Determination of nicotine and its metabolites by capillary electrophoresis and mass spectrometry.

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REFERENCE

Determination of Nicotine and its Metabolites by Capillary Electrophoresis and Mass Spectrometry

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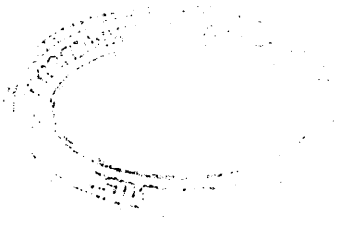

Abstract

In England an estimated, 284,000 patients are admitted to NHS hospitals each year due to disease caused by smoking. It is estimated that half of all teenagers who are currently smoking will die from diseases caused by tobacco if they continue to smoke. An estimated one quarter of smokers will die after 70 years of age and one quarter before, with those dying before 70 losing on average 23 years of life. It is the addictive nature of nicotine that exacerbates the toxicities of the other components of cigarette smoke. The Royal College of Physicians has affirmed that the way in which nicotine causes addiction is similar to drugs such as heroin and cocaine. Thus studies of nicotine metabolism are of great importance since they determine the extent of which the addictive nature of nicotine can influence smoking behaviour and hence the onset of smoking related illnesses.

In this area of research capillary electrophoresis (CE) is still in its infancy. But with its high resolution and high number of theoretical plates achieved, CE makes for an attractive separating device for coupling with a mass spectrometer (MS). The overall aims of this work were to produce a sensitive, highly selective, and simple CE-sample stacking/MS assay for the measurement of nicotine and its metabolites in urine, to develop a highly sensitive transient isotachophoretic/MS method, that yields detection limits comparable to that observed by HPLC/MS and with a separation efficiency to match that of CE-sample stacking/MS, and finally to characterise the metabolic activity of cytochrome P450 (e.g. CYP2D6) in the placenta, with respect to nicotine, from a representative in-vitro human trophoblast-like cell line, BeWo, via HPLC/MS and CE/UV.

Analysis of urine samples was accompanied by sample clean up via SPE to ensure the appropriate removal of inorganic salts. An optimised hydrodynamic and electrokinetic injection method (HE injection) was used for CE-sample stacking/ MS. HE-sample stacking/MS brought about lower detection limits (LODs of nicotine and cotinine, by CE-sample stacking/ MS (via HE injection), were found to be 0.11 and 2.25 μg/ mL, respectively) when compared to sample stacking/MS via hydrodynamic or electrokinetic injection alone. The added selectivity that the selected ion monitoring mode of MS provided ensured the clear identification of nicotine and its metabolites in urine. A counterflow transient isotachophoresis (tITP) method was developed, with MS detection, for the analysis of even lower analyte concentrations. Limits of detection for both nicotine (0.03μg/ mL) and cotinine (0.34 μg/ mL), via HE-tITP/MS, were considerably lower than those obtained by HE injection-sample stacking/MS, suggesting that HE-tITP/MS could be used complementary to HE injection-sample stacking/MS. When HPLC/MS and CE/UV were used to analyse cytochrome p450 activity in the human trophoblast-like BeWo cell line, it was clear from our data that nicotine metabolism was observed. Thus it is probable that CYP mediated processes play an important role in nicotine metabolism in the placenta. When compared to HPLC/MS both HE-sample stacking/MS and HE-tITP/MS exhibited higher resolutions and peak efficiencies; with HE-tITP/MS exhibiting comparable limits of detection and quantitation to those obtained by HPLC/MS with respect to nicotine, but not cotinine. The major improvements to the coaxial interface performance and sensitivity enhancements, has made CE/MS an attractive alternative to HPLC/MS for the determination of nicotine and its' metabolites from biological matrices.

Candidates Statement

The overall aims of this work were to produce a sensitive, highly selective, and simple CE-sample stacking/MS assay for the measurement of nicotine and its metabolites in urine, to develop a highly sensitive transient isotachophoretic/MS method, that yields detection limits comparable to that observed by HPLC/MS and with a separation efficiency to match that of CE-sample stacking/MS, and finally to characterise the metabolic activity of cytochrome P450 (e.g. CYP2D6) in the placenta, with respect to nicotine, from a representative in-vitro human trophoblast-like cell line, BeWo, via HPLC/MS and CE/UV. The work described in this thesis was carried out by the author at the School of Science and Mathematics, between December 2000 and July 2003, with contributions on Chapter 5 made by Miss Riyam Al-Khaily and Miss Kirsty Lees. The work displayed in this thesis is original except where acknowledged by reference.

Edward Emmanuel Kweku Baidoo

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Chapter 1

Introduction

1.1. A brief history of nicotine and tobacco

In 1492 Christopher Columbus discovered tobacco being smoked in the New World and later introduced the habit to Spain; and later in 1550 Jean Nicot, an ambassador to Portugal, sent tobacco and tobacco seeds to Paris from Portugal (and nicotine was named after him many years later) [1,2].

Native Indians smoked the dried leaf of *nicotiana tábacum*, which was indigenous to tropical America, at the time of Columbus. *Tábacum*, as it was termed, was subsequently used by the Italians, with the English obtaining their tobacco directly from America. Soon after, the rest of the world became involved with tobacco use and abuse [3].

Nicotiana tábacum is now cultivated throughout the world for preparation of, cigars, cigarettes, pipe and chewing tobacco. Nicotine constitutes approximately 2-8 % of the dry weight of the cured leaf (although a much larger range exists in some species), and is the major tobacco alkaloid. Other minor tobacco alkaloids, that are present in much lower amounts, include anabasine, anatabine and nornicotine (which itself is a metabolite of nicotine) [3].

Since Langley and Dickenson noted the antagonistic behaviour of nicotine towards autonomic ganglia, in 1889, this compound has contributed greatly to the understanding of pharmacological responses within the human body [3].

In 1950 Sir Richard Doll and A Bradford Hill published the first evidence of a link between lung cancer and smoking in the British Medical Journal and in 1964 the US Surgeon General Luther Terry announced that smoking causes lung cancer. The British government later banned cigarette advertisements on television in the UK, in 1965, and in the same year the Federal Cigarette Labelling and Advertising Act required the US Surgeon General's warning on cigarette packets [4]. Since then a variety of studies were carried out to determine the health effects associated with smoking and to improve upon the various smoking cessation strategies, which include nicotine replacement therapy.

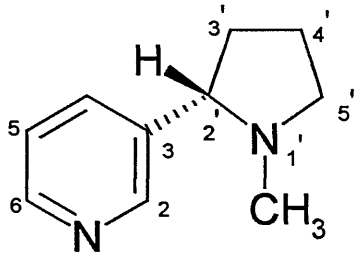
1.2. Chemical properties of nicotine

Nicotine was isolated from tobacco, in 1828, by Posselt and Reimann and its empirical formula described, in 1843, by Melsens. Pictet and Crepieux synthesized it, in 1893, and Pictet and Rotschy described its chemical isomerism, in 1904. In 1978, Pitner *et al.* identified the special orientation of natural (S)-nicotine [3].

Nicotine has the systematic name 3-(1-methyl-2-pyrrolidinyl)pyridine, 1-methyl-2-(3-pyridyl)pyrrolidine, and is also known as β-pyridyl-α-N-methylpyrrolidine. It is a 2-(3)-substituted analog of N-methylpyrrolidine and is related both structurally and biosynthetically to nornicotine, cotinine and norcotinine. It has an empirical formula of $C_{10}H_{14}N_2$, a molecular weight of 162.23, and a density of 1.0097 g/mL. In the majority of alkaloids, one heterocyclic ring is aromatic, and the other is saturated, or contains a single

double bond, with additional conformation flexibility within the saturated ring. Nicotine is thought to exist in solution primarily in a conformation in which the N'-methyl group is trans to the pyridine ring, with the pyrrolidine ring in an envelope conformation, and the relative orientation of the pyridine and pyrrolidine ring is essentially orthogonal [3].

Figure 1.1. Nicotine (adapted from Gorrod and Jacob III) [3].



Nicotine exists primarily in the S-(-)-enantiomeric form in nature but, to a lesser extent, may also be present as the R-(+)-isomer in nicotiana plant species. Both enantiomers are pharmacologically active, but the effects and potency of the (+)-2'R-nicotine enantiomer are still relatively unknown. Smokers may be exposed to both enantiomers, due to pyrolytic racemisation of the S-(-)-isomer during smoking. As much as 11 % of the R-(-)-isomer has been found in smoke condensate from cigarettes [3].

With two tertiary amino centres, nicotine is reported to have relative pKas of approximately 3.0-4.0, for the pyridino N, and 8.0-9.0 for the pyrrolidino N. The lower pKa value for the aromatic pyridino N is due to sp^2 hybridisation on base strength, since it is more towards 's' in character and hence electrons in that orbital are bound more tightly to the nucleus. The opposite is true for the pyrrolydino N. Under physiological conditions the N-1-protonated form does not occur, due to the considerably lower basicity of the pyridyl

nitrogen compared to the pyrrolidine nitrogen, while the N-1, N-1'-diprotonated species are formed predominantly in acidic media (pH < 4.0). At pH 7.4, nicotine exists in both its uncharged (lipophilic) and charged (hydrophilic) forms. Nicotine exists with a ratio of charged to uncharged forms of approximately 2 to 1 at pH 7.4. The lipophilic form diffuses through lipoprotein membranes, whilst the hydrophilic form does not. Thus the charge status of nicotine is of great importance when considering its biological actions [3].

1.3. Cigarette and tobacco smoke composition

Nicotine is contained in the moisture of the tobacco leaf. Thus when the cigarette is lit, it evaporates, attaching itself to minute droplets in the tobacco smoke inhaled by the smoker; and is absorbed by the body very quickly, reaching the brain within 10-19 seconds [5].

Cigarette tobacco is blended from two main leaf varieties: yellowish 'bright', also known as Virginia where it was originally grown, contains 2.5-3% nicotine; and 'burley' tobacco which has a higher nicotine content (3.5-4%). US blends are thought to contain up to 10% of imported 'oriental' tobacco, which is aromatic but relatively low (less than 2%) in nicotine. Cigarettes also contain 'fillers' which are made from the stems and other bits of tobacco, which would otherwise be waste products [6].

Additives are used to make tobacco products more acceptable to the consumer. They include humectants (moisturisers) to prolong shelf life; sugars to make the smoke seem milder and easier to inhale; and flavourings such as chocolate and vanilla [6].

In response to the emergence of concern regarding the health problems associated with smoking, tobacco companies began to introduce filters. Filters are made of cellulose acetate

and trap some of the tar and smoke particles from the inhaled smoke. Filters also cool the smoke slightly, making it easier to inhale [6].

Tobacco smoke is made up of "sidestream smoke" from the burning tip of the cigarette and "mainstream smoke" from the filter or mouth end. Tobacco smoke is composed of thousands of different chemicals, which are released into the air as particles and gases. Many of these toxic chemicals are present in higher concentrations in sidestream smoke than in mainstream smoke (with nearly 85% of the smoke in a room resulting from sidestream smoke) [6]. The particulate phase includes nicotine, "tar" (itself composed of many chemicals), benzene(a)anthracene and benzo(a)pyrene (table 1.1). The gas phase includes carbon monoxide (which combines with haemoglobin causing a reduction in oxygen capacity in the blood), ammonia, dimethylnitrosamine, formaldehyde, hydrogen cyanide and acrolein. Some of these compounds have marked irritant properties, whilst others, including benzo(a)pyrene and dimethylnitrosamine, have been shown to cause cancer [7].

Table 1.1. The major components of fresh, undiluted mainstream smoke of non-filtered cigarettes (adapted from K.L. Chambers, PhD thesis, Sheffield Hallam University, 2002) [8].

Vapour phase	Amount	Particular 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	Benz(a)pyrene	20-40 ng
Pyridine	16-40 μg	Qinolone	0.5-2 μg
Hydrogen cyanide	400-500 μg	2-Toluidine	160 ng
Nitrogen oxides	100-600 μg	Cadmium	100 ng
Acetic acids	330-810 μg	Polonium-210	0.03-0.5 pCi
Methyl chloride	150-600 μg	Benzoic acid	14-28 μg

In recent decades there has been a shift from the smoking of high-nicotine and high-tar cigarettes to lower yield brands. The main reason for smokers choosing light cigarettes instead of traditional types is that they think such cigarettes are safer or less addictive than high-nicotine and high-tar cigarettes. The addition of ventilation holes and the use of reconstituted tobacco, porous cigarette papers, and chemical additives in cigarettes are all modifications that tobacco companies "claim" to bring about low yield cigarettes [9]. However, a reduction in tar levels does not necessarily lead to the assumed health benefits due to the tendency of smokers to compensate for the reduction in nicotine (cigarettes lower in tar also tend to be lower in nicotine) by smoking more or inhaling more deeply, which would increase the delivery of carcinogens such as nitrosamines and polycyclic aromatic

hydrocarbons to the peripheral lung, thereby increasing the risk of lung adenocarcinoma [9]. Thus, low-tar cigarettes may cause different forms of cancer but not necessarily fewer cases.

The size of the cigarette, ventilation characteristics, nicotine and tar ratios, and nicotine content influence the way a person smokes the cigarette and, thus, presumably the dose of nicotine obtained from the cigarette. The duration of tobacco smoke inhalation, urinary pH, tobacco deprivation, stress, and blood nicotine levels are also factors that affect the puffing pattern or the number of cigarettes smoked [10].

