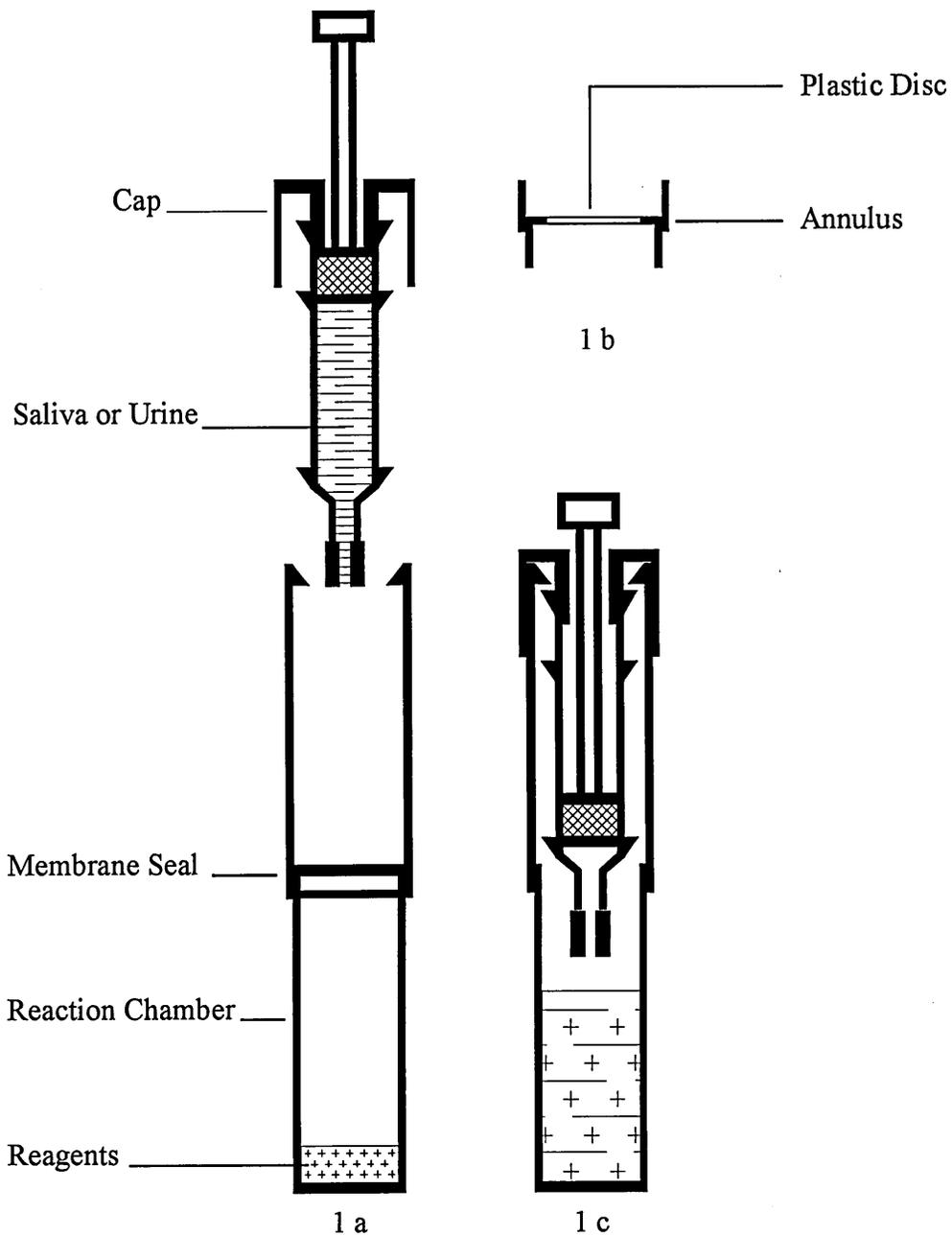
Figure 5.1 (a) SmokeScreen device before use, comparing a sampling syringe, and reaction chamber, containing dry reagents: (b) an enlargement of the seal, with the chloramines-T isolated between the two plastic discs: (c) the assembled device after addition of the sample (Cope et al 1996)

118

Cope et al (1996) reported that qualitatively the SmokeScreen produced a sensitivity and specificity of 89 percent and 100 percent, respectively and a sensitivity and specificity of 89.9 percent and 98.9 percent, respectively for quantitative results. The device was also reported to have a lower limit of detection of 0.6 μg/mL (3.4 μmol/L) and the cut off point to differentiate between smokers and non-smokers was predetermined to be 10 μg/mL.

Cope et al (2000) published another paper describing a modification to the SmokeScreen to enable exposure to environmental tobacco smoke to be quantified. This device is referred to as the Passive SmokeScreen, which is the same as the SmokeScreen, except that it measures saliva and the condensing reagent has been changed from DETB to DMDD. The results given were very vague and no explanation was given as to why they had changed the condensing reagent to DMDD. One possible reason why DMDD was chosen is that it enables the sensitivity of the Passive SmokeScreen to increase, thus allowing smaller quantities of nicotine metabolites to be detected. This is in agreement with the findings in chapter 2, which suggested that DMDD produced the highest absorbance values, when comparing the results with the other three condensing reagents used.

The SmokeScreen device has been used in clinical trials to improve information feedback to pregnant women and the Passive SmokeScreen has been used in pilot studies to measure passive smoking in bar staff and children with asthma. However, the disadvantage of both the SmokeScreen and Passive SmokeScreen is that they involve the use of potassium cyanide.

Eswara et al (1996) reported that they had developed a simple strip test referred to as the NicCheck. The advantage of the NicCheck over both the SmokeScreen and Passive SmokeScreen is that it uses the less toxic compound potassium thiocyanate instead of potassium cyanide. The NicCheck strip consists of a paper strip consisting of four regions, with each region containing a different reagent (acetate buffer, potassium thiocyanate, chloramine-T and DETB). The test strip is placed in a urine sample and as

119

the urine diffuses up the test strip the reagents dissolve and then react together. After 30 minutes the colour of the test strip and urine sample remaining in the container are observed, and a positive result is confirmed by the test strip turning pink. (The sensitivity and specificity of the NicCheck was reported to be 98.7 percent and 99.4 percent respectively.) However, from the results obtained in this current study (5.3), it was observed that the absorbance obtained when using potassium thiocyanate was reduced by up to 90 percent compared to that when using potassium cyanide. Therefore, it was concluded that potassium thiocyanate could only be realistically used to distinguish heavy smokers from non-smokers and it would be extremely difficult, if not impossible, to differentiate light smokers from non-smokers. No other publications have been found relating to this test, suggesting that it has not found wide usage or acceptability.

Esteve-Romero et al (1995) reported that they had improved the König reaction to determine (using aniline as the condensing reagent) the concentration of pyridine, pyrrolyl-methylpyridine and nicotinic acid in water with the addition of a surfactant to the reaction mixture. This study looked at three different types of surfactant, an anionic surfactant, sodium dodecyl sulphate (SDS) (figure 5.2), a cationic surfactant, N-cetylpyridinium chloride (NCPC) (figure 5.2), and a non-ionic surfactant, Triton X-100 (figure 5.2) to determine which type of surfactant, if any, would enhance the overall absorbance of the reaction. They concluded that when SDS was added to the reaction mixture the absorbance obtained had increased, consequently increasing the molar absorptivities.

CH$_3$(CH$_2$)$_{10}$CH$_2$O—S(=O)(=O)—ONa

sodium dodecyl sulphate (SDS)

N-cetylpyridinium chloride (NCPC)

H$_3$C—C(CH$_3$)(CH$_3$)—CH$_2$—C(CH$_3$)(CH$_3$)—⬡—O(CH$_2$CH$_2$O)$_n$-H

n = ~ 10

Triton X-100
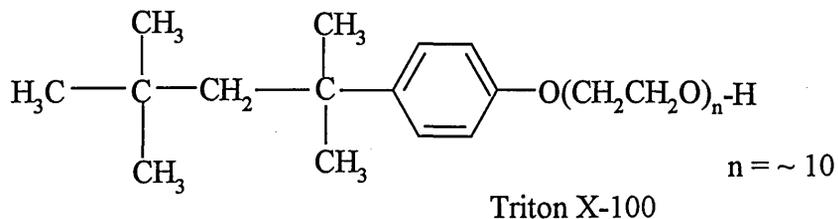
Figure 5.2 structures of surfactants

A surfactant describes a species that accumulates at the interface of two fluids and modifies the properties of the surface. A typical surfactant molecule is made up of two parts, a long hydrophobic hydrocarbon tail that can dissolve in hydrocarbon and other non-polar materials and a hydrophilic head that dissolves in a polar solvent. When a surfactant is added to a solvent, such as water, the surfactant molecules organise themselves so that their hydrophobic tails have as little contact with the water molecules as possible. The surfactant molecules are held together by hydrogen bonds and once the concentration of the surfactant exceeds the critical micelle concentration (cmc), the surfactant molecules spontaneously start to form into micelles (Figure 5.3) (Harris 1999). The shapes of micelles can vary with the concentration of surfactant used, although at the cmc spherical micelles usually form, whereas at higher concentrations the micelles more commonly form flattened spheres and rod-like shapes.

Non-polar compound
dissolved in interior of
micelle

Hydrophilic head
interacting with a
polar solvent

Hydrophobic
hydrocarbon tail
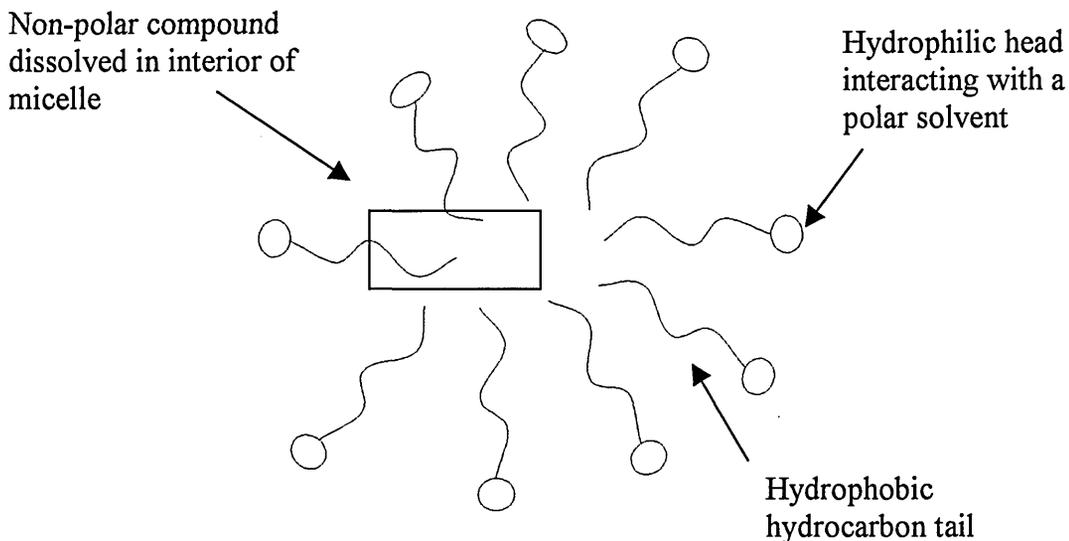
Figure 5.3 Possible structure of a micelle

Micelles are known to exhibit several properties that can facilitate analytical measurements. These include their ability to solubilise and concentrate analyte, alter acidity and polarity of solutes and modify reaction rates (Egekeze et al 1997). Micelles are also able to solubilise, within their local environment, compounds that are insoluble or sparingly soluble in water, due to the interior of the micelle resembling a non-polar organic solvent (Egekeze et al 1997). This is important as surfactants can be used instead of organic solvents.

Esteve-Romero et al (1995) suggested that since at pH 4.5 aniline is protonated (positively charged) it is therefore attracted by ionic interactions to the anionic (negatively charged) SDS micelles. They also reported that when NCPC was added to the reaction mixture the absorbance increased slowly with the concentration of surfactant, but at pH 4.5 both the aniline and the glutaconic aldehyde of nicotinic acid will be protonated and therefore should be repelled by the cationic NCPC micelles. However, they suggested that this unexpected result might be due to hydrophobic interactions, which are independent from the charge of the surfactant, and allow the association between the non-ionic glutaconic aldehyde and NCPC micelles to occur.

Therefore, the association between the glutaconic aldehyde and surfactant will increase as the concentration of NCPC increases.

Egekeze et al (1997) reported that they had enhanced the determination of cyanide ions in organic sample matrices using the cationic surfactant cetyltrimethylammonium bromide (CTAB) (figure 5.4). The determination of cyanide ions involved a spectroscopic method they described as the pyridine-barbituric acid method. This method is in fact a modification of the König reaction, where a known amount of pyridine is used to allow determination of the concentration of cyanide ions, (the reverse of the work described in chapter 2). The condensing reagent they used was barbituric acid and the surfactant CTAB was mixed with sodium hydroxide, to ensure the solution was alkaline. In an alkaline solution the glutaconic aldehyde would be deprotonated and attracted to the cationic surfactant by ionic interactions.

$$CH_3(CH_2)_{14}CH_2-\overset{\overset{\displaystyle CH_3}{|}}{\underset{\underset{\displaystyle CH_3}{|}}{N^+}}-CH_3 \quad Br^-$$
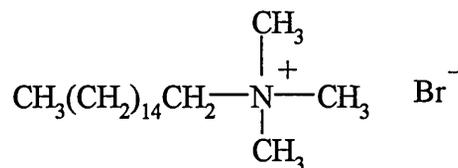
Figure 5.4 Structure of cetyltrimethylammonium bromide (CTAB)

Capella-Peiró et al (2001) reported a simple flow injection method based on the König reaction to detect nicotinic acid in commercial pharmaceuticals. The effects of the four surfactants, NCPC, CTAB, Triton X-100 and SDS with various condensing reagents, aniline, p-toluidine, and p-sulphanilic acid were studied. The reaction was carried out at pH 7.0 and they reported what they described as absorbance enhancement factors for aniline, p-toluidine and p-sulphanilic acid as 3.3, 5.2 and 4.3 for NCPC, and 2.6, 3.4 and 4.1 for CTAB respectively.