1.4. Environmental tobacco smoke (ETS)

The size of one's dwelling affects the internal dose of ETS exposure if the majority of exposure occurs in an enclosed area such as one's home. The health risk of parental tobacco consumption might be increased in families living in small dwellings [11]. In a study carried out on 295 children, Köhler *et al.* [12] found passive smoke exposure (via analysis of nicotine, cotinine, and trans-3'-hydroxycotinine in urine) in 66 % of the children, with the frequency in the younger children (82 %) significantly higher than that in children over 5 years (52 %).

However, parents may be reluctant to consult their physicians about the true extent of their smoking habits especially when their children suffer from respiratory tract diseases. Thus data obtained from questionnaires has to be supported by environmental and biochemical markers used to measure ETS and ETS exposure [13].

Other factors that may influence the extent of passive smoking include room ventilation, proximity of non-smokers to smokers, number of cigarettes smoked by the smoker, and

individual differences in sensitivity to, and/or concern about, the adverse effects of environmental tobacco smoke [13].

When a person breathes in air contaminated by tobacco smoke, he or she is described as having smoked passively. Passive smoking is associated with increased incidence of lower respiratory tract infections in young children and may increase the risk of lung cancer in the nonsmoker [14]. It has been established that long-term exposure to environmental tobacco increases the risk of lung cancer formation [15,16].

Active and passive smoking expose an individual to the same substances but may differ in the relative concentrations of the substances they inhale. Cotinine, the major metabolite of nicotine, can be used as a biomarker of environmental tobacco smoke exposure [13]. Wall *et al.* [17] reported higher levels of cotinine in the urine of passive smokers compared to non-smokers. They were also able to distinguish active smokers from passive and non-smokers using serum, saliva, and urine. In this study the greatest concentrations of cotinine were found in urine (table 1.2).

Table 1.2. Mean cotinine levels in serum, saliva, and urine of nonsmokers, passive smokers, and active smokers (adapted from Wall *et al.*) [17].

	N	Serum (ng/mL)	Saliva (ng/mL)	Urine (ng/mL)	Mean urine cotinine + by urine creatinine
Nonsmokers	28	*	*	6.0	3.6
Passive smokers	20	*	*	9.2	6.3
Active smokers 10cgts/day	9	78	66.9	646.8	6.73.4
Active smokers > 10 cgts/day	40	301.2	283.7	1100.7	1325.5

*Too few subjects with detectable levels for calculation of a mean. N is the number of subjects.

1.5. Other routes of administration.

Nicotine can also be introduced to the human body via oral snuff, nasal snuff, food, and nicotine medication. The chemistry of nicotine shows that at pH 7.4 approximately 24 % of it is in its non-ionised form, which is more permeable through lipophilic cell membranes. Since moist oral snuff has an alkaline pH, nicotine is easily absorbed via the buccal mucosa. The amount of nicotine absorbed by the snuff user is dependent upon its availability in the product as well as the consumption pattern such as the size of the pinch, the site of placement, and the oral treatment of the pinch. Nicotine is absorbed more slowly from smokeless tobacco than from tobacco smoke, but peak venous blood concentrations after single doses of oral snuffs or chewing tobacco are similar to those for tobacco smoke. After smoking nicotine levels rapidly decline, while during and after the use of smokeless tobacco nicotine concentrations plateau. Higher levels of cotinine have been observed in smokeless tobacco users when compared to those of smokers [3].

Unlike oral snuff, the absorption of nicotine from nasal snuff is fairly rapid, and levels of plasma nicotine concentrations shown by habitual snuff takers are comparable to the average increase obtained from a single cigarette by a group of heavy smokers [3].

When ingesting foods, nicotine absorption occurs via the gastrointestinal tract. Foods, however, contain only small amounts of nicotine, with probably certain vegetables and teas containing the most [3,18]. Thus food is generally considered an insignificant source of nicotine exposure [3]. However, because urinary cotinine concentrations may range from only 0.6 to 6.2 ng/ mL, the use of urinary cotinine as a biomarker of exposure to environmental tobacco smoke may be compromised by the emergence of cotinine as a

result of ingesting nicotine containing foods [19]. Thus some workers have suggested that background cotinine must be corrected for when analysing environmental smoke exposure [20].

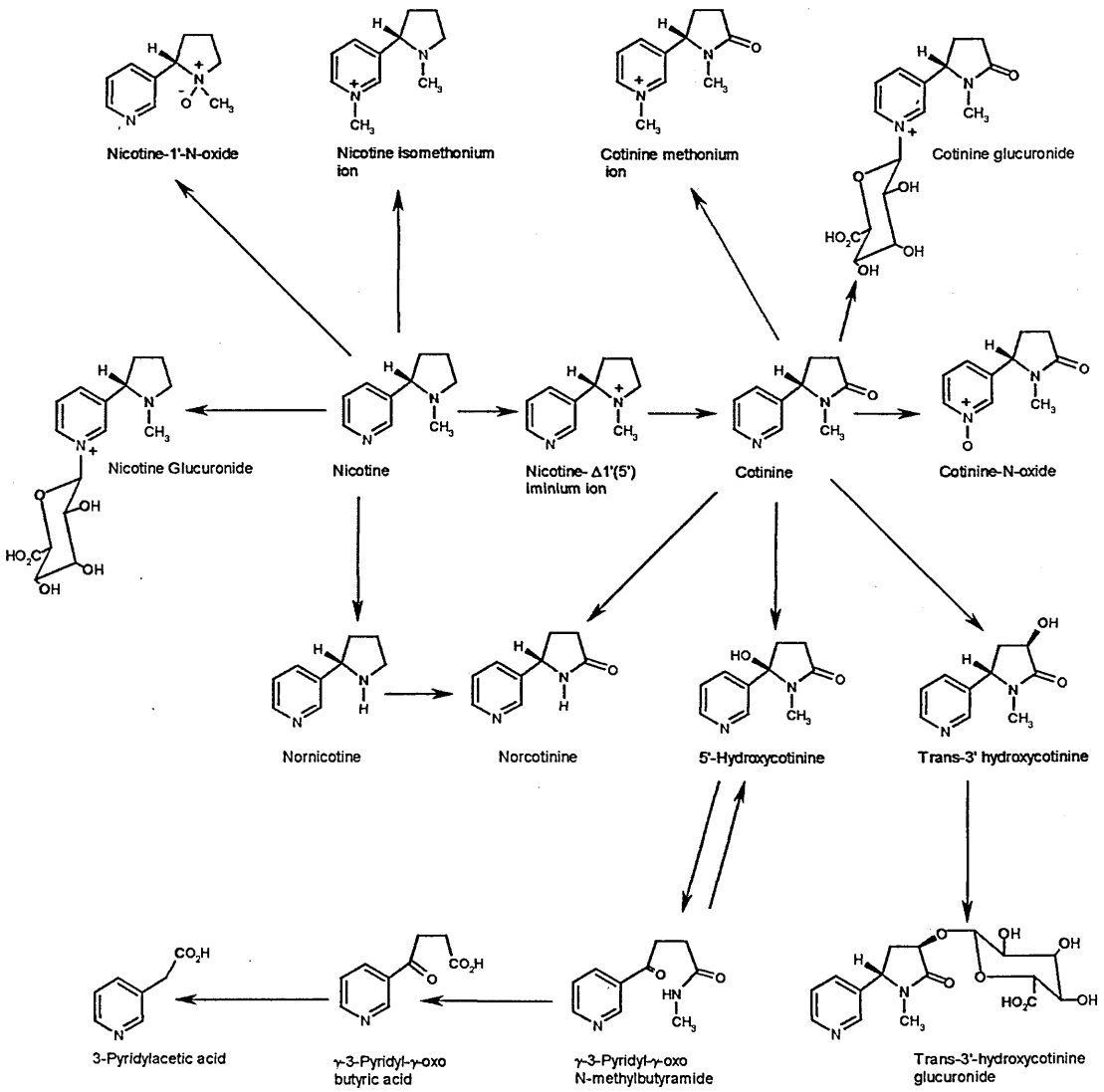
Nasal spray, inhaler, nicotine gum, and nicotine patch are all forms of nicotine replacement therapy. These therapies are primarily designed to wean people off tobacco smoking and habitual consumption of oral and nasal snuffs [21].

1.6. The Metabolism of nicotine

The purpose of metabolism is to decrease the extent to which an exogenous compound (such as a drug, dietary chemical, or pollutant) or an endogenous compound is retained within the body, by converting that compound into a more water-soluble metabolite, which increases the amount that is excreted in the urine or bile.

Metabolism of drugs is carried out by numerous enzymes, which are involved in the metabolism of endogenous compounds such as bile acids, fatty acids, steroid hormones, and bilirubin. The majority of these enzymes predominate in sites exposed to high concentrations of xenobiotics in organs such as the liver, lungs, intestines, kidneys, nasal mucosa and even the placenta. By far the greatest knowledge exists in relation to the cytochrome P450 (CYP) superfamily of oxidative enzymes [22]. The most prominent CYP involved nicotine metabolism is the CYP2A6. Below is a diagram showing the various pathways for nicotine metabolism including the conversion nicotine to cotinine via CYP2A6 (figure 1.2).

Figure 1.2. A diagram showing nicotine metabolism (adapted from Gorrod and Jacob III, 1999, [3]).



1.6.1. The role of the cytochrome P450

Cytochromes P450 (CYP) are haem-containing enzymes with iron protoporphyrin IX as the prosthetic group. CYP mediates biotransformation to polar metabolites, which can be excreted by the kidneys [23]. They are thought to bring about the hydroxylation of literally hundreds of structurally diverse drugs and chemicals, whose only common feature appears to be a reasonably high degree of lipophilicity. CYP is the terminal oxidase component of an electron transfer system, present in the endoplasmic reticulum, responsible for many drug oxidation reactions. The most intensively studied drug metabolism reaction is the cytochrome P450 catalysed mixed-function oxidation reaction [24]

$$NADPH + H^+ + O_2 + RH \xrightarrow{CYP} NADP^+ + H_2O + ROH$$

where NADPH is reduced nicotinamide adenine dinucleotide phosphate and $NADP^+$ is nicotinomide adenine dinucleotide phosphate.

CYP consists of a family of closely related isoenzymes that exist in multiple forms of monomeric molecular weight of approximately 45000 to 55000 Da. The name cytochrome P450 is derived from the fact that the cytochrome exhibits a spectral absorbance maximum at 450 nm when reduced and complexed with carbon monoxide [24].

Cytochrome P450 superfamily

CYP genes are designated a family number and an asterisk followed by a number for each allelic variant (e.g. CYP2A6*2) and are categorised into their families and sub families based on their sequence similarities. Those with amino acid sequences showing greater than 40 % homology belong to the same family, while those greater than 55 % belong to the

same subfamily. CYP1, CYP2, and CYP3 are mainly involved in the metabolism of exogenous compounds [25].

CYP2A6 activity

Approximately 80 % of nicotine is converted to cotinine in vivo by C-oxidation [26]. It is thought that CYP2A6 is the major enzyme catalysing this reaction in the liver [26] although there are also minor contributions from other CYPs such as CYP2B6 [25]. There are numerous studies showing the high expression of this enzyme in liver microsomes [26-28]. However, Pelkonen *et al.* [29] reported no such expression of CY2A6 in the kidney, duodenum, lung, alveolar macrophages, peripheral lymphocytes, placenta and uterine endometrium.

The ability of CYP2A6 to mediate nicotine C-oxidation at both high (500 μM) and low substrate concentrations (50 μM), in human liver microsomes, emphasises the major role of this enzyme in nicotine conversion to cotinine [30]. Also deletion of the CYP2A6 gene results in deficient cotinine formation from nicotine [31].

CYP2A6 is further reported to catalyse the conversion of cotinine to its primary metabolites trans-3'-hydroxycotinine [27, 32], 5'-hydroxycotinine, and possibly norcotinine [32]. Coumarin, a specific and selective CYP2A6 substrate, appears to competitively inhibit cotinine formation [26]. Specific inhibitors of CYP2A6 include methoxsalen [33], psoralen, naphthoflavone, pilocarpine, and menthofuran [25]. Methoxsalen is an effective in vivo inhibitor of CYP2A6 activity [34] and can therefore be used in the treatment of nicotine addiction [35-37]. Tyndale and Sellers [37] reported that subjects who received the methoxsalen and oral nicotine combination smoked significantly less than those who received a placebo. It should be noted that in this study, the methoxsalen/placebo

combination was more effective than the placebo/nicotine combination (the rank order of response was metheoxsalen/nicotine > methoxsalen/placebo > placebo/nicotine > placebo/placebo) [37].

When considering inhibitors, there is also the potential for enzymic inducers (e.g. phenobarbital, rifampin, antiepileptics) or repressors (e.g. methoxsalen). Both are important since the ingestion of these compounds can lead to altered enzyme activity in individuals with fully functional alleles [25].