123

Capella-Peiró et al (2001) concluded that at pH 7.0 nicotinic acid and *p*-sulphanilic acid are deprotonated and negatively charged, whereas aniline and *p*-toluidine are neutral. Therefore, there is a strong association between the various condensing reagents and the glutaconic aldehyde of nicotinic acid and the cationic NCPC or CTAB micelles by electrostatic and/or hydrophobic forces. As SDS is an anionic surfactant, there would be a repulsion between the anionic nicotinic acid or *p*-sulphanilic acid and the SDS micelles, which is probably the reason why a lower absorbance was observed.

The aim of the work in this chapter was to determine if the addition of surfactants, such as SDS could be used to enhance the sensitivity of the König reaction using potassium thiocyanate. If the sensitivity of the König reaction could be enhanced, then it might be possible to manufacture an improved device to determine an individual's smoking status. This possible device would then have the advantage over both the SmokeScreen and Passive SmokeScreen of using a less toxic compound than potassium cyanide, and could also have a greater sensitivity compared to the NicCheck. The surfactants that were to be used were sodium dodecyl sulfate (SDS), dodecyl benzene sulfonic acid (DBS) and Triton X-200 (anionic surfactants), N-cetyl pyridium chloride (NCPC) and tetradecyl trimethyl ammonium bromide (TTAB) (cationic surfactants) and Triton X-100 (non-ionic surfactant).

5.2 Materials and methods

The surfactants sodium dodecyl sulphate, dodecyl benzene sulfonic acid, Triton X-200, N-cetyl pyridium chloride, tetradecyl trimethyl ammonium bromide and Triton X-100 were purchased from Sigma (Poole, Dorset, UK). Potassium thiocyanate was also purchased from Sigma The concentration of the surfactant used was 0.5 M, unless no RMM was available for a particular surfactant then 2×10^5 ppm was used instead. Absorbance measurements were determined using a Cecil 9500 Super Aquarius.

5.2.1 The effects of the addition of surfactants on the König reaction

The individual absorbance maximum was determined for each condensing reagent with and without the addition of one of the six surfactants under investigation. A standard cotinine solution (1 mL, 10 μg/mL) was taken and allowed to react with sodium acetate buffer (200 μL, 4 M, pH 4.7), surfactant (200 μL, 0.5 M or 2×10^5 ppm) or distilled water (control sample, 200 μL), potassium cyanide or potassium thiocyanate (200 μL, 10 % w/v) and chloramine-T (200 μL, 10 % w/v). The reaction mixture was mixed well before the addition of a condensing reagent (1 mL, 1% w/v, DETB and DMDD 50:50 acetone: water v/v, DBTB and MPED, 100 % acetone). The reaction mixture was again mixed well and allowed to stand for 20 minutes, before being transferred to a cuvette. Measurements were then taken between 450 nm and 550 nm to determine the maximum absorbance.

Once the maximum absorbance had been determined for each sample, the procedure was repeated in duplicate again, except measurements were taken at the appropriate maximum absorbance (table 5.1).

	POTASSIUM THIOCYANATE		POTASSIUM CYANIDE	
Condensing reagent	Surfactant (concentration)	Maximum absorbance	Surfactant (concentration)	Maximum absorbance
DETB	Control	530	Control	525
DETB	NCPC (0.5 M)	Cloudy	NCPC (0.5 M)	526
DETB	TTBB (0.5 M)	538	TTBB (0.5 M)	528
DETB	Triton X-100 (0.5 M)	539	Triton X-100 (0.5 M)	539
DETB	SDS (0.5 M)	535	SDS (0.5 M)	Cloudy (pink)
DETB	DBS ($2x10^5$ ppm)	534	DBS ($2x10^5$ ppm)	534
DETB	Triton X-200 ($2x10^5$ ppm)	Cloudy	Triton X-200 ($2x10^5$ ppm)	530
DBTB	Control	532	Control	531
DBTB	NCPC (0.5 M)	538	NCPC (0.5 M)	537
DBTB	TTBB (0.5 M)	538	TTBB (0.5 M)	535
DBTB	Triton X-100 (0.5 M)	532	Triton X-100 (0.5 M)	540
DBTB	SDS (0.5 M)	535	SDS (0.5 M)	535
DBTB	DBS ($2x10^5$ ppm)	Cloudy	DBS ($2x10^5$ ppm)	Cloudy
DBTB	Triton X-200 ($2x10^5$ ppm)	Cloudy	Triton X-200 ($2x10^5$ ppm)	Cloudy
DMDD	Control	496	Control	496
DMDD	NCPC (0.5 M)	Cloudy	NCPC (0.5 M)	505
DMDD	TTBB (0.5 M)	495	TTBB (0.5 M)	502
DMDD	Triton X-100 (0.5 M)	497	Triton X-100 (0.5 M)	500
DMDD	SDS (0.5 M)	496	SDS (0.5 M)	497
DMDD	DBS ($2x10^5$ ppm)	496	DBS ($2x10^5$ ppm)	496
DMDD	Triton X-200 ($2x10^5$ ppm)	493	Triton X-200 ($2x10^5$ ppm)	498
MPED	Control	498	Control	499
MPED	NCPC (0.5 M)	502	NCPC (0.5 M)	503
MPED	TTBB (0.5 M)	502	TTBB (0.5 M)	500
MPED	Triton X-100 (0.5 M)	500	Triton X-100 (0.5 M)	500
MPED	SDS (0.5 M)	498	SDS (0.5 M)	500
MPED	DBS ($2x10^5$ ppm)	Cloudy	DBS ($2x10^5$ ppm)	Cloudy (yellow)
MPED	Triton X-200 ($2x10^5$ ppm)	Cloudy	Triton X-200 ($2x10^5$ ppm)	Cloudy (yellow)

Table 5.1: Maximum absorbance values for the different condensing reagents with and without the addition of surfactants.

5.3 Results

5.3.1 The effect of surfactants on the König reaction when using potassium thiocyanate

The results for the various condensing reagents using potassium thiocyanate are shown in figures 5.5– 5.8. They suggest the addition of a surfactant to the reaction mixture can either cause the absorbance value to increase, decrease or stay the same as the control absorbance.

The addition of the cationic surfactants, NCPC and TTAB, overall reduced the absorbance values obtained, except, when NCPC was used with the condensing reagent MPED, where the absorbance value increased by 143 percent. The addition of the non-ionic surfactant, Triton X-100, had no real effect on the absorbance value when DETB and DMDD were used. However, the absorbance decreased when DBTB and MPED were used by 71 percent and 86 percent respectively.

The anionic surfactant, SDS caused a significant increase when either DMDD or MPED were used, with an increase of 135 percent and 278 percent respectively. Other anionic surfactants, DBS and Triton X-200, were studied but they did not have the same effect on absorbance value as SDS. The addition of Triton X-200 to the reaction mixture caused the solution to become cloudy for all samples, except when DMDD was used. The addition of DBS caused an increase in the observed absorbance when DETB and DMDD were used by 22 percent and 142 percent respectively, although the solution turned cloudy when DBTB and MPED were used. The reason why the solution turned cloudy could be due to the increased amount of acetone present in the DBTB and MPED solutions. This may be because DBS is only soluble when a small amount of acetone is present, and as DBTB and DMDD are in 100 percent acetone, DBS is no longer soluble in the reaction mixture causing the solution to turn cloudy.
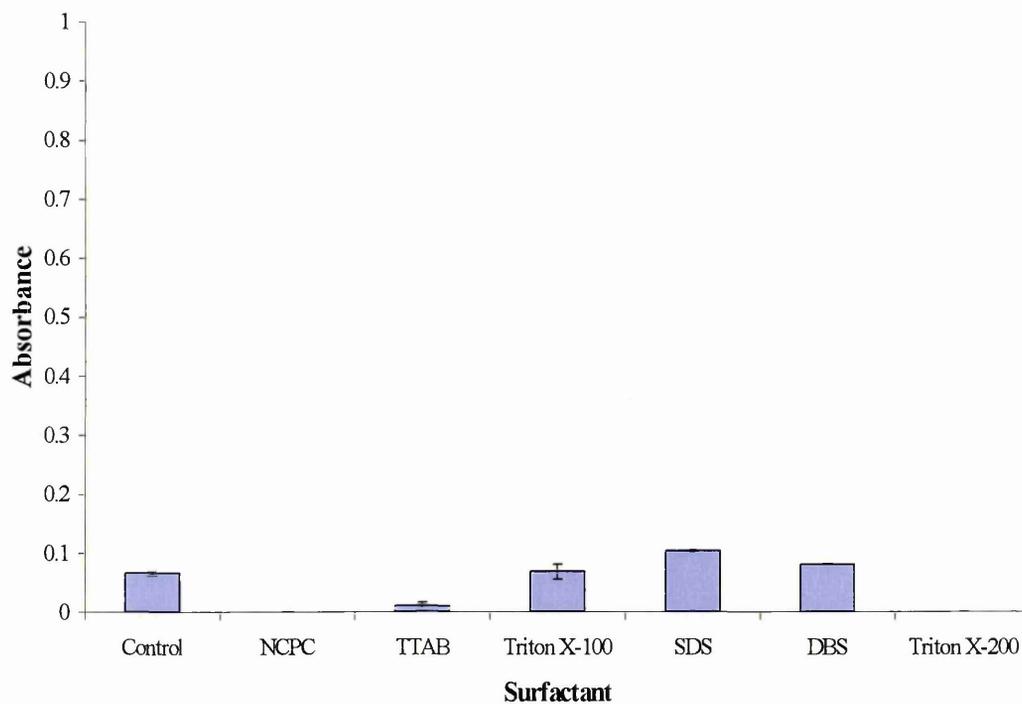
Figure 5.5 The effects of different surfactants on the König reaction using DETB and potassium thiocyanate
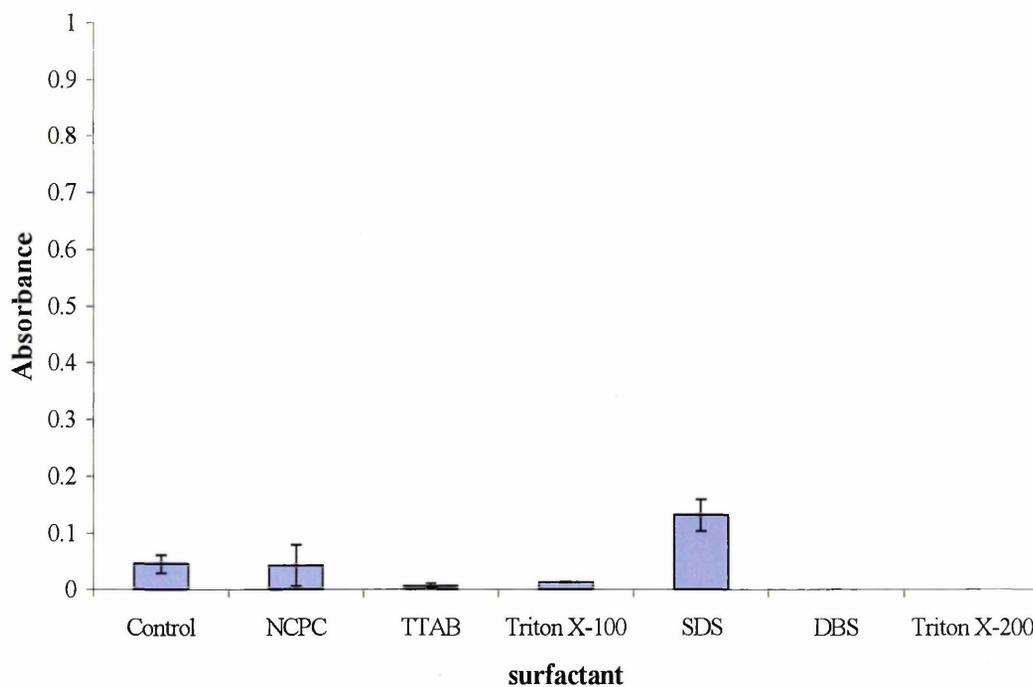
Figure 5.6 The effects of different surfactants on the König reaction using DBTB and potassium thiocyanate
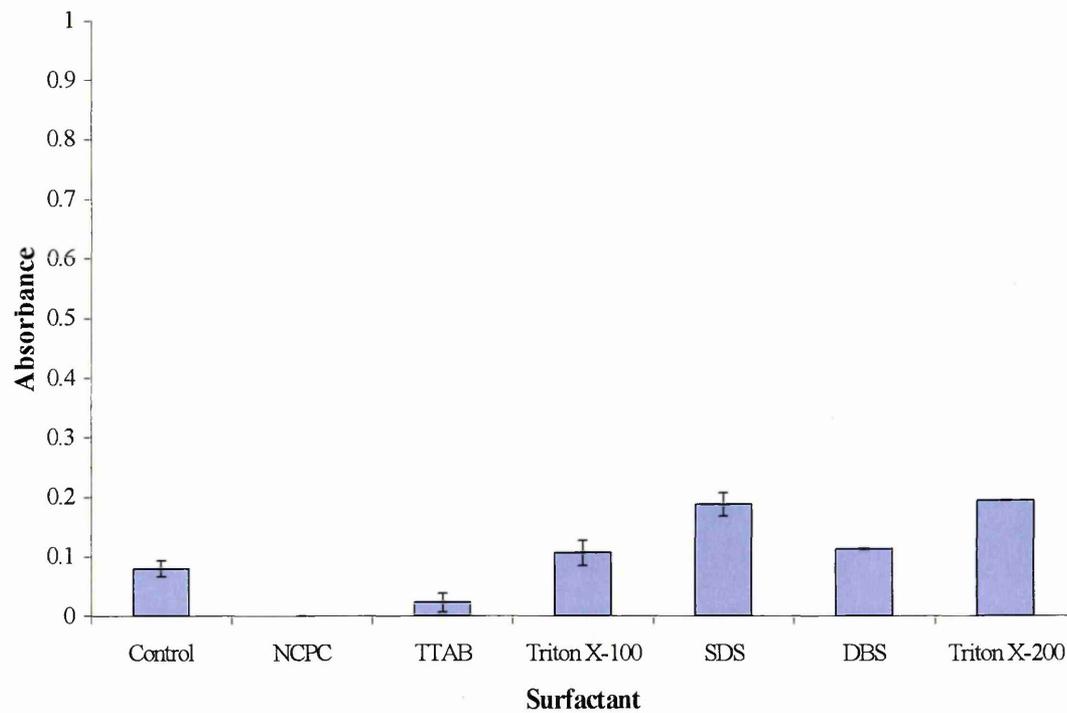
Figure 5.7 The effects of different surfactants on the König reaction using DMDD and potassium thiocyanate
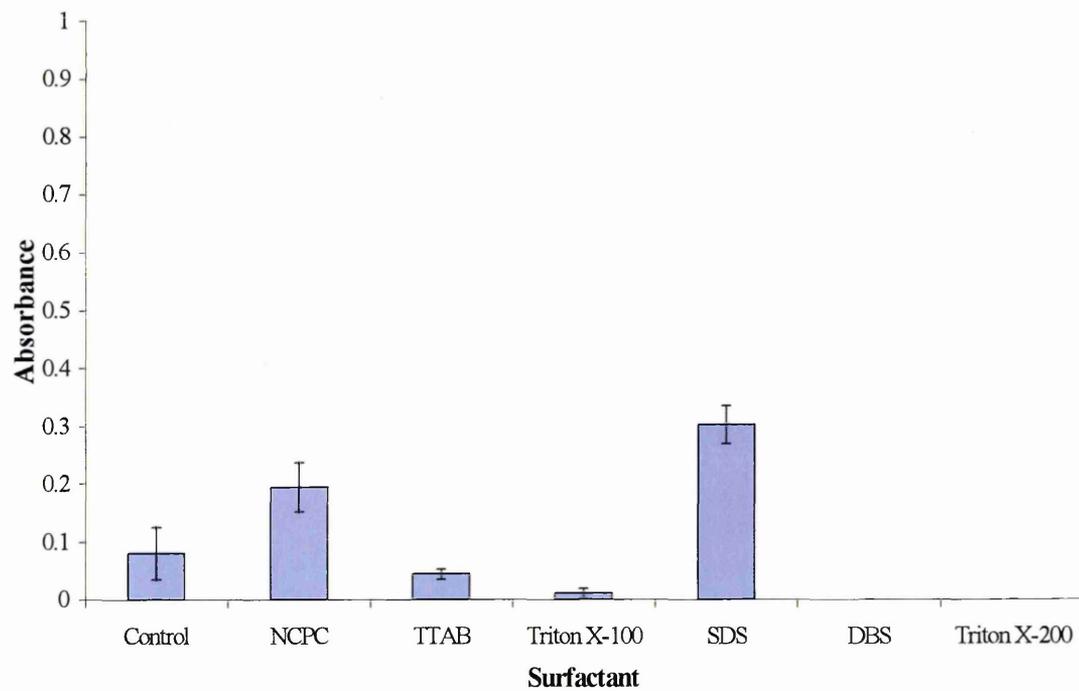
Figure 5.8 The effects of different surfactants on the König reaction using MPED and potassium thiocyanate

129

5.3.2 The effect of surfactants on the König reaction when using potassium cyanide

The results for the various condensing reagents using potassium cyanide are shown in figures 5.9– 5.12. The addition of the cationic surfactant TTAB caused the absorbance to decrease, except, when the condensing reagent DMDD was used, where a slight increase of 12 percent was observed. However, the addition of NCPC caused an increase of 20 percent and 23 percent in the absorbances observed when DETB and DBTB were used respectively, and a slight increase of 11 percent when DMDD was used.

The addition of Triton X-100 caused the absorbance value to increase when DETB and DBTB were used by 37 percent and 19 percent respectively, although no significant change was observed using DMDD or MPED.

The addition of Triton X-200 caused all samples to form a precipitate, except when DMDD was used. The addition of DBS caused the absorbance to increase when DETB and DMDD were used but when the condensing reagents DBTB and MPED were used the solution turned cloudy. The addition of SDS caused the absorbance to increase by 45 percent when DBTB was used, although when used with the condensing reagent DETB the solution turned cloudy.
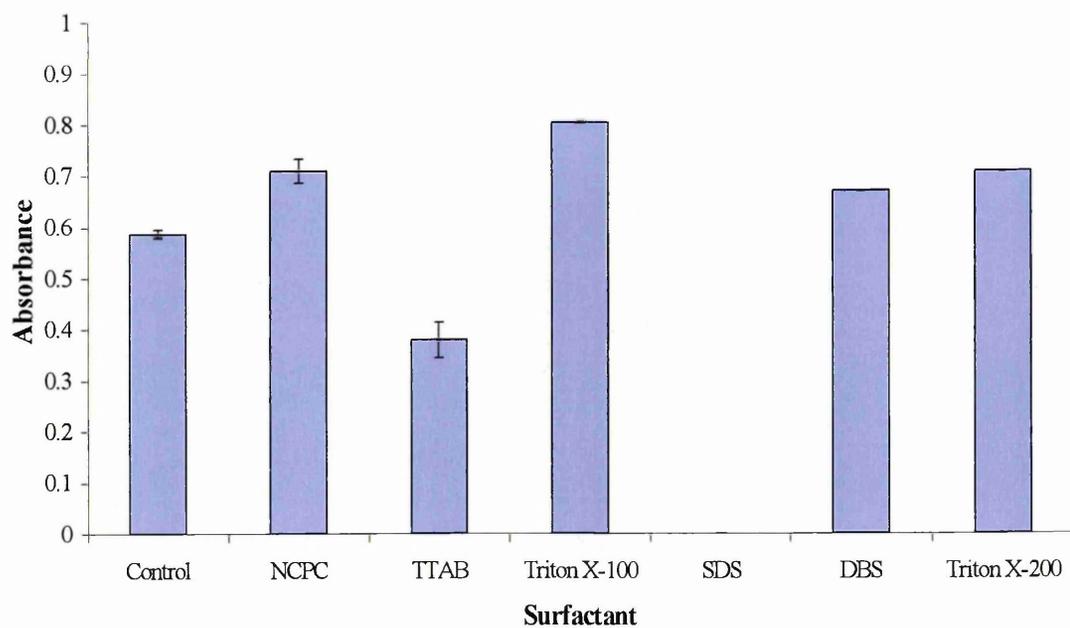
Figure 5.9 The effects of different surfactants on the König reaction using DETB and potassium cyanide
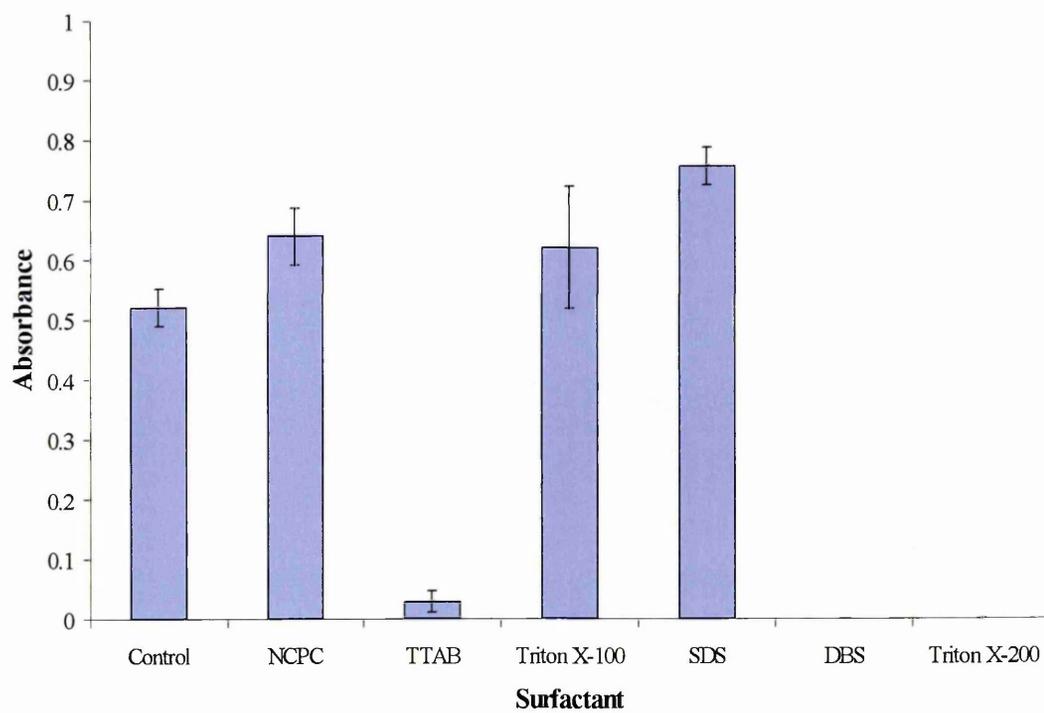
Figure 5.10 The effects of different surfactants on the König reaction using DBTB and potassium cyanide

131
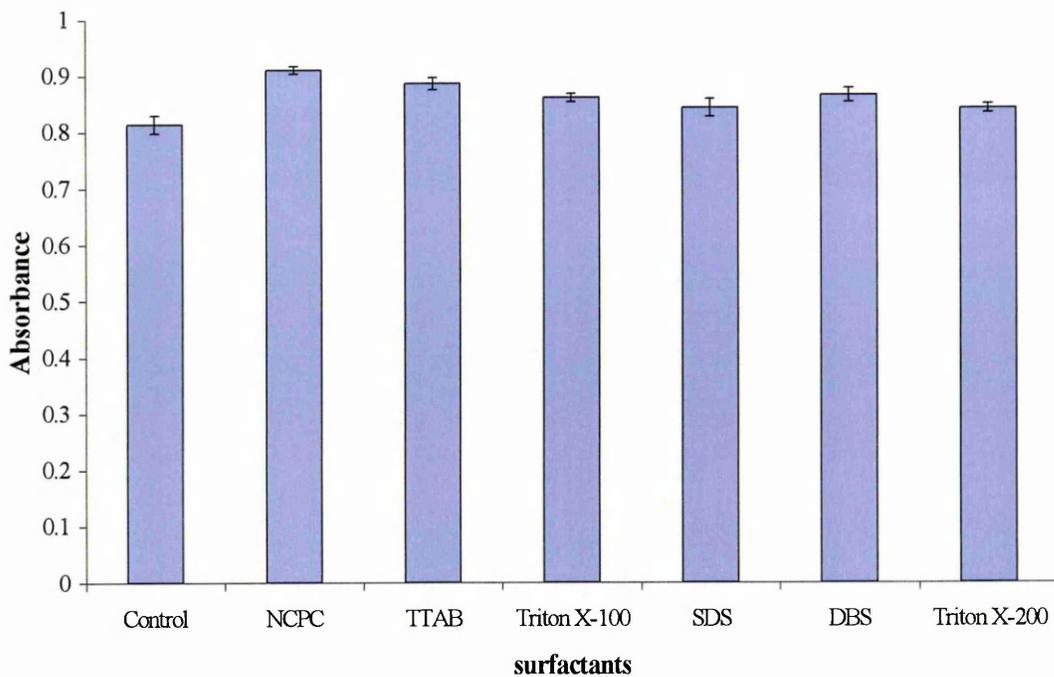
Figure 5.11 The effects of different surfactants on the Konig reaction using DMDD and potassium cyanide
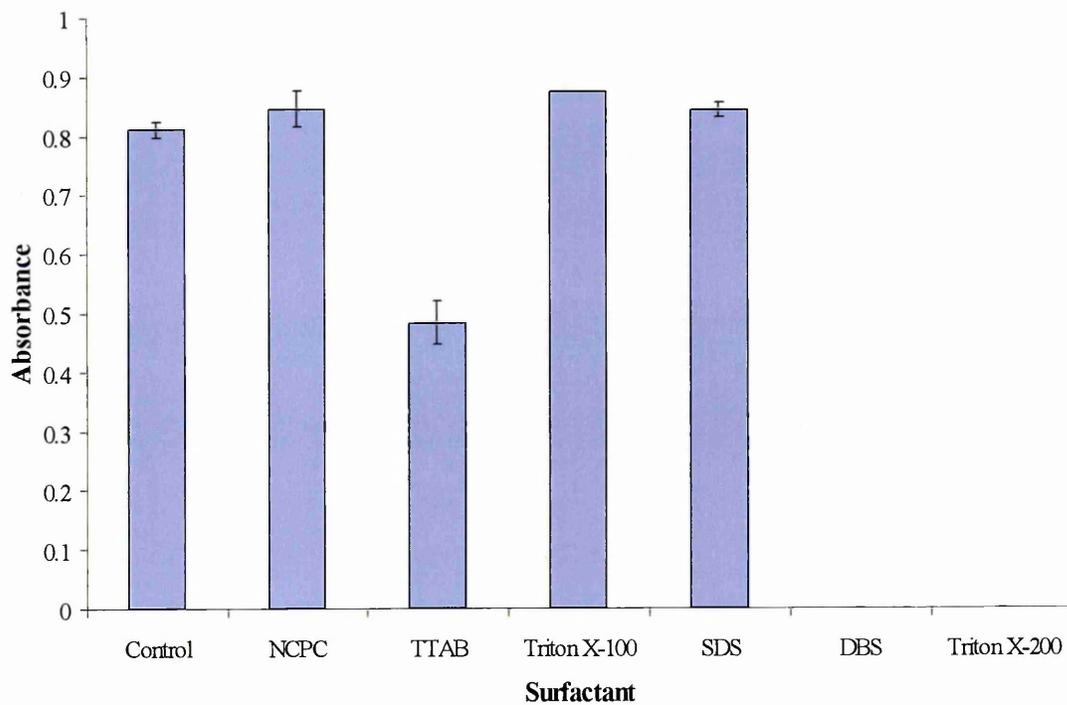
Figure 5.12 The effects of different surfactants on the König reaction using MPED and potassium cyanide