Inter-individual variability in CYP2A6 activity

Polymorphisms in the CYP2A6 gene are thought to be mainly responsible for the observed variation in CYP2A6 activity, but environmental factors, such as inducers could also contribute. Thus a change in the gene sequence via a point mutation, deletion, or a gene conversion, may lead to the overproduction, underproduction, malfunction or absence of the enzyme [25]. Poor metabolisers of nicotine (individuals with two inactive gene variants) are homozygous for the inactive alleles CYP2A6*4/*4 [38] or CYP2A6*2/*2 or CYP2A6*6/*6 or CYP2A6*7. Thus the deficient metabolism of nicotine can result from being homozygous for 1 of several CYP2A6 alleles with gene mutations [39]. Extensive metabolisers have one or two copies of the active gene (i.e CYP2A6*1/*1), while fast metabolisers have two or more copies of the active gene (i.e. gene duplication CYP2A6*1/*1 × 2) [39]. Therefore CYP2A6 fast metabolisers that carry more than two copies of the active CYP2A6 alleles metabolise nicotine more rapidly, whilst very little cotinine is produced from poor metabolisers [25,38,40].

CYP2A6 and nicotine metabolism

As well as inter-individual variation, the frequency of CYP2A6 alleles varies between different ethnic groups which may result in inter-ethnic differences in the average ability to metabolise nicotine [40]. African Americans have a much reduced nicotine to cotinine conversion, clearance of cotinine, and metabolic clearance of nicotine to cotinine compared with Caucasians [25,41]. Chinese Americans metabolise nicotine more slowly than Caucasians [42]. Oscarson *et al.* [42] reported that high allele frequency of the CYP2A6 gene deletion [42], found in Chinese subjects (15 % of Chinese subjects expressed the CYP2A6del allele), were in part responsible for their slower metabolism of nicotine [43,44]. Frequency of the defective CYP2A6 allele of 1-3 % has been observed in Finish, Spanish, and Swedish populations [45]. The metabolic ratio of cotinine to nicotine was greater in Koreans than the Japanese [25,46]. Kwon *et al.* [46] showed there were ethnic differences in the allele frequencies of CYP2A6*1A, CYP2A6*1B, CYP2A6*4 and CYP2A6*5 between Koreans (45.7 %, 42.8 %, 11.0 %, and 0.5 %) and Japanese (42.4 %, 37.5 %, 20.1 %, and 0%). Thus it would appear that inter-individual difference in nicotine metabolism is closely related to the genetic polymorphism of CYP2A6 [46].

Both inter-ethnic and inter-individual variation in nicotine or cotinine metabolism may affect smoking behaviour [37,44], treatment of nicotine addiction, and the effectiveness of nicotine replacement therapies [25]. Inter-ethnic and inter-individual variation in nicotine or cotinine metabolism may also influence the risk of tobacco related cancers [45]. It is also known that CYP2A6 activates a number of structurally unrelated precarcinogens including many nitrosamines and aflatoxin B1 [44]. CYP2A6 poor metabolisers may be at lower risk of lung cancer development due to a decreased risk of becoming tobacco dependent and

less smoking if tobacco dependent, since smoke exposure is exponentially related to cancer risk [24]; and also because of their inability to metabolise pre-carcinogens.

CYP2A6 and smoking

It has been reported that genetics is responsible for approximately half of smoking initiation. The pharmacology of nicotine suggests that it is the primary compound in tobacco that establishes and maintains tobacco dependence. CYP2A6 variants can alter the rate of nicotine metabolism and consequently the amount of nicotine maintained in the body. Thus it is possible that individuals who are slow metabolisers, who have one or more inactive CYP2A6 alleles, differ in their risk for various aspects of smoking behaviour such as rates and severity of tobacco dependence, amount smoked regularly, and successful cessation. Nicotine levels will remain high for longer periods in slow metabolisers, thereby prolonging the adverse affects of nicotine and hence making these individuals less likely to become tobacco dependent. However, tobacco dependent slow metabolisers, who smoke less, have been shown to experience nicotine withdrawal at greater intervals of time. Reports suggest that individuals with extra copies of the active CYP2A6 alleles compensate for increased nicotine metabolism by inhaling their cigarettes with greater intensity. The reverse is true of slow metabolisers. Further more, it has been demonstrated that individuals with inactive CYP2A6 alleles tend to smoke at a later stage (3 years later than fast metabolisers) and for a shorter period of time (approximately 9 years) on average. Even though these studies have demonstrated the involvement of the CYP2A6 in the regulation of smoking behaviour, not all studies have found the same association [25].

Other CYPs that may be involed in nicotine metabolism

CYP2B6 has been reported to exhibit nicotine C-oxidation activity [22,47]. CYP2B6 mediates nicotine C-oxidation more effectively at higher substrate concentration (500 μM), in the human liver, than a lower substrate concentration (50 μM) [31]. However, CYP2B6 is not constitutively expressed in human liver and is therefore relatively unimportant in the metabolism of nicotine in most individuals [47]. CYP2D6 also appears to be capable of mediating the conversion of nicotine to cotinine [30,47]; and similarly exhibits C-oxidation more effectively at higher substrate concentration (500 μM) than a lower one (50 μM) [31]. This enzyme is constitutively expressed in the liver and is therefore more likely to contribute to the C-oxidation of nicotine in humans [47]. 5-10 % of Caucasians exhibit a defective gene that codes for this enzyme, which renders them as poor metabolisers of many xenobiotics (e.g. coumarin) [47]. CYPs 2E1, 2C19, 1A2, 2C8, 3A4, 2C9, and 1A1 were all reported to catalyse nicotine C-oxidation, but only at high substrate concentration (500 μM) [30]. However, there were no measurable activities for CYPs 1B1, 2C18, 3A5, and 4A11, even at 500 μM [30].

It is probable that the inter-individual variability of these enzymes, especially CYP2D6 [23,48], could have important implications for the ability of individuals to metabolise nicotine to cotinine [47].

1.6.2. Phase 1 Metabolism

Nicotine contains both aromatic and aliphatic carbon and nitrogen atoms within its structure, providing numerous sites for metabolic oxidation (phase 1) and conjugation

(phase 2) reactions and in some cases nicotine metabolites may also undergo reductive reactions.

Nicotine

The biotransformation of nicotine to cotinine in mammals is a two-step process, the first of which suggests the catalysis of nicotine to the intermediate compound nicotine-$\Delta^{1'(5')}$-iminium ion [49]. This may be brought about by the incorporation of oxygen at the 5′ position of nicotine, thereby converting the basic pyrrolidine moiety into a cyclic amide [49]. This initial metabolic reaction of nicotine may involve electron and hydrogen abstraction to produce the intermediate nicotine-$\Delta^{1'(5')}$-iminium ion, which reacts with water (which provides oxygen) to yield cotinine (figure 1.2). It has been shown that nicotine can form three isomeric iminium ions leading to additional metabolic products [50,51]. Thus the formation of cotinine appears more complex than was originally thought and may involve electron abstraction to form an aminium radical [52], which may be further oxidized to isomeric iminium ions. But even though electron abstraction may be carried out by a single cytochrome isozyme, it is possible that the oxidation of isomeric iminium ions may involve more than one isozyme. CYP2A6 appears to mediate the formation of nicotine-$\Delta^{1'(5')}$-iminium ion and is the same enzyme responsible for the 3′-hydroxylation of cotinine. (figure 1.2) [27].

In the second step, the cytosolic molybdenum containing aldehyde oxidase is thought to mediate the oxidation of nicotine-$\Delta^{1'(5')}$-iminium ion to yield cotinine (figure 1.2) [53]. However, it is less clear as to which enzymes are involved in the oxidation of nicotine-$\Delta^{1'(2')}$- and $\Delta^{1'(6')}$-iminium ions, although the latter process is known to yield the N-demethylated alkaloid, nornicotine [50].

Cotinine

Cotinine can be excreted unchanged in most species, but is vulnerable to oxidation at both carbon and nitrogen centres. Hydroxylation at the 3′- position yields both *cis* and *trans*-3′-hydroxycotinine, with the latter form predominating over the former. Thus the *trans* form represents the major urinary nicotine metabolite in humans [54]. Hydroxylation at the 5′-position may also give rise to 5′-hydroxycotinine [55]. This metabolite predominates over its open chain tautomer 4-(3-pyridyl)-4oxo-N-methylbutyramide [3]. Cotinine and 5′-hydroxycotinine are converted to the corresponding N-oxides by oxidation of the pyridyl nitrogen (refer to figure 1.2 for cotinine-N-oxide [3].

Cotinine may undergo N-demethylation to give norcotinine in various species, but no such activity has been observed in man. It has been reported that cotinine undergoes hydroxylation of the N-methyl group to form 1′-*N*-hydroxymethylnorcotinine. This metabolite is thought to give rise to norcotinine. However, 1′-*N*-hydroxymethylnorcotinine appears to be metabolically stable and does not give rise to norcotinine in vitro. It is possible that the major route to norcotinine might be by oxidation of nornicotine [3].

Norcotinine

Regardless of its route of formation, the metabolism of norcotinine has been observed, to some extent, prior to excretion, to yield 4-(3-pyridyl)-4-oxo-butyramide [56]. This metabolite is the open chain form of 5′-hydroxynorcotinine, a compound found in the urine of rats treated with (±)-nicotine [57] but not in the urine of rats treated with (-)-nicotine [22]. In human urine the corresponding dehydrated product, demethylcotinine-$\Delta^{4'(5')}$-enamine was found with a long half-life of excretion [58]. Norcotinine has been detected in

the urine of smokers and nicotine treated rats where it has shown to be present at approximately 1-2 % of the total nicotine metabolite content [3].

Nornicotine

The metabolism of nornicotine has been studied both in vitro [59] and in vivo [60]. The corresponding acid, 4-(3-pyridyl)-4-aminobutyric acid, has also been isolated from the urine of dogs treated with nornicotine [60]. It is also possible that during the course of this oxidation process a reactive intermediate, analogous to the $\Delta^{1'(3')}$-iminium ion in cotinine formation, is produced. Nornicotine may also be sequentially oxidised at the pyrrolidine nitrogen to give nornicotine $\Delta^{1'(2')}$-nitrone via the intermediate 1′-*N*-hydroxynornicotine [59].

Pyridylbutyric acid derivatives

Cotinine and norcotinine give rise to metabolites possessing a pyridyl moiety attached to a modified butyric acid residue [57]. 4-(3-Pyridyl)-4-hydroxybutyric acid has been detected in the urine of smokers, as 5 % of the total nicotine metabolites excreted as well as the urine of (S)-(-)-nicotine treated rats [61].

The presence of 3-pyridylacetic acid was detected in the urine of animals or man following cotinine, nicotine and 4-(3-pyridyl)-4-oxobutyric acid administrations. Thus 3-pyridylacetic acid is now generally accepted as an end product of nicotine phase 1 metabolism in mammalian systems (figure 1.2). It could possibly be derived from 4-(3-pyridyl)-4-hydroxybutyric acid by dehydration to 4-(3-pyridyl)-3-butenoic acid followed by reduction to 4-(3-pyridyl)-butyric acid and fatty acid degradation [3].

Oxidation at alicyclic nitrogen

The two nitrogen centres that nicotine possesses can be oxidised chemically to produce isomeric nicotine-N-oxides. However, in biological systems only the acyclic pyrrolidine is susceptible to metabolic oxidation (figure 1.2) [3]. Nicotine can be oxidised, at the 1′-N-position, at the 1′R- and 1′S- configuration, which leads to the formation of two diastereoisomers for each nicotine enantiomer [3]. Thus the natural S-(-)-nicotine is converted to 1′S, 1′R,2′S- and 1′S,2′S-nicotine-1′-N-oxide with the N-methyl group in the *cis*- and *trans*-position to the pyridine ring, respectively [3]. The *trans* stereoisomer is the major stereoisomer produced in man [62].

Oxidation is mediated by microsomal flavin containing monooxygenases (FMO). In humans it is carried out by FMO3 [62]. The reduction of nicotine-1′-N-oxide to nicotine occurs via the hepatic and extra hepatic systems and by the bacteria of the gastrointestinal tract in man [3].

1.6.3. Phase two metabolism of nicotine

Metabolites can be further metabolised by enzymatic transfer to a molecule or molecular substructure from endogenous compounds thus forming metabolites with higher molecular weights. These are often highly polar, water-soluble compounds and thus aid urinary excretion of foreign compounds and their phase 1 metabolites. The main enzymes involved are methyltransferases, glucuronosyltransferases, sulfotransferases, glutathione transferases, and acetyltransferases [3].

Methylation

From the urine of dogs Mckennis *et al.* [63] isolated a quarternary ammonium compound derived from nicotine. They identified this metabolite as N-methylnicotinium ion, methylated at the pyridine nitrogen. They also isolated, from dogs as well as from the urine of a non-smoking man who had orally received large quantities of S-cotinine, N-methylcotininium ion, which is also methylated at the pyridine nitrogen [3].

Metabolic N-methylation, in the guinea pig, involves a stereo-specific as well as regio-specific pathway for only the R-(+)-enantiomer of nicotine and only methylation at the pyridine nitrogen [3]. N-methylated nicotine metabolites account for 15 to 20 % of the total urinary metabolites in this species. N-methylnicotinium ion was found as a human nicotine metabolite in vivo in the urine of smokers as well as in vitro by incubation of either R- or S-nicotine with human liver cytosol [3].