5.4 Discussion

Overall, the results suggest that certain surfactants can affect the absorbance value of cotinine, when derivatized with various condensing reagents. However, there appears to be no set pattern confirming that a particular type of surfactant, for example anionic or cationic, or condensing reagent will overall enhance the observed absorbance. Unlike the other studies described earlier in the chapter (Esteve-Romero et al 1995, Egekeze et al 1997 and Capella-Peiró et al 2001) the derivative of cotinine formed in this current study will not carry a charge at pH 4.7 and therefore would not be expected to be attracted to either anionic or cationic micelles. Therefore, the observed increase in absorbance with the addition of various surfactants may be due to hydrophobic interactions. This observation was also reported by Esteve-Romero et al (1995) as an explanation for the increased absorbance when NCPC was added to a nicotinic acid and aniline solution.

The observed absorbance for potassium thiocyanate did increase especially when the anionic surfactant, SDS and the condensing reagent MPED (sample 1) were used. However, when comparing the absorbance of the control sample for potassium cyanide and MPED (sample 2), the results clearly showed that the absorbance for sample 1 was 77 percent less than sample 2. Therefore, the results in this study suggest that when using potassium thiocyanate and a surfactant such as SDS, the improvement in the sensitivity is unequal to the sensitivity when using potassium cyanide alone, although overall the increase in absorbance was greater for potassium thiocyanate than potassium cyanide. The maximum increase in the absorbance when using potassium cyanide was 45 percent using SDS and DBTB compared to 278 percent for potassium thiocyanate with SDS and MPED. However, the results obtained when using potassium thiocyanate do suggest that it may be possible to distinguish urine samples taken from non-smokers and heavy smokers, although it can be concluded that it is unlikely that an alternative device will be manufactured as it would be extremely difficult to distinguish non-smokers from light or social smokers.

In this current study the experiments performed focused on determining if a particular type of surfactant; anionic, cationic or non-ionic surfactant could overall increase the observed absorbance, regardless of which condensing reagent was used. Further work could be carried out to determine the effects of changing the pH of the reaction, as it would be expected that if the derivatized metabolite was protonated it would be attracted to anionic micelles, such as SDS, whereas a deprotonated derivatized metabolite would be attracted to cationic micelles, such as NCPC. The concentration of surfactant could be altered to determine if an increase in the concentration increased the sensitivity of the reaction. The mode of delivery of the surfactant could also be changed. This could involve changing the order in which the surfactant was add to the reaction mixture, as it may be more appropriate to add the surfactant in a different step and also investigating the possibility of adding the surfactant to one or more of the reagents, thus reducing the number of steps involved.

Chapter 6

Conclusion

The work described in this study relates to a body of fundamental work regarding the use of the König reaction for the colorimetric determination of nicotine and its metabolites. The first part of the study involved the synthesis of two novel non-polar condensing reagents, DBTB and MPED, and a detailed analysis of the König reaction in relation to these and other condensing reagents. Molar absorptivities and partition coefficients were determined for nicotine and 10 of its metabolites using four condensing reagents (DETB, DBTB, DMDD and MPED). The results suggested that, contrary to the traditional assumptions, each metabolite had its own unique molar absorptivity which varied depending on the condensing reagent used. This finding has profound implications for the interpretation of the reaction when used quantitatively as a test for "Total Nicotine Metabolites" since it is increasingly recognised that nicotine metabolism may differ between individuals, ethnic groups and during different physiological states. In addition its possible utility as a colorimetric derivatisation procedure for the determination of nicotine metabolite concentrations by HPLC may also be compromised if cotinine alone is used for standardisation purposes.

Partition coefficients were determined for the Konig derivatives of nicotine and its metabolites when extracted into ethyl acetate. It was originally hypothesised that the synthesis of the more non-polar derivatives would improve that partitioning between the coloured derivatives and ethyl acetate, and thus enhance the analytical sensitivity of the reaction. The results showed that there was an increase in the relative partition coefficients when the more non-polar condensing reagents, especially MPED, was used. Overall it is possible that MPED has the potential to increase the sensitivity of the König reaction and may also prove to be a more suitable condensing reagent when carrying out analytical procedures such as HPLC or for the design of a near patient test.

The novel derivative DBTB was investigated in relation to a specific radioimmunoassay (RIA) for cotinine and the ability of the test to discriminate between smokers and non-smokers in clinical samples. When the König reaction was used qualitatively DBTB overall was the most efficient reagent. Quantitative results suggested that the optimum clinical sensitivity and specificity was achieved using DETB. However, the clinical sensitivity and specificity for DBTB was only 2 to 3 percent less than that observed using DETB. Therefore, the difference in sensitivity and specificity may be due to one or two urine samples with a cotinine concentration around the cut off point being misclassified. Nonetheless, an excellent correlation was found between the RIA and the use of the König reaction when using both condensing reagents. However, the use of the simple colourimetric procedure has several distinct advantages over the conventional RIA. It is considerably cheaper, quick, and gives almost perfect discrimination between smokers and non-smokers using no more sophisticated equipment than the human eye.

There has been considerable debate as to the precise nature of the derivatives formed during the König reaction. Two structures have been proposed, by O'Doherty et al (1990) and Rustemeier et al (1993). However, O'Doherty et al gave no evidence as to why they had suggested their structure, whereas Rustemeier et al isolated the nicotine derivative and subjected the compound to various analytical procedures before they decided on a final structure. In this current study the nicotine derivative formed when using DETB was isolated and analysis using mass spectrometry, IR and NMR was carried out. The results strongly suggested that the only evidence was for the structure proposed by Rustemeier et al and thus we can now report with some confidence the precise nature of the products formed in this reaction.

The work concludes with a consideration of the possible use of surfactants to enhance the absorbance obtained in the reaction and enable the use of potassium thiocyanate rather than potassium cyanide. Several combinations of anionic, cationic and neutral surfactants were investigated with the largest increase in absorbance being observed when using potassium thiocyanate with the anionic surfactant SDS. Despite this finding

the absorbance remained disappointingly less than when potassium cyanide was used on its own.

Overall, this current study has pressed the König reaction to its limits, leaving very few avenues left to investigate. However, since the relative partition coefficients were significantly higher when MPED was used, MPED may yet prove to be a more appropriate condensing reagent by increasing the sensitivity of the reaction, perhaps enabling nicotine and its metabolites to be detected in saliva where the levels are an order of magnitude less than in urine. In addition there may yet be some merit in designing another barbiturate derivative incorporating some of the features of the MPED molecule.

Chapter 7

References

6.1 Bibliography

W N Aldridge (1944), "A new method for the estimation of micro quantities of cyanide and thiocyanate", *Analyst*, **69**:262-265.

G Aislaitner, Y Li and J W Gorrod (1992), "*In vitro* metabolic studies on (-)-(S)-nornicotine", *Medical Science Research*, **20**:897-899.

J Baranowski, G Pochopién and I Baranowska (1998), "Determination of nicotine, cotinine and caffeine in meconium using high-performance liquid chromatography", *Journal of Chromatography B*, **707**:317-321.

Robert D Barlow, Rossana B Stone, Nicholas J Wald and Eino V J Puhakainen (1987), "The direct barbituric acid assay for nicotine metabolites in urine: a simple colorimetric test for the routine assessment of smoking status and cigarette smoke intake", *Clinica Chimica Acta*, **165**: 45-52.

C E Bartecchi, T D MacKenzie and R W Schrier (1994), "The human cost of tobacco (1) ", *The New England Journal of Medicine*, **330**:(13):907-912.

Leland B Baskin, Rodger W Anderson, Joel R Charlson, Richard D Hurt and George M Lawson (1998), "A solid phase extraction method for determination of nicotine in serum and urine by isotope dilution gas chromatography/mass spectrometry with selected ion monitoring", *Annals of Clinical Biochemistry*, **35**:522-527.

Allan B Becker, Jure Manfreda, Alexander C Ferguson, Helen Dimich-Ward, Wade T A Watson and Moira Chan-Yeung (1999), "Breast-feeding and environmental tobacco smoke exposure", *Archives of Pediatrics and Adolescent Medicine*, **153**:(7):689-691.

A H Beckett and E J Triggs (1966), "Determination of nicotine and its metabolite, cotinine, in urine by gas chromatography", *Nature*, **211**:1415-1417.

Neal L Benowitz, Eliseo J Perez-Stable, Irving Fong, Gunnard Modin, Brenda Herrera, and Peyton Jacob III (1999), "Ethnic differences in N-glucuronidation of nicotine and cotinine", *The Journal of Pharmacology and Experimental Therapeutics*, **291**:(3):1196-1203.

Neal L Benowitz, Shoshana Zevin and Peyton Jacob III (1998), "Suppression of nicotine intake during *Ad Libitum* cigarette smoking by high-dose transdermal nicotine", *The Journal of Pharmacology and Experimental Therapeutics*, **287**:(3):958-287.

Neal L Benowitz, Peyton Jacob III, Irving Fong and Suneel Gupta (1994), "Nicotine metabolic profile in man: comparison of cigarette smoking and transdermal nicotine", *The Journal of Pharmacology and Experimental Therapeutics*, **268**:(2):296-303.

Neal L Benowitz (1993), "Nicotine replacement therapy. What has been accomplished –
can we do better? ", *Drugs*, **45**:(1):157-170.

Neal L Benowitz (a) (1988), "The human pharmacology of nicotine", *Research Advances in
Alcohol and Drug Problems*, **9**:1-52.

Neal L Benowitz (b) (1988), "Pharmacologic aspects of cigarette smoking and nicotine
addiction", *The New England Journal of Medicine*, **319**:(20):1318-1330.

Neal L Benowitz, Sharon M Hall, Ronald I Herning, Peyton Jacob III, Reese T Jones and
Abdel-Latif Osman (1983), "Smokers of low-yield cigarettes do not consume less
nicotine", *New England Journal of Medicine*, **309**:139-142.

F W P J van den Berkmortel, G P Bootsma, Y F Heijdra, P I van Spiegel and H
Wollersheim (2000), "Smoking cessation: which aids are worthwhile?", *The Netherlands
Journal of Medicine*, **57**:198-205.

Abraham Bohadana, Fredrik Nilsson, Thomas Rasmussen and Yves Martinet (2000),
"Nicotine inhaler and nicotine patch as a combination therapy for smoking cessation, a
randomized, double blind, placebo-controlled trial", *Archive of International Medicine*,
160:3128-3134.

E R Bowman (1968), "Studies on the excretion of 5-(3-pyridyl)-tetrahdrofuanone-2 and its
intermediary role in the metabolism of nicotine", *Virginia Journal of Science*, **19**:113-121.

Edward R Bowman, Eskil Hansson, Lennox B Turnbull, Herbert McKennis Jr. and Carl G Schmiterlöw (1964), "Disposition and fate of (-)-cotinine-H^3 in the mouse", *Journal of Pharmacology and experimental Therapeutics*, **143**:301-308.

Gary D Byrd, Kuo-Mei Chang, Jackie M Green, and J Don deBethizy (1992), "Evidence for urinary excretion of glucuronide conjugates of nicotine, cotinine, and tran-3'-hydroxycotinine in smokers", *Drug Metabolism and Disposition*, **20**:(2):192-197.

W S Caldwell, J M Greene, G D Byrd, K M Chang, M S Uhrig, J D deBethizy, P A Crooks, B S Bhatti, and R M Riggs (1992), "Characterization of the glucuronide conjugate of cotinine: A previously unidentified major metabolite of nicotine in smokers' urine", *Chemistry Research in Toxicology*, **5**:280-285.

Elisa Capella-Peiró, Llorenç Monferrer-Pons, Celia García-Alvarez-Coque, and Josep Esteve-Romero (2001), "Flow-injected spectrophotometric determination of nicotinic acid in micellar medium of *N*-cetylpyridinium chloride", *Analytica Chimica Acta*, **427**:93-100.