Glucuronidation

Nicotine and cotinine are metabolised by N-glucuronidation to yield N-glucuronides of both compounds [41], whereas trans-3'-hydroxycotinine is metabolised by O-glucuronidation (figure 1.2) [3]. Studies have shown glucuronides to account for up to 40 % of the metabolites excreted in urine [3]. There appear to be ethnic differences in N-glucuronidation of nicotine and cotinine [41]. Benowitz *et al.* [41] found blacks to excrete significantly less nicotine as nicotine-N-glucuronide and less cotinine as cotinine-N-glucuronide than whites, but there was no difference in the excretion of trans-3'-hydroxycotinine-O-glucuronide. In this study glucuronidation appeared to be polymorphic, with evidence of slow and fast N-glucuronide formers among blacks but unimodal

behaviour predominating with fast conjugators among whites [41]; and high inter-individual variation in the urinary metabolite pattern has been observed [64]. Slower cotinine metabolism in blacks could be due to both slower oxidative metabolism (via CYP2A6) and slower N-glucuronidation [41].

Thus far N-glucuronides of *cis* and *trans*-3'-hydroxycotinines have not been found in the urine of smokers, even though trans-3'-hydroxycotinine-O- β-D-glucuronide was identified by both mass spectrometry and NMR, in urine. However, glucuronides of nicotine and cotinine have been isolated from smokers' urine [65].

Cleavage of glucuronides may be obtained by the use of glucuronidase. UDP-glucuronidetransferases, of which there are at least two families, comprising at least forty isoenzymes, may catalyse glucuronide formation [66]. The isozymes, which are involved in N-glucuronidation of nicotine and cotinine and O-glucuronidation of trans-3-hydroxycotinine have yet to be identified. High inter-individual variation in glucuronide synthesis and the observation that the extent of conjugation of nicotine and cotinine is highly correlated, but that neither is correlated with the conjugation of trans-3-hydroxycotinine, suggests that the same UGT isozyme may be involved in the formation of N-glucuronides of nicotine and cotinine [64]. However, the formation of nicotine-N-glucuronide, but not cotinine-N-glucuronide, has been observed in marmoset microsomal preparations [67], indicating that nicotine and cotinine are not common substrates of a single isozyme.

1.7. Metabolite distribution

Nicotine enters the body through the lungs, and subsequently the blood stream. It is then circulated to various body organs, including the liver and kidneys. It is in the liver where major metabolism occurs, after which a small percentage, usually 5-10 percent, of nicotine is excreted unchanged into the urine [64]. Approximately 70-80 % of nicotine is converted to cotinine, of which only 10-15 % is excreted. The remainder of cotinine is converted to other metabolites, particularly cotinine glucuronide, trans-3'-hydroxycotinine and trans-3'-hydroxycotinine glucuronide (refer to figure 1.3 and table1.3).

Figure 1.3. Quantitative scheme of nicotine metabolism. Circled compounds indicate excretion in urine and associated numbers indicate percent of systemic dose of nicotine. Adapted from Benowitz *et al.* [64].

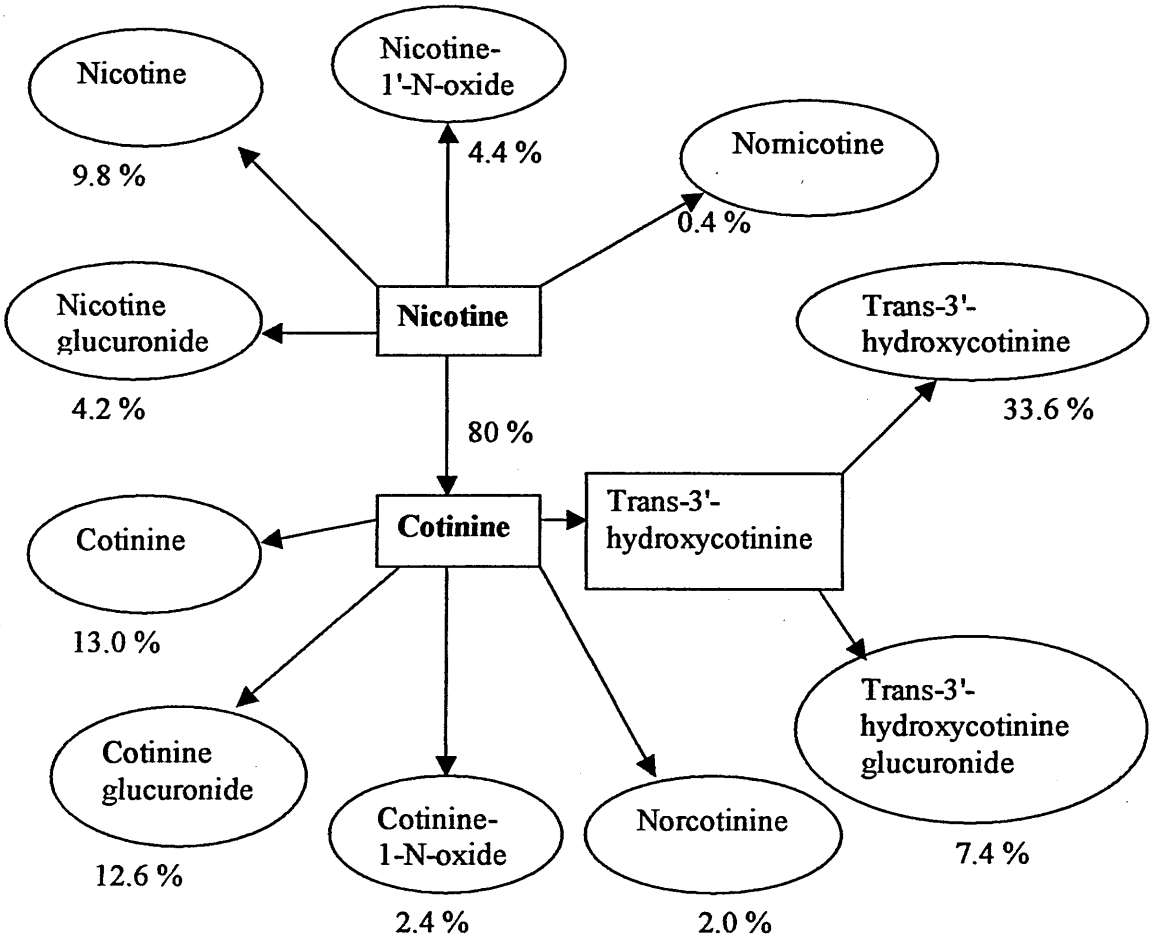


Table 1.3. Distribution of urinary nicotine metabolites as percentage of the total amount of metabolites excreted during 24 hour (average and standard deviation).

Nicotine Metabolites	Smokers[a] (n = 91)	Smokers[b] (n = 12)	Smokeless tobacco users (n = 54)	Transdermal nicotine (n = 12)
Nicotine	9.4(5.7)	10.4(4.4)	8.3(5.7)	11.1(4.3)
Nicotine-GlcA	4.5(2.5)	4.6(2.9)	3.0(1.8)	5.3(3.3)
Cotinine	9.2(2.6)	13.3(3.1)	7.9(2.2)	14.9(4.6)
Cotinine-GlcA	14.0(5.4)	15.8(7.8)	8.9(4.6)	15.4(7.9)
3HC	36.1(10.6)	39.1(12.5)	41.6(10.6)	37.0(10.8)
3HC-GlcA	22.8(10.0)	7.8(5.9)	19.4(11.0)	7.9(4.7)
NNO	3.0(2.1)	3.7(0.9)	8.6(6.9)	2.7(1.2)
CNO	0.9(0.9)	4.5(1.5)	2.5(2.3)	5.2(1.5)
Nornicotine		0.65(0.15)		0.41(0.12)

Table 1.3 was adapted from Gorrod and Jacob III [3].
GlcA – glucuronide
NNO – Nicotine-1'-N-oxides
CNO – Cotinine-1-N-oxide
3HC – Trans-3'-hydroxycotinine
Superscripts a and b denote results obtained from two different studies.

Since similar excretion profiles were found in smokers, smokeless tobacco users and users of transdermal nicotine, it seems likely that the metabolism of nicotine is independent of route of administration (table 1.3). However, these profiles of nicotine metabolism have not included all metabolites. For example, it is known that 5'-hydroxycotinine is a metabolite of cotinine. But to what extent is cotinine metabolised to 5'-hydroxycotinine? This is one of the many questions which need to be addressed [3].

1.8. Health effects associated with smoking

In England an estimated 284,000 patients are admitted to NHS hospitals each year due to disease caused by smoking, occupying an average of 9,500 hospital beds every day, with smoking related illness accounting for 8 million consultations with GPs and over 7 million prescriptions each year [68].

It is estimated that half of all teenagers who are currently smoking will die from diseases caused by tobacco if they continue to smoke (refer to figure 1.4 for health effects associated with tobacco). One quarter will die after 70 years of age and one quarter before, with those dying before 70 losing on average 23 years of life. It is estimated that between 1950 and 2000 six million Britons, 60 million people worldwide, would have died from tobacco-related diseases [68].

Nicotine poisoning can cause increased salivation, nausea, vomiting, and cardiac palpitation leading to an increase in blood pressure [69]. Nicotine poisoning involves central and peripheral effects of the nervous system (refer to table 1.5). Rapid light-headedness, skeletal muscle weakness, prostration, tremor and convulsions are also symptomatic of this poisoning [3]. Nicotine may also be associated with cellular stress (apoptosis) [70] and cardiovascular disease [71]. Metabolites of nicotine have also been reported to be detrimental to the human body [72]. However, nicotine may in turn play a part in the beneficial and protective mechanisms against some neurodegenerative diseases [73].

As previously mentioned, nicotine is only one of many toxic compounds in cigarette smoke. It is the addictive nature of nicotine that exacerbates the toxicities of the other components of cigarette smoke. The Royal College of Physicians has affirmed that the way in which nicotine causes addiction is similar to drugs such as heroin and cocaine [74].

Table1.4. Health effects associated with tobacco smoke (adapted from ASH fact sheet number 2, www.ash.org, 2003) [68].

Increased risk for smokers	
Acute necrotizing ulcerative gingivitis (gum disease)	Muscle injuries
Angina (20 x risk)	Neck pain
Back pain	Nystagmus (abnormal eye movements)
Buerger's Disease (severe circulatory disease)	Ocular Histoplasmosis (fungal eye infection)
Duodenal ulcer	Osteoporosis (in both sexes)
Cataract (2 x risk)	Osteoarthritis
Cataract, posterior subcapsular (3 x risk)	Penis (inability to have an erection)
Colon Polyps	Peripheral vascular disease
Crohn's Disease (chronic inflamed bowel)	Pneumonia
Depression	Psoriasis (2 x risk)
Diabetes (Type 2, non-insulin dependent)	Skin wrinkling (2 x risk)
Hearing loss	Stomach ulcer
Influenza	Rheumatoid arthritis (for heavy smokers)
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 (eyes, 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 the 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 (eyes)	Optic Neuritis (eyes)
Disease more severe or persistent in smokers	
Common cold	Pneumonia
Crohn's Disease (chronic inflamed bowel)	Tuberculosis
Influenza	

Lung cancer

Lung cancer can be brought about by exposing the bronchial epithelium to environmental carcinogenic compounds of cigarette smoke [75]. Thus the inhalation of cigarette smoke together with its harmful byproducts such as benzopyrene will act directly on the bronchial tree and the alveoli leading to metaplasia of the bronchial epithelium from a columnar pattern to a squamous one and hence dysplasia and carcinoma formation in situ. Each time a smoker inhales, the carcinogenic chemicals released touch the inside of the lungs (the bronchus, bronchioles and alveoli) leading to the mutation of epithelial cells which keep on dividing and eventually form a cancer [76,77]. The disease can spread very quickly to other parts of the body and therefore surgery or radiation therapy, targeting localized disease, may be relatively ineffective in forestalling mortality. It is usually treated with a combination of chemotherapy drugs, and if it has not spread beyond the lung at the time of diagnosis, a combination of surgery and chemotherapy may be used [78].

Lung cancer is reported to be three times more prevalent in men than in women [79] and is the most common cancer in men [80]. Whilst it is on the increase in females it is now decreasing in men due to changes in smoking habits in recent decades [81,82], and by the early 1990s was said to have displaced coronary heart disease as the leading cause of death among smokers in the US [83]. It has been observed in various countries that regional variation in lung cancer mortality is difficult to explain by differences in current smoking, and in order to explain lung cancer death rates fully, one has to go further back in time [84].

Approximately 3 million people each year die from smoking in developed countries, half of them before 70 years of age [85], which in 40 years time may rise to 10 million [79]. There is a strong relationship between smoking and lung cancer, with about 90 % of cases may be attributed to tobacco usage [76]. But despite the overwhelming evidence for linking

lung cancer to smoking, there are other environmental and /or genetic risk factors that may be associated with the development of this disease [86].

All cancers

Cigarette smoking is the predominant cause of lung, laryngeal, pharyngeal, oesophageal, bladder [87,88], kidney, and pancreatic cancer [89]. Heavy smokers are at risk of up to 30 percent cumulative lifetime risk of developing cancer, compared to 1 percent of non-smokers [90]. A study by Schnoll *et al.* [91] on cancer patients, revealed that continued smoking and a reluctance to quit were associated with having relatives at home who smoke, a longer time between diagnosis and assessment, completion of medical treatment, greater nicotine dependence, and emotional distress.

1.9. Smoking and pregnancy

Approximately one-third of pregnant women in England smoke, and the majority of women who smoke during pregnancy are likely to be young, single, and of low social standing. The male partner is also likely to smoke. Only one in four women who smoke succeed in stopping at some time during pregnancy, and almost two-thirds of women who succeed in stopping smoking in pregnancy restart again after the birth of their baby [92].

Babies born to women who smoke are on average 200 g (8 oz) lighter than babies born to comparable non-smoking mothers. Furthermore, the more cigarettes a woman smokes during pregnancy, the greater the probable reduction in birth weight. Recent research suggests that cigarettes can reduce the flow of blood in the placenta, which limits the amount of nutrients that reach the foetus [93,94]. Low birth weight is associated with higher risks of death and disease in infancy and early childhood. Since adverse effects of smoking in pregnancy are

due mainly to smoking in the second and third trimesters, if a woman stops smoking within the first three months of pregnancy, her risk of having a low-weight baby is dramatically reduced [93].

The rate of spontaneous abortion (miscarriage) is substantially higher in women who smoke. On average, smokers may experience more complications of pregnancy such as premature detachment of the placenta, bleeding during pregnancy, and premature rupture of the membranes; there may also be a link between smoking and ectopic pregnancy and congenital defects in the offspring of smokers [92].

Exposure of the mother to passive smoking has also been associated with lower birth weight, a higher risk of perinatal mortality (still birth) and spontaneous abortion. Still-birth is increased by about one-third in babies of smokers. More than one-quarter of the risk of death due to Sudden Infant Death Syndrome (cot death) is attributable to maternal smoking (equivalent to 365 deaths per year in England and Wales) [92].

Smoking during pregnancy may also have implications for the long-term physical growth and intellectual development of the child. It has been associated with a reduced height of children of smoking mothers as compared with non-smoking mothers. There is also evidence that smoking interferes with women's hormonal balance during pregnancy and that this may have long-term consequences on the reproductive organs of her children [92,95].

It has been known for some time that the offspring of women who smoke during pregnancy are likely to have abnormal lung function and associated higher incidences of lower respiratory disorders [96-99]. The identification of nicotinic acetylcholine receptors (nAChR) in foetal monkey lung suggests that a direct interaction between nicotine and nAChR in foetal lung may predispose such infants to postnatal pulmonary abnormalities [100].

Fukuda *et al.* [101] 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 non-smoking couples. It was 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 [102] suggest that it may be possible for genotoxic and carcinogenic components of tobacco smoke to pass through to the placenta and hence the developing foetus. The exposure of BeWo cells (a human trophoblast-like cell line) to benzo(a)pyrene, from cigarette smoke, has been associated with placental toxicity [103].

Animal models have also implicated both nicotine and cotinine in Sudden Infant Death Syndrome [104].

1.10. Tobacco policy

The control of lung cancer has been a goal of policies over the years since the 1960s. Over time, nicotine replacement therapy (NRT), the development of counter-advertising and the introduction of tobacco taxes for the promotion of health has become a part of these policies. Many countries were unable to legislate comprehensively but nevertheless achieved substantial effects from enthusiastic and active public campaigns. This is most evident in the decline in male lung cancer observed in the UK. However, for reasons which can be largely attributed to specific advertising targeting women, the decline in male rates has been accompanied by an almost universal increase in smoking by women, and

predictably an increase in the number of female deaths by lung cancer. By 2002, lung cancer exceeded breast cancer as a cause of death in women [81].

It was therefore logical for governing bodies to implement the policy of reducing tar and nicotine yields, since there was a dose response between cigarette dose and cancer risk as well as a reduction of risk with cessation. However, changes in the qualitative nature of tar led to increased levels of nitrate (e.g. nitrosamines) in tobaccos and a downward trend in benzo(a)pyrene. All this meant was that a decline in squamous cell carcinoma, and an increase in adenocarcinoma were observed. Ventilation filters were also introduced, but to no avail (as previously described). Nevertheless, the reduction of yields has some beneficial effect, but has not delivered the substantial benefits that were expected [81].

An increase in the amount of nicotine was meant to decrease compensatory smoking, but did nothing to reduce nicotine addiction. Higher levels of nicotine should have reduced inhalation of carcinogens and toxins, but would have been very difficult to sell publicly. A reduction in the amount of nicotine should have reduced the addictiveness of cigarettes, but would have increased compensatory smoking [81].

A review, which appeared in the 20 May (1998) issue of *New Scientist* [106] revealed that "in 106 papers published" the major factor associated with concluding that passive smoking is not harmful was whether an author was affiliated with the tobacco industry. Therefore it can be said that the major problem faced with trying to link smoking with lung cancer is not the lack of evidence, but is the power of the major tobacco companies to influence research. If these companies recognize the relationship between smoking and lung cancer (or any other cancer or disease for that matter) they will have to pay compensation to nonsmokers who have developed the disease and bans on smoking in public places will be enforced, both of which will cause severe losses to businesses [105]. However, the influence of the

tobacco industry, together with its credibility, is decreasing, probably as a result of numerous large compensation payouts to families in the U.S. [81].

Today nicotine replacement therapies (NRTs), such as patches and nasal sprays, help ease the withdrawal symptoms many smokers experience when they try to quit and studies have shown these products to be useful to those smokers who are have a strong nicotine addiction. Smoking in the UK is now less socially acceptable than ever, with the number of adult nonsmokers outnumbering those who do by approximately two to one. Long-term policy should include a reduction in the attractiveness of the cigarette and as much replacement as possible by clean nicotine products (implying the control of additives and demonstration of safety in both burnt and unburnt form) [81].

A cessation policy should involve the use of psychological and therapeutic support [106]. Unfortunately not all G.P.s are forthcoming with advice. The health service should do more to promote NRTs, as such products are widely available in most countries. In Europe, of the 40-80 % of patients who want to quit, only one-third are advised by their doctor, with slightly greater than 10 % given advice as to how to go about it. Despite this set back, the epidemic of tobacco-induced mortality is on the decrease in some developed countries, but is on the increase in the developing world (where NRTs are too expensive for consumers) [81].

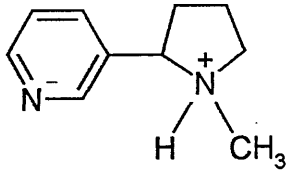
1.11. Pharmacology

Nicotine distributes rapidly into many tissues of the body including foetal tissues as shown by studies with pregnant mice [3]. Nicotine initially concentrates in the brain and spinal cord and within 15 minutes its concentration in the liver increases. Within 30 minutes of injection the majority of nicotine is in the liver, the adrenals, the stomach and bone marrow, but only a small amount is left in the central nervous system [3].

Nicotine is reported to have some similar actions to acetylcholine, as well as parasympathetic and sympathetic nerve stimulation. Acetylcholine, a neurotransmitter, affects both muscarinic and nicotinic receptors. The charge similarities of nicotine and acetylcholine may account for their similar actions. The pyridino N of nicotine is an electron donor that is similar to the keto oxygen of the acetyl group of acetylcholine, and the positive charge of the quarternary nitrogen of acetylcholine is similar to the positive charge of the pyrrolidino nitrogen (refer to structures in figure 1.4) [3].

Figure 1.4. Charge similarity of nicotine and acetylcholine [3].

Nicotine



Acetylcholine

There are at least five muscarinic receptor subtypes (M_1-M_5) with transduction mechanisms to alter second messengers. Specific genes for each of the five subunits of several nicotinic cholinergic receptors (nAChRs), which encode a variety of protein subunits similar to the skeletal muscle receptor, have been isolated [3].

Since (S)-nicotine is usually more potent than (R)-nicotine it is most probable that nAChRs have stereo-selective preference. However, nAChRs do not appear to be the main targets of cotinine, the major metabolite of nicotine [107]. The fact that all major organs of the body possess nAChRs makes it possible for nicotine to affect all of these. As multiple ligand-gated ion channels, these receptors are very complex, involving many presynaptic and postsynaptic sites, especially in the central nervous system. An influx of Ca^{2+} and Na^{+}, intracellularly, provides the ionic basis for the release of multiple chemical messengers upon receptor depolarisation. But repeated nicotine exposure leads to a rapidly diminishing receptor response and to eventual receptor desensitisation (tachyphylaxis), which could probably lead to receptor desensitisation to acetylcholine. This may account for the mixed stimulant/ depressant actions of nicotine [3].

Behavioural and neurochemical effects of nicotine via the mesolimbic dopaminergic system have been documented by Balfour and Benwell [108]. The pharmacological antagonism of dopaminergic receptors results in the blockade of locomotor stimulant and reinforcing effects of nicotine [109,110].

Table 1.5. Dose-dependent pharmacological actions of nicotine (adapted from Gorrod and Jacob III, 1999) [3].

Pharmacological sites	Pharmacological actions of nicotine
Central nervous system	Stimulation of brain stem- arousel or wake-up effect. +ve cognitive effects increasing memory, teaching behaviour etc. Relaxation. Mixed stimulant/ depressant actions. Increased orienting response. Improved gating of relevant stimuli. Decreased monosynaptic reflexes (e.g. patellar reflex). Reinforcing self administration behaviour. Dependence, addiction, withdrawal. Stimulation of chemoreceptor trigger zones- nausea and vomiting. Tremor, convulsions and death.
Peripheral nervous system	Stimulation and blockade of sympathetic and parasympathetic autonomic ganglia. Peripheral afferent stimulant involving taste and smell. Aortic and carotid body chemoreceptor stimulation. Stimulation of gastrointestinal, pulmonary and skeletal muscle spindle afferents.
Cardiovascular system	These include: Low concentrations- increased heart rate. High concentrations- decreased heart rate (poisoning). Increase in systolic and diastolic blood pressure. Cutaneous vasoconstriction.
Therapeutic potential of nicotine	Treatment of tobacco addiction and dependence. Parkinsons disease. Alzheimer's disease. Schizophrenia.

Nicotine may also affect the gastrointestinal system, the endocrine system, and hepatic and renal systems and lead to a decrease in body weight, an increase in energy expenditure and decrease energy consumption [3].

Since many of the metabolites of nicotine are structurally similar it is logical that some would also be pharmacologically active. Currently the metabolites of nicotine thought to be pharmacologically active are cotinine (to a much lesser extent) and nornicotine. The former was reported to oppose the effects of nicotine on the cardiovascular system, but its effects were not blocked by antagonists of nicotinic, muscarinic, histaminic or adrenergic receptors [107]. The latter was reported to be behaviourally active, since it has locomotor stimulant and reinforcing effects similar to that of nicotine in rats [111]. Nornicotine, like nicotine, desensitises nicotinic receptors, but with approximately 12-fold lower potency. It is able to bring about cross-desensitization, suggesting the involvement of common nicotinic receptor subtypes [111]. Additionally nornicotine was found to increase synthesis and metabolism of dopamine, providing evidence that it plays a contributory role in tobacco dependence [112]. This is entirely reasonable as nornicotine has a longer half-life than nicotine in plasma and brain [113] and therefore reaches a three-fold higher concentration in brain and plasma than nicotine [114].

1.12. Nicotine in biological samples

Most biological materials are complex mixtures consisting of many different components. These may include proteins, salts, lipids, nucleic acids, etc [3]. Most of these constituents of biological matrices may compromise the analytical procedure [3]. Blood or plasma levels are important in pharmacokinetic/ pharmacodynamic studies, since they are likely to correlate with concentrations at the site of action [115]. The longer half-life of cotinine ensures that its concentration in blood is greater and more stable throughout the day than that of nicotine [115]. Cotinine concentration, whether measured in plasma, saliva or urine has been shown to be the best indicator of smoking status [3].

The most widely used biological materials for nicotine and cotinine measurements are whole blood, plasma (or serum), and urine [3]. Other biological materials which have been studied include saliva, cerebrospinal fluid, bile, sweat, breast milk, hair, and seminal plasma. Cerebrospinal fluid is one of the least problematic fluids to analyse while whole blood and tissue are the most difficult [3]. Saliva and urine samples are utilised most frequently in tobacco cessation studies and epidemiological studies, due to their less invasive nature. Generally concentrations of nicotine and cotinine are greater in urine than in blood, plasma or oral fluid [17,115].

Whole blood

Blood is made up of a complex mixture of proteins, dissolved fats and solids, and suspended cells, the centrifugation (at low speeds) of which aids removal of red blood cells

and leaves a clear solution, the plasma. Lysing of red blood cells may prove hazardous to most experiments, since components released could interfere with analysis. Generally drugs or metabolites are not extracted from whole blood, but from plasma and serum [3].

Plasma and Serum

Drugs and metabolites present in both serum and plasma can bind to proteins in these samples, and by doing so they may compromise the quality of data produced, especially when considering HPLC and capillary electrophoresis. Thus numerous methodologies for the removal of protein from the sample and the release of protein-bound drugs have been developed. These include precipitation by heating or freeze-thawing cycles, the denaturation and precipitation of protein, solvent extraction, and solid phase extraction (SPE) [116].