Ralph S Caraballo, Gary A Giovino, Terry F Pechacek, Paul D Mowery, Patricia A Richter, Warren J Strauss, Donald J Sharp, Michael P Eriksen, James L Pirkle and Kurt R Maurer (1998), "Racial and ethnic differences in serum cotinine levels of cigarette smokers. Third national health and nutrition examination survey 1988-1991", *Journal of the American Medical Association*, **280**:(2):135-139.

Graham Cope, Pamela Nayyar, Lucy Wilkinson, and Roger Holder (2000), "Simple colorimetric test to quantify exposure to environmental tobacco smoke: occupational health study", *Annals of Clinical Biochemistry*, **37**:795-796.

Graham Cope, Pamela Nayyar, Roger Holder, John Gibbons, and Roger Bunce (1996), "A simple near-patient test for nicotine and its metabolites in urine to assess smoking habit", *Clinica Chimica Acta*, **256**:135-149.

Peter A Crooks, Balwinder S Bhatti, Alain Ravard, Robert M Riggs and William S Caldwell (1992), "Synthesis of N-glucuronic acid conjugates of cotinine, *cis*-3-hydroxycotinine and *trans*-3-hydroxycotinine", *Medical Science and Research*, **20**:881-883.

K Michael Cummings, Samuel J Markello, Martin Mahoney, Arvind K Bhargava, Peter McElroy and James R Marshall (1990), "Measurement of current exposure to environmental tobacco smoke", *Archives of Environmental Health*, **45**:(2):74-79.

M Curvall, E Kazemi-Vala, G Englund and C R Enzell (1989), "Urinary excretion of nicotine and its major metabolites", 43rd Tobacco chemists research conference, Richmond, USA, Abstract 54, 29th September- October 3rd.

Margaret Curvall, Eeva Kazemi-Vala and Curt R Enzell (1982), "Simultaneous determination of nicotine and cotinine in plasma using capillary column gas chromatography with nitrogen-sensitive detection", *Journal of Chromatography B*, **232**: 283-293.

Ermias Dagne, Larry Gruenke and Neal Castagnoli Jr. (1974), "Deuterium isotope effects in the *in vivo* metabolism of cotinine", *Journal of Medicinal Chemistry*, **17**:(12):13301333.

Ermias Dagne and Neal Castagnoli Jr. (1972), "Cotinine-N-oxide, a new metabolite of nicotine", *Journal of Medicinal Chemistry*, 15:(8):840-841.

Delia Dempsey, Peyton Jacob III and Neal L Benowitz (2000), "Nicotine metabolism and elimination kinetics in newborns", *Clinical Pharmacology and Therapeutics*, 67:(5):458-465.

D A Dempsey, P Jacob III, and N L Benowitz (1998), "Accelerated nicotine and cotinine metabolism during pregnancy", *Clinical Pharmacology and Therapeutics*, 63:(2):202 PII-120.

D Demetriou, K Rustemeier, P Voncken and G Schepers (1992), "Two radiochromatographic high pressure liquid chromatography methods for the determination of nicotine metabolites", *Medical Science Research*, 20:873-875.

Richard Doll, Richard Peto, Keith Wheatley, Richard Gray, and Isabelle Sutherland (1994), "Mortality in relation to smoking: 40 years' observations on male British doctors", *British Medical Journal*, 309:901-911.

Richard Doll and (1976), "Mortality in relation to smoking: 20 years' observation on male British doctors", *British Medical Journal*, 2:1525-1536.

E F Domino, M Hariharan, T VanNoord and T Demana (1992), "Current experience with HPLC and GC-MS analysis of nicotine and cotinine", *Medical Science Research*, 20:859-860.

John O Egekeze, Thomas M Dowling, Nelu Grinberg, Holly J Perpall, and Gary R Bicker (1997), "Micellar enhanced cyanide ion determination in samples from synthetic organic processes", *Talanta*, **44**:1203-1209.

N Eldirdiri, M Ulgen, P Jacob III and J W Gorrod (1997), "The *in vitro* metabolism of norcotinine and related biotransformation products by microsomal preparations", *European Journal of Drug Metabolism and Pharmacokinetics*, **22**:(4):385-390.

Gordon A Ellard (1995), "Colorimetric assessment of smoking status and relative daily nicotine intake", *Clinical Chemistry*, **41**:(11):1673-1674.

G A Ellard, Patricia T Gammon, and Susan M Wallace (1972), "The determination of isoniazid and its metabolites acetylisoniazid, monoacetylhydrazine, diacetylhydrazine, isonicotinic acid and isonicotinylglycine in serum and urine", *Biochemical Journal*, **126**: 449-458.

J S Esteve-Romero, Ll Monferrer-Pons, G Ramis-Ramos, and M C Garcia-Alvarez-Coque (1995), "Enhanced spectrophotometric determination of nicotine acid in a sodium dodecyl sulphate micellar medium", *Talanta*, **42**:(5):737-745.

Amruta R Eswara, Saraswathy V Nochur, and Donald J Mossman 1996, "Detection of nicotine and its metabolites in urine", *American Journal of Health Behavior*, **20**:(5):333-345.

Karl-Olov Fagerström (1978), Measuring degree of physical dependence to tobacco smoking with reference to individualization of treatment", *Addictive Behaviors*, 3:(3-4):235-241.

S E Falkman, I E Burrows, R A Lundgren and BF Page (1975), "A modified procedure for the determination of nicotine in blood", 100:(1187):99-104.

Pedro Fernandez-Salguero, Susan M G Hoffman, Suzanne Cholerton, Harvey Mohrenweiser, Hannu Raunio, Arja Rautio, Olavi Pelkonen, Jin-ding Huang, William E Evans, Jeffrey R Idle and Frank J Gonzalez (1995), "A genetic polymorphism in coumarin 7-hydroxylation: sequence of the human CYP2A genes and identification of varient CYP2A6 alleles", *American Journal of Human Genetics*, 57:651-660.

C Feyerabend and M A Russell (1979), "Improved gas chromatographic method and micro-extraction technique for the measurement of nicotine in biological fluids", *The Journal of Pharmacy and Pharmacology,* 31:(2):73-76.

Lois A Fingerhut, Joel Kleinman, and Juliette S Kendrick (1990), "Smoking before, during and after pregnancy", *American Journal of Public Health*, 80:(5):541-544.

A M Flammang, H V Gelboin and T Aoyama, Gonzalez F J and G D McCoy (1992), "Nicotine metabolism by cDNA-expressed human cytochrome P450s", *Biochemical Archives*, 8:1-8.

Misao Fukuda, Kiyomi Fukuda, Takashi Shimizu, Claus Yding Andersen and Anne Grete Byskov (2002), "Parental periconceptional smoking and male: female ratio of newborn infants", *The Lancet*, **359**:1407-1408

Omar A Ghosheh, Debra Browne, Thea Rogers, José de Leon, Linda P Dwoskin and Peter A Crooks (2000), "A simple high performance liquid chromatographic method for the quantification of total cotinine, total 3'-hydroxycotinine and caffeine in the plasma of smokers", *Journal of Pharmaceutical and Biomedical Analysis*, **23**:543-549.

P A Gillies, B Wilcox, C Coates, F Kristmundsdottir and D J Read (1982), "Use of objective measurement in the validation of self-reported smoking in children aged 10-11 years: saliva thiocyanate", *Journal of Epidemiology and Community Health*, **36**:205-208.

Goodman and Gilman (1990), 8th Edition, "The pharmacological basis of therapeutics", Mc Graw-Hill Inc, Health professions division, 192-193, 565-566.

D H Gonzales, M A Nides, L H Ferry, R P Kustra, B D Jamerson, N Segall, L A Herrero, A Krishen, A Sweeney, K Buaron, and A Metz (2001), "Bupropion SR as an aid to smoking cessation in smokers treated previously with bupropion: A randomised placebo-controlled study", *Clinical Pharmacology and Therapeutics*, **69**:(6):434-444.

John W Gorrod and Peyton Jacob III (1999), "Analytical determination of nicotine and related compounds and their metabolites", Elsevier, Amsterdam, pp. 1-4, ch. 2.

J W Gorrod and A R Hibberd (1982), "The metabolism of nicotine-$^{\Delta}1$'(5')-iminium ion, *in vivo* and *in vitro*", *European Journal of Drug Metabolism and Pharmacokinetics*, 7:(4):293-298.

Klaus M Hahn, Alan S Waggoner, and D Lansing Taylor (1990), "A calcium-sensitive fluorescent analog of calmodulin based on a novel calmodulin-binding fluorophore", *The Journal of Biological Chemistry*, **265**:(33):20335-20345.

M Hariharan and T VanNoord (1991), "Liquid-chromatographic determination of nicotine and cotinine in urine from passive smokers: comparison with gas chromatography with nitrogen-specific detector", *Clinical Chemistry*, **37**:(7):1276-1280.

Daniel C Harris (1990), 5th Edition, "Quantitative Chemical Analysis", W H Freeman and Company, New York, pp. 291-292.

Todd F Heatherton, Lynn T Kozlowski, Richard C Frecker and Karl-Olov Fagerström (1991), "The Fagerström test for nicotine dependence: a revision of the Fagerström tolerance questionnaire", *British Journal of Addiction*, **86**:1119-1127.

Jean Hee, Françoise Callais, Isabelle Momas, Ann Marie Laurent, Sreng Min, Patricia Molinier, Madeleine Chastagnier, Jean Roger Claude and Bernard Festy (1995), "Smokers' behaviour and exposure according to cigarette yield and smoking experience", *Pharmacology Biochemistry and Behavior*, **52**:(1):195-203.

Nikolaus Hengen and Michaela Hengen (1978), "Gas-liquid chromatographic determination of nicotine and cotinine in plasma", *Clinical Chemistry,* **24**:(1):50-53.

Jack E Henningfield (1995), "Nicotine medications for smoking cessation", *The New England Journal of Medicine,* **333**:(18):1196-1203.

B Holmstedt (1988), "Toxicity of nicotine and related compounds", *ICSU Symposium Series,* **9**:61-88.

Charles F Huebner (1951), "Paper chromatography of pyridine derivatives", *Nature,* **167**:119-120.

Michael G R Hull, Kate North, Hazel Taylor, Alexandra Farrow, W Christopher L Ford and the Avon longitudinal study of pregnancy and childhood study team (2000), "Delayed conception and active and passive smoking", *Fertility and Sterility,* **74**:(4):725-733.

P Jacob III, L Yu, M Wilson and N L Benowitz (1991), "Selected ion monitoring method for determination of nicotine, cotinine and deuterium-labeled analogs: absence of an isotope effect in the clearance of (S)-nicotine-3′,3′-d2 in humans", *Biological Mass Spectrometry,* **20**:(5):247-252.

Peyton Jacob III, Alexander T Shulgin and Neal L Benowitz (1990), "Synthesis of (3′R,5′S)-trans-3′-hydroxycotinine, a major metabolite of nicotine. Metabolic formation of 3′hydroxycotinine in humans is highly stereoselective", *Journal of Medicinal Chemistry,* **33**:1888-1891.

Peyton Jacob III, Margaret Wilson and Neal L Benowitz (1981), "Improved gas chromatographic method for the determination of nicotine and cotinine in biological fluids", *Journal of Chromatography B*, **222**:60-70.

Kirby E Jackson (1941), "Alkaloids of tobacco", *Chemistry Reviews*, **29**:123-197.

M Jarvis, H Tunstall-Pedoe, C Feyerabend, C Vesey and Y Salloojee (1984), "Biochemical markers of smoke absorption and self-reported exposure to passive smoking", *Journal of Epidemiology and Community Health*, **38**:335-339.

Richard Jones and Brian Payne (1997), "Clinical Investigation and Statistics in Laboratory Medicine", ABC Venture Publications, pp. 89-91.

Martin Kharrazi, David Epstein, Barbara Hopkins, Richard Kreutzer, Gwendolyn Doebbert, Robert Hiatt, Shanna Swan, Brenda Eskenazi, James L Pirkle, and John T Bernert (1999), "Evaluation of four maternal smoking questions", *Public Health Reports*, **114**:60-70.

Kyoko Kitagawa, Naoki Kunugita, Takahiko Katoh, Mihi Yang and Toshihiro Kawamoto (1999), "The significance of the homozygous CYP2A6 deletion on nicotine metabolism; a new genotyping method of CYP2A6 using a single PCR-RFLP", *Biochemical and Biophysical Research Communications*, **262**:146-151.

J Klein and G Koren (1999), "Hair analysis – a biological marker for passive smoking in pregnancy and childhood", *Human and Experimental Toxicology*, **18**:279-282.

George J Knight, Phillip Wylie, Mary S Holman, and James E Haddow (1985), "Improved ^{125}I radioimmunoassay for cotinine by selective removal of bridge antibodies", *Clinical Chemistry*, **31**:(1):118-121.

W König (1904), "Untersuchungen aus dem organischen laboratorium der technischen hochschule zu dresden", *Journal fur Prakische Chemie Chemiker Zeitung*, **70**:19-56.

G A Kyerematen, L H Taylor, J D deBethizy and E S Vesell (1988), "Pharmacokinetics of nicotine and 12 metabolites in the rat. Application of a new radiometric high performance liquid chromatography assay", *Drug Metabolism and Disposition*, **16**:125-129.

G A Kyerematen, L H Taylor, J D deBethizy and E S Vesell (1987), "Radiometric-high-performance liquid chromatographic assay for nicotine and twelve of its metabolites", *Journal of Chromatography B*, **419**:191-203.

Gabriel A Kyerematen, Michael D Damiano, Barry H Dvorchik and Elliot S Vesell (1982), "Smoking-induced changes in nicotine disposition: application of a new HPLC assay for nicotine and its metabolites", *Clinical Pharmacology and Therapeutics*, **32**:(6):769-779.

John J Langone, Hilda B Gjika and Helen Van Vunakis (1973), "Nicotine and its metabolites. Radioimmunoassays for nicotine and cotinine", *Biochemistry*, **12**:(24):5025-5030.

George M Lawson, Richard D Hurt, Lowell C Dale, Kenneth P Offord, Ivana T Croghan, Darrell R Schroeder and Nai-S Jiang (1998), "Application of urine nicotine and cotinine

excretion rates to assessment of nicotine replacement in light, moderate, and heavy smokers undergoing transdermal therapy", *Journal of Clinical Pharmacology*, **38**:510-516.

Edgar Leifer, Wright H Langham, Joseph F Nyc, and Herschel K Mitchell (1950), "The use of isotopic nitrogen in a study of the conversion of 3-hydroxyanthanilic acid to nicotinic acid in neurospora", *Journal of Biological Chemistry*, **184**:589-592.

Bruce N Leistikow, Mai Tran and Christina Milano (1999), "Tobacco as pesticide, tobacco as cigarette: a history and comparison of health warnings", *Society of Research on Nicotine and Tobacco Conference*, PO A57.

D C Mariner, J Moore, K Cornelissen and N M Sinclair (1992), "Urinary recovery of nicotine and five metabolites after smoking conventional cigarette", *Medical Science Research*, **20**:861-862.

N W McCracken, S Cholerton and J R Idle (1992), "Cotinine formation by cDNA-expressed human cytochromes P450", *Medical Science Research*, **20**:877-878.

H McKennis, S L Schwatz and E R Bowman (1964), "Alternative routes in the metabolic degradation of the pyrrolidine ring of nicotine", *Journal of Biological Chemistry*, **239**:3990-3996.

Merck Index (1996), 12th Edition, "An Encyclopedia of Chemicals, drugs and biologicals", Merck Research laboratories, Division of Merck and Co Inc, Whitehouse Station, New York, USA.

E S Messina, R F Tyndale and E M Sellers (1997), "A major role for CYP2A6 in nicotine C-oxidation by human liver microsomes", *The Journal of Pharmacology and Experimental Therapeutics*, **282**:(3):1608-1614.

J Moore, M Greenwood, and N Sinclair (1990), "Automation of a high-performance liquid chromatographic assay for the determination of nicotine, cotinine and 3-hydroxycotinine in human urine", *Journal of Pharmaceutical and Biomedical Analysis*, **8**:(8-12):1051-1054.

Patrick J Murphy (1973), "Enzymatic oxidation of nicotine to nicotine $\Delta^{1'(5')}$ Iminium ion. A newly discovered intermediate in the metabolism of nicotine", *The Journal of Biological Chemistry*, **248**:(8):2796-2800.

Per Nafstad, Grete Botten, Jørgen A Hagen, Kolbjørn Zahlsen, Odd G Nilsen, Terje Silsand and Johny Kongerud (1995), "Comparison of three methods for estimating environmental tobacco smoke exposure among children aged between 12 and 36 months", *International Journal of Epidemiology*, **24**:(1):88-94.

Miki Nakajima, Toshinori Yamamoto, Yukio Kuroiwa and Tsuyoshi Yokoi (2000), "Improved highly sensitive method for determination of nicotine and cotinine in human plasma by high-performance liquid chromatography", *Journal of Chromatography B*, **747**:211-215.

Miki Nakajima, Toshinori Yamamoto, Ken-Ichi Nunoya, Tsuyoshi Yokoi, Kazuo Nagashima, Kazuaki Inoue, Yoshihiko Funae, Noriaki Shimada, Tetsuya Kamataki and Yukio Kuroiwa (1996), "Role of human cytochrome P4502A6 in C-oxidation of nicotine", *Drug Metabolism and Disposition*, **24**:1212-1217.

Georg B Neurath, Michael Dünger, Dorothea Orth and F Gerhard Pein (1987), "Trans-3'-hydroxycotinine as a main metabolite in urine of smokers", *International Archive of Occupational Environmental Health*, **59**:199-201.

T-L Nguyen, L D Gruenke and N Castagnoli (1976), "Metabolic *N*-demethylation of nicotine. Trapping of a reactive iminium species with cyanide ion", *Journal of Medicinal Chemistry*, **19**:1168-1169.

K I Nunoya, T Yokoi, Y Takahashi, K Kimura, M Kinoshita and T Kamataki (1999), "Homologous unequal cross-over within the human CYP2A gene cluster as a mechanism for the deletion of entire CYP2A6 gene associated with the poor metabolizer phenotype", *Journal of Biochemistry*, **126**:407-407.

Christine Oddoze, Jean Christophe Dubus, Monique Badier, Xavier Thirion, Anne Marie Pauli, Jean Pastor and Bernard Bruguerolle (1999), "Urinary cotinine and exposure to parental smoking in a population of children with asthma", *Clinical Chemistry*, **45**:(4):505-509.

C Oddoze, A M Pauli and J Pastor (1998), "Rapid and sensitive high-performance liquid chromatographic determination of nicotine and cotinine in nonsmoker human and rat urines", *Journal of Chromatography B*, **708**:95-101.

S O'Doherty, M Cooke, and D J Roberts (1990), "Enhancing the LC analysis of nicotine and its metabolites in urine using meldrums acid as a complexing agent", *Journal of High Resolution Chromatography*, **13**:74-77.

Mikael Oscarson, Roman A McLellan, Harriet Gullstén, Qun-Yin Yue, Matti A Lang, Maria Luisa Bernal, Blanca Sinues, Ari Hirvonen, Hannu Raunio, Olavi Pelkonen and Magnus Ingelman-Sundberg (a) (1999), "Characterisation and PCR-based detection of a CYP2A6 gene deletion found at a high frequency in a Chinese population", *Federation of European Biochemical Society Letters*, **448**:105-110.

Mikael Oscarson, Roman A McLellan, Harriet Gullstén, José A G Agúndez, Julio Benítez, Arja Rautio, Hannu Raunio, Olavi Pelkonen and Magnus Ingelman-Sundberg (b) (1999), "Identification and characterisation of novel polymorphism in the CYP2A locus: implications for nicotine metabolism", *Federation of European Biochemical Society Letters*, **460**:321-327.

Mikael Oscarson, Harriet Gullstén, Arja Rautio, Maria Luisa Bernal, Blanca Sinues, Marja-Liisa Dahl, Jari H Stengård, Olavi Pelkonen, Hannu Raunio and Magnus Ingelman-Sundberg (1998), "Genotyping of human cytochrome P450 2A6 (CYP2A6), a nicotine C-oxidase", *Federation of European Biochemical Society Letters*, **438**:201-205.

R Pacifici, S Pichini, I Altieri, M Rosa, L Paris and P Zuccaro (1992), "Pharmacokinetics interactions in epileptic smokers", *Medical science Research*, **20**:905-907.

Hedley Peach, G A Ellard, P J Jenner, and R W Morris (1985), "A simple, inexpensive urine test of smoking", *Thorax*, **40**:351-357.

Eliseo J Pérez-Stable, Brenda Herrera, Peyton Jacob III, Neal L Benowitz (1998), "Nicotine metabolism and intake in black and white smokers", *Journal of the American Medical Association*, **280**:(2):152-156.

George Phillipou, Stephen K James, Christopher J Seaborn, and Patrick J Phillips (1994), "Assessment of a simple colorimetric procedure to determine smoking status in diabetic subjects", *Clinical Chemistry*, **40**:(7):1296-1298.

Michael L Pianezza, Edward M Sellers, and Rachel F Tyndale (1998), "Nicotine metabolism defect reduces smoking", *Nature*, **393**:750.

Andreas Pickert, Thomas Lingenfelser, Christiane Pickert, Niels Birbaumer, Dietrich Overkamp, and Manfred Eggstein (1993), "Comparison of a mechanized version of the "König" reaction and a fluorescence polarization immunoassay for the determination of nicotine metabolites in urine", *Clinica Chimica Acta*, **217**:143-152.

T Phil Pitner, William B Edwards III, Ronald L Bassfield and Jerry F Whidby (1978), "The solution conformation of nicotine. A ^{1}H and ^{2}H nuclear magnetic resonance investigation", *Journal of the American Chemical Society*, **100**:(1):246-251.

Eino V J Puhakainen, Robert D Barlow, and Jukka T Salonen (1987), "An automated colorimetric assay for urine nicotine metabolites: a suitable alternative to cotinine assay for the assessment of smoking status", *Clinica Chimica Acta*, **170**:255-262.

Marisa Rebagliato, Francisco Bolúmar, Charles du V Florey, Martin J Jarvis, Santiago Pérez-Hoyos, Ildefonso Hernández-Aguado and M José Avinó (1998), "Variations in cotinine levels in smokers during and after pregnancy", *American Journal Obstetrics and Gynaecology*, **178**:(3):568-571.

C Reichardt (1992), "Solvatochromism, thermochromism, piezochromism, halochromism, and chiro-solvatochromism of pyridinium *N*-phenoxide betaine dyes", *Chemical Society Reviews*, **21**:(3):147-153.

Klaus Rustemeier, Demetrios Demetriou, Georg Schepers, and Peter Voncken (1993), "High-performance liquid chromatographic determination of nicotine and its urinary metabolites via their 1,3,diethyl-2-thiobarbituric acid derivatives", *Journal of Chromatography B*, **613**:95-103.

David L Sackett, R Brian Haynes, Gordon H Guyatt and Peter Tugwell (1991), 2nd Edition *Clinical epidemiology, A basic science for clinical medicine*, Little Brown and Company, USA, pp. 112-118.

Y Sai and J W Gorrod (1995), "Metabolic interconversion of acid of acid metabolites of cotinine *in vitro*", *Drug Metabolism and Drug interactions*, **12**:93-103.

Annie J Sasco and Harri Vainio (1999), "From *in utero* and childhood exposure to parental smoking to childhood cancer: a possible link and the need for action", *Human and Experimental Toxicology*, **18**:192-201.

G Schepers, D Demetriou, R Stabbert, B Diehl and J I Seeman (1999), "5'Hydroxycotinine-*N*-oxide, a new nicotine metabolite isolated from rat urine", *Xenobiotica*, **29**:793-801.

G Schepers, K Rustemeier, R-A Walk and U Hackenberg (1993), "Metabolism of S-nicotine in noninduced and Aroclor-induced rats", *European Journal of Drug Metabolism and Pharmacokinetics*, **18**:187-197.

G Schepers, D Demetriou, K Rustemeier, P Voncken and B Diehl (1992), "Nicotine phase 2 metabolites in human urine – structure of metabolically formed *trans*-3'-hydroxycotinine glucuronide", *Medical Science Research*, **20**:863-865.

M J Seaton, E S Vesell, H Luo and E M Hawes (1993), "Identification of radiolabeled metabolites of nicotine in rat bile. Synthesis of S-(-)-nicotine N-glucuronide and direct separation of nicotine-derived conjugates using high performance liquid chromatography", *Journal of Chromatography B*, **621**:49-53.

R W Sillett, M B Wilson, R E Malcolm and K P Bull (1978), "Deception among smokers", *British Medical Journal*, **2**:1185-1186

Celia L Smith, Michael Cooke and D J Roberts (1989), "Determination of nicotine metabolites by HPLC complexation with diethylthiobarbituric acid", *Analytical Proceedings*, **26**:348-351.

Celia L Smith and Michael Cooke (1987), "Evaluation of diethylthiobarbituric acid as a spectrophotometric reagent for tobacco alkaloids", *Analyst*, **112**:1515-1518.

Robert F Smith, Hugh M Mather, and Gordon A Ellard (1998), "Assessment of simple colorimetric procedures to determine smoking status of diabetic subjects", *Clinical Chemistry*, **44**:(2):275-280.

Jin Tang, Malcolm Law and Nicholas Wald (1994), "How effective is nicotine replacement therapy in helping people to stop smoking?", *British Medical Journal*, **308**:21-26.

Shiho Taniguchi, Masato Niitsuya, Yoko Inoue, Hiroshi Katagiri, Takehiro Kadowaki and Yoshiharu Aizawa (1999), "Evaluation of passive smoking by measuring urinary trans, trans-muconic acid and exhaled carbon monoxide levels", *Industrial Health*, **37**:88-94.

Gwendolyn H Thompson and Dee A Hunter (1998), "Nicotine replacement therapy", *The Annals of Pharmacotherapy*, **32**:1067-1075.

J A Tracey, N Cassidy, P B Casey and I Ali (2002), "Bupropion (Zyban) toxicity", *Iris Medical Journal*, **95**:(1):23-24.

Rachel F Tyndale and Edward M Sellers (2001), "Variable CYP2A6-mediated nicotine metabolism alters smoking behavior and risk", *Drug Metabolism and Disposition*, **29**:548-552.

Johan B Ubbink, Jennifer Lagendijk, and W H Hayward Vermaak (1993), "Simple high-performance liquid chromatographic method to verify the direct barbituric acid assay for urinary cotinine", *Journal of Chromatography B*, **620**:254-259.