Urine

Urine is relatively free from proteins and lipids and can usually be extracted with organic solvents, or analysed directly. Its composition is largely dependent upon diet and diuresis. Extraction of lipid-soluble drugs from urine can yield an extract which is relatively free from potentially interfering endogenous components, as such components in urine are water-soluble [3]. Urine that is allowed to stand, for relatively long periods of time, slowly loses its carbon dioxide content and becomes more alkaline due to bacterial conversion of urea to ammonia [3]. The alkaline conditions result in the precipitation of inorganic salts such as phosphates, and possible co-precipitation, or degradation of analytes. One of the most popular and simplest methods for analyte extraction from urine is SPE. To date there are various examples of SPE carried out on the urine of smokers [116,117].

Urine analysis may also be affected by temperature. Hagan *et al.* [118] found that as storage temperature increased, cotinine levels in urine also dramatically increased, possibly due to hydrolysis of cotinine-N-glucuronide. This would have an adverse effect on analysis especially when trying to quantify the amount of conjugated or unconjugated cotinine present in urine. That is, a false positive could be achieved in many cases.

Oral fluid

Oral fluid, like urine, is relatively free from interfering substances. Even though it is a relatively viscous material, saliva can be easily extracted with organic solvents, and can be analysed directly (especially when considering immunoassays). But direct introduction into a HPLC or GC column or a fused silica capillary may prove difficult because of the viscous nature of this matrix. For some drugs, levels of drug in saliva are thought to reflect the concentration of non-protein bound analytes in the blood (e.g. non-ionised drugs such as steroids readily pass from plasma to saliva). Analysis of nicotine and its metabolites has been reported after a simple centrifugation step followed by dilution with water. However, drugs may bind to mucoid proteins in saliva. The pH of saliva is approximately 7.0 to 7.8 and any change in pH will affect the distribution of drugs in saliva, especially those bound to proteins. Thus it is imperative that the investigator be made aware of the pH prior to analysis [3].

Cerebrospinal fluid and Bile

Analysis of cerebrospinal fluids is important for centrally acting drugs, since it provides concentrations of the drugs and their metabolites in the vicinity of the receptor and/or enzyme active sites responsible for the drug's pharmacological properties. Even though

cerebrospinal fluids can be difficult to obtain, extraction of analytes from such fluids may be brought about by the same extraction procedures for serum and plasma. Bile, like cerebrospinal fluids, is difficult to obtain but techniques that have been developed for the analysis of drugs in plasma and serum are generally applicable to this type of matrix [3].

Breast milk

As for breast milk, the major problem is the presence of large amounts of fat (approximately 4.5 % in mature human breast milk). Treatment of this fluid with lipase prior to HPLC analysis has been reported for some drug analyses, but generally a fat removal step (e.g. washing 0.5 mL breast milk, at pH 3.0-4.0, with 10 mL *n*-hexane) is recommended. The importance of breast milk analysis is realised when one needs to determine if drug molecules can be transferred from mother to infant via this route [3].

Hair

Analyses of scalp hair from smokers have also revealed that inhaled nicotine becomes fairly concentrated in the hair shaft (an average of 15.5 μg/ mL in smokers) [3]. An average concentration of 2.42 μg/ mL of nicotine, from the scalp hair of 10 non-smokers, suggests that the use of this biological material would be ideal for the analysis of environmental smoke exposure. However, contamination of the exterior of the hair shaft by environmentally derived nicotine and various hair treatments could compromise the analysis [3]. A further draw back is the harsh extraction procedures that are required for hair analysis [119].

1.13. Analytical methodology for the determination of nicotine and its metabolites

Numerous methods have been used for the analysis of nicotine and its metabolites. These range from highly sensitive and accurate mass spectrometric methods to qualitative or semi-quantitative colorimetric methods. In general the more sensitive and accurate methods are less available and more costly. New instrumentation suitable for GC/MS determination of nicotine and cotinine is likely to cost about £ 31,000. State of the art mass spectrometers can cost in excess of £ 125,000 [115].

Gas chromatography coupled to mass spectrometric detection (GC/MS) is well suited to nicotine and cotinine determination and has proved to be very sensitive and accurate. Nicotine and cotinine are readily detected by gas chromatography coupled with nitrogen-phosphorus detection (GC/NPD), with excellent precision and accuracy (and instrumentation can cost up to $ 15,000) [115].

A polarisation fluoroimmunoassay (carried out on the Abbot TDx analyser) was able to discriminate active smokers from non-smokers (including passive smokers) by applying a cut-off at 0.5 mg/ L of total urinary cotinine [120]. Radio-immunoassays (RIA) and enzyme-linked immunosorbent assays (ELISA) have frequently been used for measuring nicotine and cotinine in plasma with good precision and accuracy. GC, HPLC and immunoassay methods used for the determination of nicotine and cotinine, in plasma, are usually applicable to urine. Colorimetric methods for measuring urine levels of nicotine, cotinine, and their metabolites without distinguishing individual compounds have also been developed [115].

Chromatographic methods (GC and HPLC), especially when coupled with mass spectrometry, usually provide great sensitivity and specificity. However, they are generally

the most costly due to the expensive instrumentation, the need for computational interpretation of results, and highly trained personnel. Chromatographic methods have been successfully applied to pharmacokinetic studies, in which accurate measurement of low levels of nicotine and its metabolites are needed to determine half-lives [115].

Immunoassays

Immunoassays are fast and economical for large numbers of samples and in most cases have good sensitivity, accuracy, and precision. Antibodies to nicotine and its metabolites can be coupled to an enzyme, which reacts with a substrate to yield a coloured product (as in the case of ELISA) which is measured spectrophotometrically [121,122] or is radiolabelled (as in RIA) and read by a radiometric detectors [123-125].

Benkirane *et al.* [126] observed similar detection limits for both ELISA and RIA (0.1 μg/ mL of cotinine). For the analysis of serum and saliva, non-smoker values ranged from 0.1 to 17 μg/ L by ELISA and 0.1 to 27.5 μg/ L by RIA, whereas smoker values ranged from 50 to 1000 μg/ L (ELISA) and from 70 to 800 μg/ L (RIA) [126]. Comparative analysis of 96 human sera revealed a good correlation between ELISA and RIA methods [126].

When ELISA results were compared with those obtained by GC, a correlation coefficient $r = 0.886$ was obtained [122]. For samples containing > 50 ng/ mL of cotinine, RIA gave results on average 60 % higher than GC [127]. This was thought to be due to RIA cross-reactivity of cotinine antibodies with other nicotine metabolites [127]. However, samples containing < 7 ng/ mL of cotinine gave no significant correlation [127].

Thus a major disadvantage of immunoassays is the possibility of interference by endogenous compounds or other drugs. It is possible for compounds with similar structures to interfere with immunoassays due to their having significant affinity to the antibody.

Schepers and Walk [121] found cotinine metabolites to cross-react with antibodies used in certain immunoassays, which led to higher values than expected, when compared to chromatographic methods [128,129]. However, cross-reactivity only poses a problem if quantitative information about a particular substance is required (as in pharmacokinetic studies), but not if the assay is being used for verification of smoking status.

Colorimetric methods

Colorimetric methods for estimating nicotine and nicotine metabolite levels have been reported [115]. The speed of analysis and simplicity and inexpensiveness of the equipment used make this an attractive alternative to the chromatographic and immunoassay methods. In most cases a crude sample, such as urine, is used, which means no sample clean up is required [130-133]. The general principle behind these methods is the reaction of the pyridine ring of nicotine and its metabolites, with reagents that generate a coloured product that is measured spectrophotometrically. Probably the best known of these methods is the König reaction, in which the pyridine ring is cleaved with cyanogen bromide or chloride and the resulting compound is condensed with an aromatic amine [131-133]. Recently Chambers *et al.* [134] coupled this reaction mechanism to HPLC.

Pickert *et al.* [135] achieved a limit of detection of 0.5 mg/ L for cotinine via the König reaction. However, in the same study a fluorescence polarization immunoassay achieved even lower limits of detection for cotinine (0.05 mg/ L) [135]. Quantitative methods, such as the NicCheck I®, use test strips that develop a particular colour in the presence of nicotine and its metabolites, and are commercially available [131,133].

Like immunoassays, sources of interference can compromise the effectiveness of colorimetric methods [115]. Drugs or endogenous substances having a pyridine ring are a

potential source of interference [115]. Thus a false positive can be obtained from the urine of persons consuming large amounts of, the pyridine-containing compound, niacin [115].

Gas chromatography

Gas chromatography (GC) separates substances that are volatile enough to be vaporised. It consists of an injection system for introducing the sample, a column in which the separation occurs, a carrier gas (usually helium), and a detector. Separation occurs by partitioning of the substances in a mixture between the vapour phase moving through the system and the stationary phase. Compounds that are less volatile and/or have greater affinity for the column material move more slowly, bringing about separation [3].

In 1966 Beckett and Triggs [136] used GC for the determination of nicotine and cotinine in urine. Since then there have been numerous studies for the determination of nicotine and its metabolites in urine [137-139]. GC has also been used for the analysis of nicotine and its metabolites in blood [3], plasma [137,140-142], saliva [137], breast milk [142], and hair [143,144].

Hariharan and Van Noord [139] used a GC coupled to a nitrogen-specific detector (NSD), for the determination of nicotine and cotinine in urine from passive smokers. In this study the correlation coefficient for nicotine and cotinine, between GC/NSD and HPLC/UV, were 0.934 and 0.987 respectively. Limits of detection for nicotine by GC/NSD have been observed well below 1 ng/ mL [139,142]. GC coupled to a nitrogen-phosphorus detector has also been used to determine the amount of nicotine in hair from environmental tobacco smoke [143]. The NPD has similar detection limits to the NSD [140,141]. Other detectors used for GC analysis of nicotine include UV and flame ionisation [136,137]. The suitability

of the former has been questioned since it brings about the decay of nicotine (which is light sensitive).

Mass spectral analysis of nicotine and its metabolites is becoming increasingly popular. One of the earliest examples of mass spectrometric work done on nicotine and its metabolites was performed by Horning *et al.* in the early 1970s [3]. Here the mass spectra of nicotine and its major metabolites, cotinine and trans-3'-hydroxycotinine, obtained from extracts of smokers' urine, were recorded. The volatile nature of compounds separated by GC meant that this separation technique was initially one of the most attractive and simplest ways of introducing samples into the mass spectrometer. It is for this reason that sample introduction to MS by GC has been widely employed for over thirty years. The low carrier gas flow rates required by capillary GC columns and the development of more efficient mass spectrometer pumping systems have allowed the direct placement of the outlet end of the column in the ionisation source with an improvement in ionisation efficiency [3].

GC/MS has been widely used for the determination of nicotine and up to nine of its metabolites. In the late 1970s Dow and Hall used glass capillaries for the development of a GC/MS method for the estimation of nicotine and obtained good resolution, sensitivity, and efficiency for both nicotine (extracted from plasma) and quinoline [3]. Nowadays capillary GC columns are utilised in most GC applications including the analysis of nicotine and its metabolites [18,146]. The advancements in computer-controlled operation of entire systems and the development of the quadrupole mass analyser, for rapid scanning, have ensured the storage of vast amounts of data produced by full scanning GC/MS. This has allowed highly sensitive GC/MS applications to become ever-present in the majority of analytical laboratories.

The limit of detection of nicotine in biological fluids, by GC/MS, has been found at picogram levels [18]. The low limits of detection achieved have allowed GC/MS to be used for monitoring the extent of environmental tobacco smoke via urine analysis of cotinine [147] and to estimate the amount of nicotine in indoor air as an indicator of passive smoking [148].

High-pressure liquid chromatography

Partition chromatography is sub-divided into liquid-liquid or bonded phase chromatography. When using the bonded phase, the stationary phase is chemically bonded to support surfaces, which may be silica or silica based compositions. Liquid chromatography requires the sample to be dissolved in a solvent. High pressure is essential for driving the mobile phase through the tightly packed stationary phase within the column [149]. In reversed phase chromatography, the stationary phase is non-polar, often a hydrocarbon, and the mobile phase is relatively polar (such as water, methanol, or acetonitrile). Elution can either be performed isocratically (with a single solvent or a constant solvent mixture) or under a gradient (from one solvent/mixture to another) [150]. Chromatography of this type usually shows the most polar molecules eluting first. By increasing the polarity of the mobile phase, the elution time, for a particular compound, is also increased [151]. Reversed phase is currently the most popular of bonded phase packings. The most common R group of the siloxane in these coatings is a C_8 (*n*-octyl) or C_{18} (*n*-octadecyl) chain. Differences in analyte elution times are dependent upon their hydrophobic interactions with the bonded reversed phase.

When using a reversed phase HPLC method for nicotine and its metabolites with a gradient that runs from a mostly aqueous to mostly non-aqueous composition, metabolites

such as glucuronides and N-oxides should elute before the parent compound. Separation of nicotine and its metabolites has been developed using reversed phase [152], and cation exchange chromatography [153-155].

A high-pressure liquid chromatographic (HPLC) method for the analysis of nicotine and cotinine in smokers' urine was first reported by Watson in 1977 [156]. This study utilised a normal phase isocratic method with UV detection. The method had shorter analysis time (with both nicotine and cotinine eluting in under 5 minutes) when compared to the existing gas chromatographic methods of the day. Later on normal-phase HPLC/UV achieved detection limits for nicotine and cotinine of 2 ng/mL [157].

In the 1980s the use of reversed phase chromatography for the analysis of nicotine and other tobacco alkaloids became widespread [158]. Since the 1980s reversed phase HPLC/UV has been used in numerous studies for the determination of nicotine [159,160] and cotinine [160-163] in urine. Quantification of nicotine, cotinine and nornicotine in rat plasma and urine, by HPLC/UV, has also been described [163].

Recently, Nakajima *et al.* [164] developed a highly sensitive method for the determination of nicotine and cotinine in human plasma by HPLC/UV. This method showed detection limits of 1 ng/mL, which was reportedly enough to carry out pharmacokinetic studies after smoking one cigarette or chewing a piece of nicotine gum. Data obtained from this method showed good precision and accuracy. Nakajima *et al.* [165] also developed a HPLC/UV method for the determination of N-glucuronidation of nicotine and cotinine in human liver microsomes. This was the first report of the in vitro determination of cotinine-glucuronide and nicotine-glucuronide in liver microsomes and would most probably form the basis of any future attempts to determine nicotine-

glucuronide and cotinine-glucuronide in biological specimens. HPLC/UV has also been used for the analysis of nicotine in medicated chewing gums [166].

Other popular detection methods for HPLC analysis of nicotine include diode array, electrochemical detection, radiometric detection, colorimetric assay, and mass spectrometry. Diode array coupled to reverse phase HPLC has been used for the analysis of nicotine and its related substances in chewing gum formulations [167], and for the assessment of foetal exposure to tobacco smoke through the measurement of nicotine and cotinine in meconium with detection limits of 10 ng/ mL [168]. Mahoney and Al-Delaimy [119] coupled reversed phase HPLC with electrochemical detection and observed limits of detection for nicotine to be lower than 0.05 ng/ mg of hair.

In a study by Kyerematen *et al.* [152] the coupling of HPLC to radiometric detection, via the administration of radiolabelled nicotine to animals, was used to determine the extent of nicotine metabolism. This radiometric assay was able to measure the concentrations of nicotine and twelve of its metabolites in urine and blood. The metabolism of nicotine proved to be complex and HPLC methods with greater resolving power were developed. Of these new methods a cation exchange method was shown to resolve numerous polar metabolites of nicotine in guinea pig urine [153] and has recently been used, with UV/vis detection, for the quantification of total cotinine and total 3'-hydroxycotnine in the plasma of smokers [154]. Demetriou *et al.* [155] used radiometric detection with ion-exchange HPLC to identify and quantify nicotine and many of its metabolites in rat urine. This method showed good reproducibility, whilst metabolites were detected above 1.5 μM. Thus radiometric detection has cemented its position as a viable detection apparatus for HPLC analysis of nicotine.

Colorimetric-HPLC assays have been applied to nicotine and its metabolites [134,169,170] and have also been used for the study of passive smoke exposure [12]. But as with radiometric detectors identification of particular metabolites can sometimes be unclear. When a situation like this arises, the coupling of HPLC to a more informative detector such as a mass spectrometer can and should be carried out.

The method developed by Nakajima *et al.* [164] for example, would be an ideal method for HPLC/MS (i.e. combining the selectivity of the MS with the efficient separation provided by HPLC). Unfortunately it requires the use of a phosphate buffer, which can have a detrimental effect on most MS ion sources and hence analyte detection, due to the readiness of this salt to form crystalline deposits that block the apertures leading to the mass analyser [3]. HPLC/MS requires the use of a volatile mobile phase since ions are sampled in the gas phase. Thus aqueous mobile phases, which tend to give the best separations for polar compounds and their metabolites, are rarely used, since evaporation of such mobile phases becomes harder. HPLC/MS is therefore somewhat compromised by its inability to utilise aqueous mobile phases efficiently. Thus most ionisation sources use volatile organic buffers, such as ammonium formate or ammonium acetate, at low concentrations (below 20 mM); with only the commercial Z-spray interface (developed by Micromass, Manchester, UK) being able to cope with harsh mobile phases such as potassium or sodium phosphate buffers) [3].

Liquid chromatography can be applied to a greater variety of samples than gas chromatography, since the latter requires the analyte to be volatilised while LC requires only the dissolution of the analyte in some solvent prior to separation [3]. Thus metabolites such as N-oxides, which are not thermally stable, cannot pass through a GC without decomposing [3]. Generally such metabolites are derivatised and converted back to the

aglycone for GC analysis. Derivatisation of trans-3'-hydroxycotinine with bis(trimethylfluorosilyl) acetamide has also been performed for GC/MS analysis [171]. But derivatisation of the analyte requires extra sample work-up and therefore a direct method is usually preferred.

To date there have been numerous studies for the determination of nicotine and its metabolites in biological fluids by HPLC/MS, which include saliva [18], urine [65,172], and plasma [3]. Applications of liquid chromatographic-tandem mass spectrometric methods (LC/MS/MS) have also been extended to quantitative analysis of polar ionic compounds in biological fluids [173]. More importantly LC/MS/MS has been used in the determination of cotinine and 3-hydroxycotinine in human saliva [174], the analysis of nicotine and its metabolites in urine of smokers [65,174] and the analysis of cotinine in serum [175]. The limit of detection of both nicotine [172] and cotinine [18,175] in biological fluids, by HPLC/MS, has been found at picogram levels.

Capillary electrophoresis

Electrophoresis is the migration of charged electrical species when dissolved, or suspended, in an electrolyte (a conductive liquid medium, usually aqueous) through which an electric current is passed. Capillary electrophoresis (CE) is therefore the separation of charged compounds usually in a narrow fused silica capillary upon the application of an electric field. Thus cations migrate toward the negatively charged electrode (cathode) and anions toward the positively charged electrode (anode). The rates at which solute ions migrate depend on their charge-to-mass ratios. Smaller ions migrate faster than larger ions of the same charge and an ion with a higher charge will migrate faster than an ion with a

lower charge, of the same size. Neutral solutes are not attracted to either electrode. The electrical driving force of solvent in CE is what is known as the electroosmotic flow [176].

CE can be used as an alternative method to both GC and HPLC. Like HPLC, CE can utilise a wide range of sample types, and with its high resolution and high number of theoretical plates achieved, it makes for an attractive separating device for MS coupling. Even though CE has been applied to nicotine analysis in tobacco products [177-180], and the study of neuropharmacological effects of cotinine atcholinergic nicotineic receptors of the superior cervical ganglion [182], there are currently no publications regarding its application to nicotine metabolism that we are aware of. So in this area of research CE is still in its infancy. Furthermore, there is only a single publication, that we are aware of, on the coupling of CE to MS for the determination of nicotine and metabolites [182]; and hence to the best of our knowledge there are no CE/MS analyses of this compound or its metabolites from the major biological matrices, which are saliva, urine, and blood. However, there are numerous publications on CE-ESI-MS (CE coupled to the MS via an electrospray ionisation source) [183,184], of which one of the latest by Stöckigt *et al.* [185] features the analysis of selected alkaloid groups. Thus the potential is there for a robust CE/MS method to be developed for application to nicotine metabolism.

CE can offer several advantages over the more established separating techniques of GC and HPLC, including high separation efficiency, extremely small injection volumes, short analysis times, speed of method development and low reagent costs. CE may produce highly-efficient separations of a wide range of sample types, and the number of theoretical plates in CE may reach several hundred thousand. The main limitation of CE is its lack of sensitivity due to its low sample loadability and the short path length of the detection window (when UV detectors are used).

Several techniques have been shown to yield on-column sample pre-concentration and to lower detection limits in CE. The most practical way to concentrate a sample is the on-line sample stacking approach, but the most effective way is by transient isotachophoresis [186-188]. Sample pre-concentration is most essential when coupling CE to MS, since the coaxial sheath flow interface requires a make-up flow, which dilutes the sample. Other ways of improving sensitivity include the use of the nanospray interface (but it is somewhat more difficult to achieve a stable spray with this interface) and sample pre-concentration via solid phase extraction (SPE).

Conclusion

The method of choice for any given application is one that is sensitive, specific, requires simple instrumentation/ equipment, and is economical. Colorimetric methods are amongst the cheapest methods for nicotine determination and are also very rapid in their analysis. If very high sensitivity is required, such as for studies of nicotine and its metabolites in biological fluids of non-smokers, mass spectrometric methods are probably most desirable; and for studies in which it is necessary to measure nicotine and cotinine specifically without interference from other nicotine metabolites, chromatographic methods, along with capillary electrophoretic methods, would be the most suitable. Greater selectivity and sensitivity are obtained from the selected-ion monitoring mode of MS and structural elucidation, from tandem mass spectrometry. The latter will also achieve higher sensitivities. However, in order to distinguish heavy smokers from non-smokers, most of the aforementioned methods would be applicable.

The high temperatures exhibited in GC analysis means that thermally labile metabolites, such as the N-oxides, would not remain intact during GC separation of the sample. Also since GC requires the analyte to be volatilised, there are a limited number of compounds it can be applied to. Thus the use of HPLC for the determination of nicotine and its metabolites may prove advantageous, since it can be applied to a wider range of compounds. However, the high resolution and high peak efficiency obtained by CE make this technique an even more attractive proposition, providing the issue of sensitivity is addressed. By combining CE with MS a potentially highly efficient electrophoretic separating method, with selective mass identification and structural elucidation, can potentially be achieved.

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Chapter 2

The Determination of Nicotine and its Metabolites, in Urine, by Solid Phase Extraction and Sample Stacking Capillary Electrophoresis/Mass Spectrometry.

2.1. Introduction

In the study of nicotine metabolism the application of capillary electrophoresis (CE) is still in its infancy. However, CE offers several potential advantages over GC and HPLC for the analysis of complex mixtures of metabolites, including high separation efficiencies, extremely small injection volumes, short analysis times, rapid method development and low reagent costs [1]. CE may be used to perform highly efficient separations of a wide range of sample types, and the number of theoretical plates in CE may reach several hundred thousand [2].

The main limitation of CE is its lack of sensitivity [2-18]. However, detection limits in CE are generally 10 - 100 times higher than in HPLC [4,5]. The relatively higher limit of detection observed in CE comes from its restricted injection volume (where typical injection volumes are in the nanolitre range) and the short path length for on–capillary detection (when coupled to a UV detector) [1,4]. Thus a major area of interest in CE is to improve its low concentration sensitivity without sacrificing resolution.

Several techniques have been shown to yield on-column sample concentration and hence to lower detection limits in CE. These include isotachophoresis [1], sample stacking [6], and field amplified injection [8]. The most practical way to concentrate a sample is in fact the on-line sample stacking approach. The sample stacking phenomenon was first introduced by Tiselius, and can be achieved through the manipulation of sample and buffer solutions with injection procedures common to any CE instrumentation.

In capillary zone electrophoresis (CZE) the sample is dissolved in the same buffer as the run buffer and hydrodynamically injected, with the width of the solute zone being the same as the width of the sample plug. Thus when a voltage is applied the sample migrates through the capillary, and elutes in a zone that is slightly wider than, and proportional to the initial width of the sample plug (figure 2.1).

Figure 2.1. A schematic representation of the capillary zone electrophoresis procedure for the separation of cations (adapted from Baker, 1995) [9].

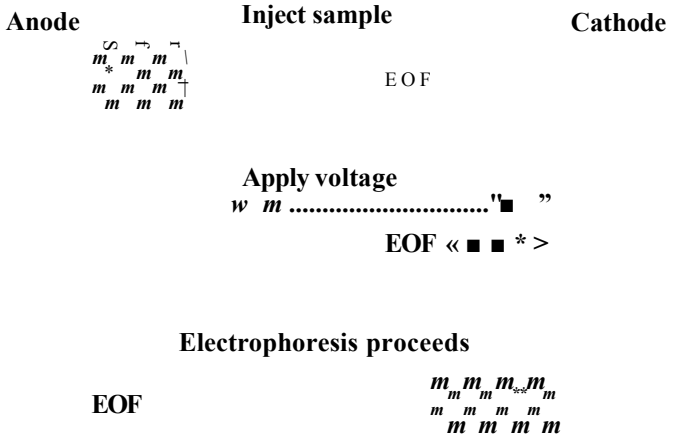
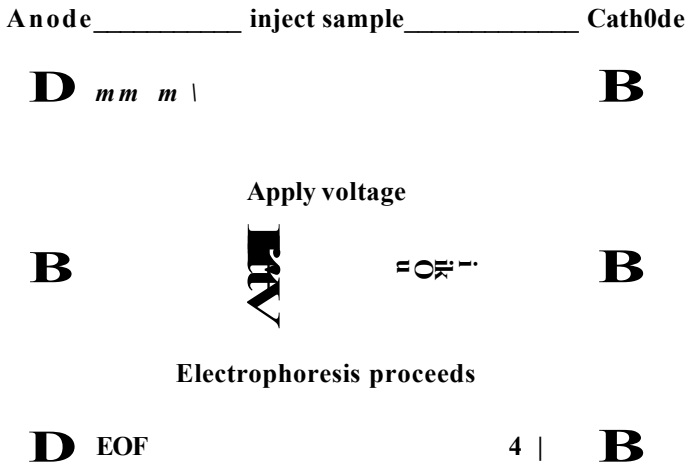


Figure 2.2. A schematic representation of the sample stacking procedure for the separation of cations (adapted from Baker, 1995) [9].