C J Vesey, Y Saloojee, P V Cole and M A H Russell (1982), "Blood carboxyhaemoglobin, plasma thiocyanate, and cigarette consumption: implications for epidemiological studies in smokers", *British Medical Journal*, **284**:1516-1518.

I Vindatiche, D Roche, F Callais, N T Lequang and F Labrousse (2000), "Analytical improvements in Barlow reaction coupled to HPLC detection of nicotine and its metabolites", *Journal of Liquid Chromatography and Related Technology*, **23**:(9):1423-1437.

Einosuke Wada, Edward R Bowman, Lennox B Turnbull and Herbert McKennis (1961), "Norcotinine (desmethylcotinine) as a urinary metabolite of nornicotine", *Journal of Medicinal and Pharmaceutical Chemistry*, **4**:(1):21-30.

Michael A Wall, Jean Johnson, Peyton Jacob III, and Neal L Benowitz (1988), "Cotinine in the serum, saliva, and urine of nonsmokers, passive smokers and active smokers", *American Journal of Public Health*, **78**:(6):699-701.

Xiaobin Wang, Ira B Tager, Helen van Vunakis, Frank E Speizer and John P Hanrahan (1997), "Maternal smoking during pregnancy, urine cotinine concentrations, and birth outcomes. A prospective cohort study", *International Journal of Epidemiology*, **26**:(5):978-987.

R G Wilcox, J Hughes and J Roland (1979), "Verification of smoking history on patients after infarction using urinary nicotine and cotinine", *British Medical Journal*, **2**:1026-1028.

A R White, H Rampes and E Ernst (2002), "Acupuncture for smoking cessation", *Cochrane Database of Systematic Reviews (Online: Update Software)*, issue 2, page CD000009.

Ernest L Wynder (1956), "Experimental production of carcinoma with cigarette tar", *Bulletin for New York Academy of Medicine*, **32**:76.

Shigeru Yamano, Jun Tatsuno and Frank J Gonzalez (1990), "The CYP2A3 gene product catalyzes coumarin 7-hydroxlation in human liver microsomes", *Biochemistry*, **29**:1322-1329.

Tsuyoshi Yokoi and Tetsuya Kamataki (1998), "Genetic polymorphism of drug metabolizing enzymes: new mutations in CYP2D6 and CYP2A6 genes in Japanese", *Pharmaceutical Research*, **15**:(4):517-524.

Shoshana Zevin and Neal L Benowitz (1999), "Drug interaction with tobacco smoking. An update", *Clinical Pharmacokinetics*, **36**:(6):425-438.

Shoshana Zevin, Steven G Gourlay and Neal L Benowitz (1998), "Clinical pharmacology of nicotine", *Clinics in Dermatology*, **16**:557-564.

Lijie Zhong, Mark S Goldberg, Marie-Élise Parent and James A Hanley (2000), "Exposure to environmental tobacco smoke and the risk of lung cancer: a meta-analysis", *Lung Cancer*, **27**:3-18.

P Zuccaro, I Altieri, M Rosa, A R Passa, S Pichini and R Pacifici (1995), "Solid-phase extraction of nicotine and its metabolites for high performance liquid chromatographic determination in urine", *Journal of Chromatography B*, **668**:187-188.

6.2 Web Addresses

Ash, basic facts No 3, February 2002, "Smoking and economics", http://www.ash.org.uk/html/factsheets/html/basic03.html.

Ash, basic facts No 4, February 2002, "Tobacco regulations", http://www.ash.org.uk/html/factsheets/html/basic04.html.

Ash, Fact sheet No 2, February 2001, "Smoking statistics: illness and death", http://www.ash.org.uk/factsheets/html/fact02.html.

Ash, Fact sheet No 4, July 2001, "Smoking and Cancer", http://www.ash.org.uk/factsheets/html/fact04.html.

Ash, Fact sheet No 6, May 2002, "Smoking, the heart and circulation", http://www.ash.org.uk/factsheets/html/fact06.html.

Ash, Fact sheet No 11, July 2001, "What happens when you stop smoking", http://www.ash.org.uk/factsheets/html/fact11.html.

Ash, Fact sheet No 12, August 2001, "What's in a cigarette?",
http://www.ash.org.uk/factsheets/html/fact12.html.

Asthma and smoking, action on smoking and health, updated June 2002,
http://www.ash.org.uk/html/passive/html/asthma.html.

BBC news, "Royal warrant stubbed out",
http://www.news.bbc.co.uk/hi/English/uk/newsid-274000/274100.stm.

British American Tobacco
http://www.bat.com/bat/WhatWD.nsf/97df56...77b49c391b86256887000ee419!OpenDocu
ment.

CNN, Focus: tobacco under attack, "A brief history of tobacco",
http://www.cnn.com/US/9705/tobacco/index.html.

Gallaher group, "The history of smoking",
http://www.gallaher-group.com/annual98/botinforhistory.htm.

Samuel Garten, "A possible mechanism to help explain the effects of menthol on tobacco
smoking", http://www.goodhealth.freeservers.com/MentholMechanism.htm.

HowStuffWorks, "How nicotine works",
http://www.howstuffworks.com/nicotine.htm.

Imperial tobacco group, "The tobacco story",
http://www.imperial-tobacco.com/History/Chapter1.asp.

Tobacco.org, "Tobacco timeline", http://www.tobacco.org/History/TobaccoHistory.html.

The university of Birmingham, Wolfson applied technology laboratory, "Smoking test
device", http://www.bham.ac.uk/WATL/Smoking.htm.

World Health organisation, "World no tobacco day history",
http://www.5.who.int/tobacco/printer_content.cfm?pid=65.

Appendix 1

Urine test for the assessment of smoking status

Urine test for the assessment of smoking status

K. L. CHAMBERS*, G. A. ELLARD†, A. T. HEWSON‡ and R. F. SMITH*

**Division of Biomedical Science and Biomedical Research Centre, Sheffield Hallam University, Sheffield S1 1WB;* †*Department of Medical Microbiology, St George's Hospital Medical School, Cranmer Terrace, London SW17 0RE;* ‡*Division of Chemistry and Biomedical Research Centre, Sheffield Hallam University, Sheffield S1 1WB, UK*

Abstract: A simple, quick and inexpensive test for smoking status would be useful in a variety of settings. The non-polar barbituric acid derivative 1,3-dibutyl-2-thiobarbituric acid (DBTB) is described as a novel derivatisation reagent for nicotine and its metabolites in the König reaction to assess smoking status. The relative performance of qualitative methods for assessing smoking status using DBTB and the previously employed derivatisation reagent 1,3-diethyl-2-thiobarbituric acid (DETB), as well as quantitatively-based methods for determining 'total nicotine metabolites' (TNMs) using these two reagents, were evaluated against a cotinine-based radioimmunoassay (RIA) as the 'gold standard'. Clinical sensitivity and specificity for all the approaches studied were in excess of 94%. Simple qualitative assessment by eye was superior to quantitatively-based measures of smoking status. Correlation between estimation of nicotine metabolites using DBTB, DETB and RIA were good. The most efficient and convenient method to distinguish between smokers and non-smokers was the simple qualitative method using the more lipophilic reagent DBTB.

Key words: Cotinine. König reaction. Smoking. Total nicotine metabolites.

Introduction

Smoking is the major preventable cause of mortality and morbidity in the western world. Recent evidence suggests that about half of all regular smokers will die from their smoking habit.[1] Whilst smoking prevalence is on the decline within the industrialised nations, it is increasing in most other parts of the world. It has been suggested[2] that the most effective strategy for reducing the incidence of cardiovascular disease, stroke and cancer is to cease smoking.

The efficiency with which smokers smoke their cigarettes varies between individuals, and smokers often deny their addiction. Smoking cessation initiatives, the monitoring of smoking during pregnancy,[3] and epidemiological studies of smoking-related illness would be facilitated if simple, reliable methods were available to identifying smokers and assess their daily intake of tobacco-related compounds.

For many years, questionnaires have provided information on individual smoking habits; however,

Correspondence to: Dr R.F. Smith.
E-mail: r.f.smith@shu.ac.uk

often they have been found to be incorrect when compared with measurements taken in a laboratory. The most widely used marker of tobacco usage has been the measurement of cotinine, the primary nicotine metabolite. This biomarker has been used largely because of its long serum half-life (about 17 hours), compared with a half-life of about two hours for nicotine.[4] Methods for cotinine measurement include gas chromatography–mass spectrometry (GC–MS),[5,6] high-performance liquid chromatography (HPLC)[7–9] and immunoassay;[10,11] these have been applied to a wide variety of biological samples including serum, saliva, urine and hair.

GC–MS is the most sensitive and specific method for the measurement of individual nicotine metabolites, but it is labour-intensive and requires very expensive equipment. HPLC lacks sensitivity and generally picks out only a few of the individual metabolites. Immunoassays can be extremely sensitive but measure only cotinine with variable cross-reactivity to other metabolites, especially the 3'- and 5'-hydroxycotinine metabolites, depending on the antibody employed.

Alternative urine-based methods to identify smokers and assess their relative nicotine intakes involve the use of modifications (or variants) of the simple and inexpensive colorimetric König reaction, in which orange or pink/red chromophores are formed from nicotine and its metabolites, using barbituric acid or 1,3-diethyl-2-thiobarbituric acid (DETB) as condensing reagents, respectively.[12] The colours that form may be read and compared with the colour produced by a known concentration of cotinine; alternatively, the colour produced may be extracted into ethyl acetate and assessed by eye in order to provide an immediate qualitative assessment of the smoking status of the individual. Using this method, Smith *et al.*[13] have reported a sensitivity of 95% and specificity of 100%, compared with radioimmunoassay (RIA), for a qualitative asessment of smoking status.

The modified König reaction also has been used to develop a simple 'near patient' test, using DETB as a condensing reagent,[14,15] for HPLC[16] and in flow-injection[17] analysis. In the original qualitative method of Peach *et al.*,[12] and subsequent HPLC procedures, the DETB-derived chromophores were extracted into ethyl acetate.

However, unlike the situation with cotinine, it was noted that some of the pink chromophores formed in smokers' urine samples were incompletely extracted into ethyl acetate. Therefore, it was argued that these probably were derived from much more polar metabolites of nicotine, and that the extractability of the reaction products might be considerably enhanced — and the sensitivity improved — if a more lipophilic barbituric acid derivative was employed. Hence the rationale for the synthesis of 1,3-dibutyl-2-thiobarbituric acid (DBTB).

Materials and methods

Potassium cyanide, chloramine-T and sodium acetate were purchased from Merck eurolab Ltd. (Lutterworth, UK). Acetic acid was purchased from Fisher Scientific Ltd. (Loughborough, UK) 1,3-dibutyl-2-thiobarbituric acid was synthesised using 1,3-dibutyl-2-thiourea and diethylmalonate, which were purchased from Aldrich (Gillingham, UK). Methanol, ethyl acetate and acetone were all of HPLC grade. The absorbencies of the urine samples derivatised with either DETB or DBTB were measured using a Cecil 9500 Super Aquarius. The identity of the synthesised DBTB was confirmed by nuclear magnetic resonance (NMR), using a Bruker AC 250.

Subjects

During a previous study, urine samples were collected from 251 patients attending the diabetic clinic at Ealing Hospital.[12] According to the gold-standard cotinine RIA method, this population was judged to comprise 130 non-smokers and 120 smokers, with one result regarded as borderline. The current study utilised aliquots of the 224 urine samples (106 smokers, 118 non-smokers) remaining from the Ealing Hospital investigation, whose volumes were sufficient for the colorimetric analyses described below.

Samples were subjected to both qualitative assessment of smoking status and quantitative analysis by comparison with a standard cotinine preparation. Quantitative measurements were made before and after extraction with ethyl acetate, although the measurements taken after extraction with ethyl acetate were carried out on only 95 of the samples from RIA-confirmed smokers and on 115 samples from RIA-confirmed non-smokers. All samples were collected in accordance with the ethical code used at Ealing Hospital. All samples were deep-frozen until analysis and all analyses were carried out 'blind', without reference to the results of the RIA.

Preparation of 1,3-dibutyl-2-thiobarbituric acid

DBTB was prepared according to the method of Hahn *et al.*[18] Briefly, 6.5 g sodium (washed with petroleum spirits and dried) was placed in a round-bottomed flask containing 75 mL methanol. 10 g N,N-dibutyl-thiourea (0.076 mol) and 17.2 g diethylmalonate (0.1 mol) were added to the flask, and the resulting reaction mixture was allowed to reflux for 36 h.

After this period, 50 mL distilled water was added and partly evaporated under pressure to remove any remaining methanol. The small amount of white precipitate (unreacted dibutylthiourea) formed during this evaporation was removed by filtration. The solution was diluted further with 100 mL distilled water, chilled on ice and then acidified with concentrated hydrochloric acid to between pH 1 and 2.

The resulting precipitate was collected and dried under reduced pressure to produce a light yellow powder of DBTB. The melting point range was between 56°C and 60°C. An NMR scan was carried out to confirm the identity of the compound — ^{1}H NMR (250 MHz; CDCl$_3$) δ ppm 0.94 (6H, t, *J* 7.5, CH$_3$ x2); 1.35 (4H, m, CH$_2$CH$_2$CH$_3$); 1.6 (4H, m, CH$_2$CH$_2$CH$_3$) 3.7 (2H, s, O=CCH$_2$C=O; 4.3 (4H, t, *J* 7.5-NCH$_2$CH$_2$-).

Cotinine radioimmunoassay

For the purpose of this study, RIA was used as the gold-standard test. This is a well-documented method that can reliably distinguish between two categories; for example, an individual that is exposed to high levels of nicotine (i.e. a smoker) and an individual who is exposed to low levels of nicotine (i.e. a non-smoker).

Urinary cotinine concentrations were determined by RIA,[11] as described previously.[12] Aliquots (10 µL) of the samples or cotinine calibrators (0.14–4.26 µmol/L) were incubated overnight with cotinine antibody and radioactive iodine ([125I])-labelled cotinine. Bound and free fractions were separated using a polyethylene-glycol-assisted second antibody technique. The radio-activity in the bound fraction was counted, and cotinine concentrations were estimated using a four-parameter logistic curve-fitting programme. Within- and between-batch imprecisions were 5.1% and 7%, respectively.

König reaction

The total nicotine metabolite concentration was determined both qualitatively and quantitatively. Briefly, this involved adding 1 mL of individual nicotine metabolite, urine, or standard cotinine solution to 400 µL sodium acetate buffer (pH 4.7). This was followed by addition of 200 µL potassium cyanide (10% w/v) and 200 µL chloramine-T (10% w/v). The solution was mixed well before the addition of condensing reagent (1 mL; DETB [1% w/v, water:acetone 50:50] or DBTB [1% w/v in acetone]). The reaction mixture was again mixed well and allowed to stand for 20 min. Absorbance measurements were made both before and after extraction with ethyl acetate (1 mL).

Qualitative assessment was by simple observation of a pink colour in the ethyl acetate layer. Absorbence of unextracted and ethyl-acetate-extracted samples was measured at 525 nm and 533 nm, respectively. A stock solution of 28 µmol/L cotinine (5 µg/mL) was used to determine the equivalent nicotine metabolite concentration. In addition, a sample blank was used, which comprised a mixture of urine samples from both self-reported smokers and non-smokers, and was assayed following the basic method, except that the chloramine-T was omitted.

Results

Urinary concentrations determined by cotinine RIA ranged from 0.06 µmol/L to 0.97 µmol/L (median:

0.1 µmol/L) for non-smokers, and from 1.48 µmol/L to 76.5 µmol/L (median: 19.3 µmol/L) for smokers.

Urinary concentrations determined using DETB ranged from <0.1 µmol/L to 12.9 µmol/L (median: 1.25 µmol/L) for non-smokers, and 4.32 to 282 µmol/L (median: 45 µmol/L) for smokers.

Urinary concentrations determined using DBTB without extraction ranged from <0.1 µmol/L to 9.8 µmol/L (median: 4.29 µmol/L) for non-smokers, and 5.17 µmol/L to 324 µmol/L (median: 30.3 µmol/L) for smokers.

When measurements were made post-extraction (DBTB only), results ranged from <0.1 µmol/L to 12.6 µmol/L (median: 0.91 µmol/L) for non-smokers, and 2.56 µmol/L to 212 µmol/L (median: 23.8 µmol/L) for smokers (Table 1).

The optimum cut-off point between smokers and non-smokers using DBTB was determined by receiver operator characteristic (ROC) curve analysis[13] from a consideration of sensitivity and specificity when compared with the gold-standard RIA determination. Sensitivity was calculated as the proportion of true positive (TP) test results obtained in subjects who smoked (i.e. TP/TP + FN [false negatives]), whereas specificity was calculated as the proportion of true negative (TN) results obtained in subjects who did not smoke (i.e. TN/TN + FP [false positives]). Overall test efficiency was calculated as TP + TN/TP + FP + TN + FN. The cut-off point for DBTB unextracted was determined as 8.5 µmol/L, compared with 4 µmol/L for samples extracted in ethyl acetate.

Using DETB, qualitative assessment of smoking status gave a sensitivity of 94% and specificity of 100%. Using DBTB, sensitivity and specificity were 100% and 99%, respectively. There was an overall improvement in efficiency from 97% with DETB to 99.5% with DBTB. When the samples were analysed quantitatively, results for DETB-unextracted samples gave a sensitivity and specificity of 98%. DBTB-unextracted samples produced a sensitivity and specificity of 95% compared with results obtained from samples using DBTB extracted in ethyl acetate, which gave a sensitivity and specificity of 96% (Table 2).

Table 1. Urinary cotinine and TNM values in smokers and non-smokers (µmol/L)

	Non-smokers		Smokers	
	Median	Range	Median	Range
RIA	0.1	0.06–0.97	19.3	1.48–76.5
DETB	1.25	<0.1–12.9	45	4.3–282
DBTB	4.29	<0.1–9.8	30.3	5.2–324
DBTB (post-extraction)	0.91	<0.1–12.6	23.8	2.6–212

	Sensitivity	Specificity
Qualitative		
DETB	94%	100%
DBTB	100%	99%
Quantitative		
DETB	98%	98%
DBTB (post-extraction)	95%	95%
DBTB	96%	96%

Amongst the smokers, correlation between urinary cotinine measurement (RIA) and the colorimetric procedures was determined using Spearman's Rank analysis. DBTB extracted ($n = 95$): rs = 0.84, P <0.01 (Figure 1); DBTB unextracted ($n = 106$): rs = 0.790, P <0.01. Correlation between DETB and DBTB unextracted ($n = 106$) gave rs = 0.871, P <0.01.

Discussion

There is a need for a quick, simple, inexpensive test to assess smoking status in a variety of settings. Several workers have attempted to employ the König reaction for this purpose. The approach employed here — that is, attempting to improve assay sensitivity by use of a more non-polar derivatisation reagent, allied to extraction of the coloured product — yielded good results. Qualitative assessment of smoking status was clearly superior to quantitative determination. The increase in partitioning between the aqueous and ethyl acetate phases, obtained using DBTB, improves distinction between non-smoker and smoker, from an overall efficiency of 97% with DETB to 99.5% with DBTB. However, this distinction is notoriously difficult to make because no individuals truly can be said to not be exposed to tobacco smoke; and in any study of this type, the numbers of subjects appearing at the cut-off point is usually small.

Use of this compound offers clear advantages, both for HPLC analysis and for use in small near-patient devices for monitoring exposure to environmental tobacco smoke.[19] Correlation between RIA and colorimetric methods is good, but the results clearly are skewed by one or two subjects who had levels of total nicotine metabolites proportionately higher compared with cotinine. The implication is that these individuals may metabolise nicotine differently to the majority.

This is in accordance with the recent finding of null alleles for the cytochrome P_{450} enzyme CYP2A6, which is important in the metabolism of nicotine.[20] Individuals who cannot metabolise nicotine, or who metabolise nicotine poorly via the P_{450} pathway, may have greater amounts of other nicotine metabolites, which are measured in the König reaction but not by RIA.

Recent work suggests that nicotine is metabolised differently in the pregnant and non-pregnant
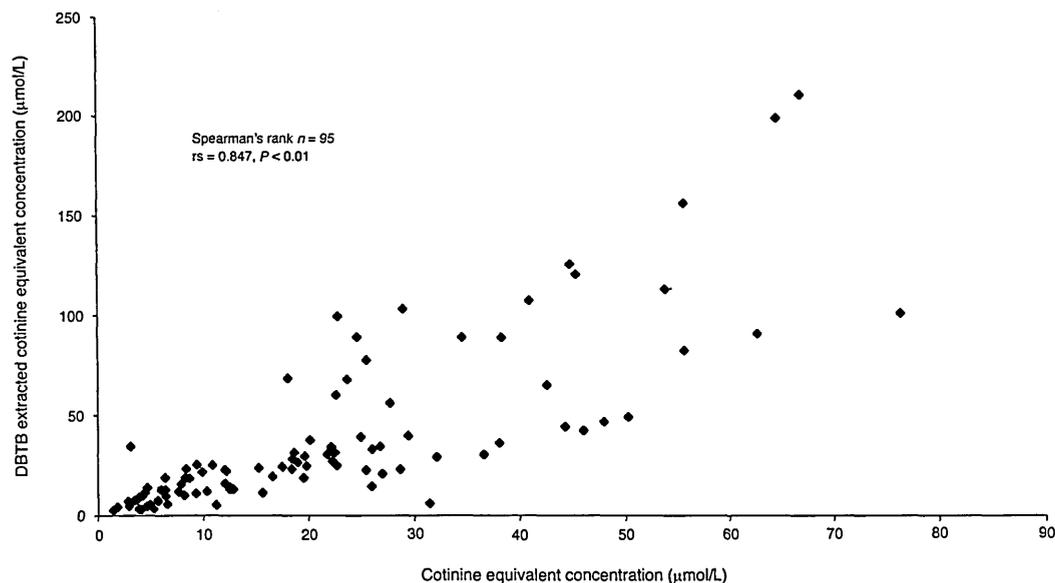
Fig. 1. Correlation between DBTB (extracted) and cotinine RIA.

individual,[21] and that there are inter-racial differences in nicotine metabolism.[22] The novel derivative described in this paper provides an improved, simple, inexpensive and near-perfect test for the determination of smoking status.

References

1 Doll R, Peto R, Wheatley K, Gray R, Sutherland I. Mortality in relation to smoking: 40 years' observation on male British doctors. *BMJ* 1994; **309**: 901–11.

2 Hays JT, Dale LC, Hurt RD, Croghan IT. Trends in smoking-related diseases. Why smoking cessation is still the best medicine. *Postgrad Med* 1998; **104**: 56–62.

3 Ellard GA, Johnstone FD, Prescott RJ, Wang, J-X, Mao J-H. Smoking during pregnancy: the dose dependence of birth-weight deficits. *Br J Obstet Gynaecol* 1996; **103**: 806–13.

4 Wall MA, Johnson J, Jacob P, Benowitz NL. Cotinine in the serum, saliva and urine of non-smokers, passive smokers and active smokers. *Am J Public Health* 1988; **78**: 699–701.

5 Jacob P, Wilson M, Benowitz NL. Improved gas chromatographic method for the determination of nicotine and cotinine in biological fluids. *J Chrom Biomed Appl* 1981; **222**: 61–70.

6 Ji A, Lawson G, Anderson R, Dale L, Croghan I, Hurt R. A new gas chromatography-mass spectrometry method for simultaneous determination of total and free trans-3'-hydroxy-cotinine and cotinine in the urine of subjects receiving trans-dermal nicotine. *Clin Chem* 1999; **45**: 85–91.

7 Kyerematen GA, Damiano MD, Dvorchik BH, Vesell ES. Smoking-induced changes in nicotine disposition: application of a new HPLC assay for nicotine and its metabolites. *Clin Pharm Ther* 1982; **32**: 769–79.

8 Moore J, Greenwood M, Sinclair N. Automation of a high-performance liquid chromatographic assay for the determination of nicotine, cotinine and 3-hydroxycotinine in human urine. *J Pharm Biomed Anal* 1990; **8**: 1051–4.

9 Ubbink JB, Lagendijk J, Vermaak WHH. Simple high-performance liquid chromatographic method to verify the direct barbituric acid assay for urinary cotinine. *J Chrom Biomed Appl* 1993; **620**: 254–9.

10 Langone JJ, Gjika HB, Van Vunkis H. Nicotine and its metabolites: radioimmunoassay for nicotine and cotinine. *Biochemistry* 1973; **12**: 5025–30.

11 Knight GJ, Wylie P, Holman MS, Haddow JE. Improved ^{125}I radioimmunoassay for cotinine by selective removal of bridge antibodies. *Clin Chem* 1985; **31**: 118–21.

12 Peach H, Ellard GA, Jenner PJ, Morris RW. A simple, inexpensive urine test of smoking. *Thorax* 1985; **40**: 351–7.

13 Smith RF, Mather HM, Ellard G. Assessment of simple colorimetric procedures to determine smoking status of diabetic subjects. *Clin Chem* 1998; **44**: 275–80.

14 Cope G, Nayyar P, Holder R, Gibbons J, Bunce R. A simple near-patient test for nicotine and its metabolites in urine to assess smoking habit. *Clin Chim Acta* 1996; **256**: 135–49.

15 Eswara AR, Nochur SV, Mossman DJ. Detection of nicotine and its metabolites in urine. *Am J Health Behav* 1996; **20**: 333–45.

16 Parvainen MT, Puhakainen EVJ, Laatikainen R, Savolainen K, Herranen J, Barlow RD. Nicotine metabolites in the urine of smokers. *J Chrom Biomed Appls* 1990; **525**: 193–202.

17 Lui J, Feng Y. Determination of nicotine by reagent injection flow injection photometric method. *Talanta* 1998; **47**: 833–40.

18 Hahn KM, Waggoner AS, Taylor DL. A calcium-sensitive fluorescent analog of calmodulin based on a novel calmodulin-binding fluorophore. *J Biol Chem* 1990; **265**: 20335–45.

19 Cope G, Nayyar P, Wilkinson L, Holder R. Simple colorimetric test to quantify exposure to environmental tobacco smoke: occupational health study. *Ann Clin Biochem* 2000; **37**: 795–6.

20 Pianezza ML, Sellers EM, Tyndale RF. Nicotine metabolism defect reduces smoking. *Nature* 1998; **393**: 750.

21 Dempsey DA, Jacob P III, Benowitz NL. Accelerated nicotine and cotinine metabolism during pregnancy. *Clin Pharmacol Ther* 1998; **63**: 202 PII–120.

22 Benowitz NL, Peiez-Stable EJ, Fong I. Ethnic differences in N-glucuronidation of nicotine and cotinine. *J Pharmacol Exp Ther* 1999; **291**: 1196–203.