In sample stacking, however, the sample is dissolved in a lower ionic strength solution than the run buffer (figure 2.2). This can be a more dilute buffer or deionised water. This will result in the sample solution having a lower conductivity than the run buffer. Thus when a voltage is applied, a higher electric field strength is generated within the sample plug than in the run buffer due to its higher resistivity. Since electrophoretic velocity is proportional to electric field strength, the solute ions will migrate rapidly through the dilute sample plug until they reach the concentration boundary between the sample plug and the run buffer. The solute ions then encounter a reduced electric field strength at this boundary and therefore slow down, forming a narrow, stacked zone. They will then proceed through the capillary, under the influence of the electroosmotic flow and their electrophoretic mobilities, as stacked zones that are narrower than the sample plug (figure 2.2). Neutrals

are not stacked as they migrate under the influence of the electroosmotic flow alone. Anions, however, will stack at the rear of the sample plug, also as narrow zones. The thin solute zones are then separated by CZE [5,6].

The degree of stacking and on-column preconcentration is proportional to λ the ratio of resistivities of the sample solution and the run buffer [7-9]

$$\lambda = C_R/C_S \qquad (2.1)$$

where C_R is the concentration of electrolyte in the run buffer and C_S the concentration of electrolyte in the sample buffer. Solute ions should be concentrated to an amount equal to λ, the enrichment factor. Thus if the sample is dissolved in water λ could be several hundred. However the expected (theoretical) λ-value may not be fully observed due to the laminar flow that is produced during the stacking process. The difference in electric field strengths, in the sample and buffer zones, results in different electroosmotic flow velocities, which create an electroosmotic pressure leading to this laminar flow. The laminar flow leads to increased solute zone width, and hence broader peaks since it produces a parabolic flow profile in contrast to the flat profile of CZE. The greater the difference in concentration between the sample and buffer zones, the broader the peaks [10]. So even though a sample dissolved in the most dilute buffer, water, should yield the highest concentration enhancement, it also gives the broadest peaks. A study carried out by Chien and Burgi [5] showed that the optimum situation, for minimising the effect of broadening and obtaining a significant concentration enhancement, was achieved by the sample solution having a concentration one tenth of the run buffer.

Sample stacking can be performed by hydrodynamic (under pressure) or electrokinetic (under voltage) injection. In the case of the former, the sample is dissolved in a low conductivity buffer and the resulting solution is introduced into the capillary under pressure. In the case of the latter, sample stacking can be performed whether the matrix is a low conductivity solution or not. When dealing with a moderately high conductivity solution, a water plug (or solvent plug without background ions) is introduced before electrokinetic injection to induce sample stacking. Thus the sudden change in electrophoretic velocity across the water plug and the background solution is responsible for the focussing effect [3]. One limitation of electrokinetic injection is that there may be a bias towards higher mobility analytes. Thus a larger amount of higher mobility ions will be introduced and focussed when compared to lower mobility ions [3]. However, electrokinetic injection does provide sensitivity enhancements in comparison to hydrodynamic injection. The volume of sample solution that can be introduced to the capillary, by the latter, can limit the injected amount of the sample; but as analyte molecules are introduced electrophoretically to the capillary, by electrokinetic injection, this does not pose as a major problem for this injection method.

Palmer *et al.* [11] recently reported the separation and detection of standards of nicotine and ten of its metabolites using CE/ MS. However, to date there are no reported studies, to this author's knowledge, on the use of CE for the determination of nicotine and its metabolites in "real samples". By combining CE with mass spectrometry (MS), selective identification and also structural elucidation can be achieved. In this study the use of solid phase extraction combined with sample stacking CE/MS for the determination of nicotine and its metabolites in urine is demonstrated.

Aims and objectives

The objective of this study was to produce a sensitive and selective CE/MS method for the determination of nicotine and its metabolites and the extraction of these compounds, from urine, by solid phase extraction.

2.2. Methods and Materials

2.2.1 Samples and Sample Preparation

Standards of nicotine and its major metabolites were obtained from P. Jacobb III, San Francisco, CA, USA. Nicotine and cotinine was also obtained from Sigma-Aldrich, Dorset, UK. These were prepared in buffer for CZE experiments or mobile phase for HPLC or one tenth of the run buffer (or the appropriate solvent mixture) for sample stacking experiments.

Urine was obtained from five male smokers (aged 21 to 39 years), A to E, and stored at -20 °C in tissue culture flasks. Two samples, labelled C_1 and C_2, were collected from the same subject, but on different days. Prior to extraction urine samples were thawed overnight at 4 °C, thoroughly mixed and filtered with a 0.45 μm Supor acrodisc filter (Pall Corporation, Portsmouth, UK).

2.2.2 Solid Phase Extraction

Solid phase extraction (SPE) was carried out using a 100 mg C18 cartridge (Varian Ltd, Surrey, UK). For conditioning purposes 3 mL of methanol (Fischer Scientific, Manchester, UK) followed by 3 mL of deionised water (Milli- Q, UK) were passed through the SPE cartridge sequentially. Then 1 mL of sample was introduced into the cartridge followed by 6 mL of deionised water. It should be noted that for 5, 10, 15 and 20 fold pre-

concentrations, 5, 10, 15, and 20 mL of sample were introduced respectively. The sample was then eluted with 1 mL of methanol. The eluted product was dried with nitrogen gas. The dried product was then reconstituted with 1 mL of mobile phase for the recovery experiments or 1 mL buffer for CZE or 1 mL one tenth buffer (or solvent mixture) for sample stacking.

Solid phase extraction (SPE) was also carried out using a 1g C18 Isolute cartridge (Jones Chromatography Ltd., UK). For conditioning purposes 6 mL of methanol (Fischer Scientific, Manchester, UK) followed by 6 mL of deionised water (Milli- Q, UK) were passed through the SPE cartridge sequentially. Then 100 mL of sample was introduced into the cartridge followed by 6 mL of deionised water. The sample was then eluted with 6 mL of methanol. The eluted product was dried with nitrogen gas. The dried product was reconstituted with 0.5 mL buffer or one-tenth buffer or solvent mixture (200 fold pre-concentration for CE/ MS) or 1 mL mobile phase (100 fold pre-concentration for recovery experiments).The reconstituted product was then subjected to ultrafiltration (via a Sorval Tc-6 centrifuge, Sorval Instruments - Dupont, CT, USA; and Vivaspin Concentrator- 3000 MW, Vivascience, Gloucestershire, UK) at 3950 rpm for 20 minutes.

2.2.3 Buffer and Mobile Phase Preparation

The required quantity of ammonium formate or acetate (Sigma-Aldrich, Dorset, UK) was dissolved in the solvent mixture (acetonitrile [Fischer Scientific, Manchester, UK]/ deionised water [Milli-Q, UK]); buffer pHs were adjusted with formic acid or HCl (Sigma-Aldrich, Dorset, UK), as appropriate, and buffers were freshly prepared. Buffers were degassed prior to analysis using a Branson 1210 ultrasonic bath (Branson Ultrasonics,

Danbury, CT, USA) and filtered through a 0.2 μm Supor acrodisk syringe filter (Pall Corporation, UK). The buffers prepared were 25 mM ammonium formate (10 % acetonitrile, 90 % deionised water), which was adjusted to pH 2.5 with formic acid, for CZE; and 10 mM ammonium formate (75 % acetonitrile, 25 % deionised water) adjusted to pH 2.5 with HCl, for sample stacking experiments.

2.2.4 Recovery Experiments

Non-smokers' urine samples were spiked with cotinine and extracted using the SPE method described above. These samples were analysed by HPLC/UV and HPLC-MS using a VG Quattro I (Manchester UK) triple quadrupole mass spectrometer operating in electrospray ionisation (ESI) and selected ion monitoring (SIM) mode (which is the same as selected ion recording [SIR]).

The HPLC conditions were as follows; Phenomenex Luna 5μ C18 column, 250 x 4.6 mm (Phenomenex, UK), 60 % mobile phase (15 % acetonitrile, 5 % methanol and 80 % buffer in deionised water [10 mM ammonium acetate- pH adjusted to 4.8 with HCl]) + 40 % methanol; flow rate 1 mL/ min. A 50:1 split post column was employed with a flow of 20 μL/ min being introduced into the mass spectrometer. The injection volume was 10 μL.

2.2.5 CE Separation

CE experiments were carried out in a 100 cm, 50 μm i.d. x 365 μm o.d., and a 100 cm, 75 μm i.d. x 365 μm o.d., untreated fused silica capillary (Composite Metal Services Ltd., Hallow, UK). The CE system used (Crystal CE System, PrinCE Technologies, Lauerlabs, Emmen, Netherlands) utilizes programmable injection with pressure and voltage. Separations were achieved using an applied voltage of +30 kV. Injections were performed

either hydrodynamically (25 mbar/ 0.2 min) or electrokinetically (30 kV/ 0.2min) or hydrodynamically + electrokinetically (HE: 25 mbar + x kV/ 0.2 min, where x is the voltage applied in kV). Initial conditioning of the capillary took place with 1 M NaOH, (5 min, 2000 mbar [Sigma-Aldrich, Dorset, UK]) followed by the buffer system (7 min, 2000mbar). The capillary was conditioned prior to each run with 1 M NaOH (2000 mbar/ 1 min) followed by buffer system (2000 mbar/ 1.5 min). The buffer system was replenished after each run cycle.

2.2.6 CE/ MS

A VG Quattro I triple quadrupole mass spectrometer (Micromass, Manchester, UK) was used throughout. To date, electrospray ionisation (ESI) is the most common technique for coupling CE to MS. In these experiments CE/ESI/MS coupling was achieved using the coaxial sheath-flow interface developed in-house and previously described by Palmer *et al.* [11]. The sheath liquid comprised either, 1:1 MeCN/H_2O + 0.1% formic acid, or run buffer and was delivered by a Harvard Model 11 syringe pump (Harvard Apparatus, Edenbridge, UK) at a flow rate of 3-5 $\mu L\ min^{-1}$.

Positive ions were generated through the application of 3.5 kV to the probe tip, with a source cone voltage of 25 V. Desolvation was aided by nebulising gas (~40 L/hr) and bath gas (~225 L/hr).

Data was acquired by selected ion recording of the analyte protonated molecules employing a 1 Da window with a dwell time of 0.08 s. Data acquisition was performed by Masslynx 3.1 (Micromass, Manchester, UK).

2.3. Results and discussion

2.3.1 CZE/MS of nicotine and its metabolites in smokers' urine (sample C2)

Before one can perform CZE/MS on a urine sample, an extensive clean-up strategy must be employed to remove the high concentration of inorganic salts present (50 to 500 mM sodium chloride) [19]. Direct CE analysis of urine is problematic since inorganic salts present in urine would increase the electrical conductivity of the sample, which could cause a distortion in the electric field, thereby reducing the overall efficiency of separation and hence leading to the observation of broad, skewed peaks, i.e., this situation would be the opposite to that in sample stacking. In other words when a voltage is applied to a urine sample a lower electric field strength will be generated within the sample plug than in the run buffer, due to its lower resistivity. Since electrophoretic velocity is proportional to electric field strength, the solute ions will migrate slowly through the dilute sample plug until they reach the concentration boundary between the sample plug and the run buffer. The solute ions will then encounter higher electric field strength at this boundary and therefore speed up, forming a broad zone leading to the poor peak shape.

If the inorganic salt content is too high then according to the Ohm's law plot, the current will also be high and the resistance low. The zeta potential, ζ, is defined as [9]

$$\zeta = 4\pi\delta e/\varepsilon \qquad (2.2)$$

where δ is the thickness of the diffuse double layer, e is the charge per unit surface area and ε is the dielectric constant. Zeta potential, which determines the EOF velocity (v_{EOF}) and can be described as [9]

$$\zeta = v_{EOF}\, 4\pi\eta/\, \varepsilon E \qquad (2.3)$$

where η is the viscosity, and E is the electric field in (volts/cm). High electrolyte concentration will lead to high current according to the Ohm's law plot [9]

$$E = IR \qquad (2.4)$$

where I is the current and R is the resistance. High currents will inevitably cause an increase in heat production via Joule heating, ΔT, (equation 2.4) [9]

$$\Delta T = (0.239Q/4k)r^2 \qquad (2.5)$$

where Q is the power density, k is the thermal conductivity, r is the tube radius. A rise in temperature will cause a reduction in viscosity, permitting even more current to flow. Increased heat in the capillary may lead to broader peaks, sample decomposition, or even boiling of the buffer, which can cause electrical discontinuity through the capillary, eventually bringing electrophoresis to an end [9].

It should also be noted that the high inorganic salt content of urine would be incompatible with the MS, due to the crystallisation of salts on the ion source. This would prevent the efficient transmission of ions through the MS. For these reasons the direct analysis of urine by CE is not practicable.

Liquid-liquid extraction and SPE are currently the most frequently applied off-line sample preparation techniques in bioanalysis. The polarity of the eluting solvent (e.g. methanol or acetonitrile) used in the latter is normally compatible with the subsequent CE separations and sample pre-concentration is a useful consequence of this method. If the elution system is aqueous or partially aqueous, then time-consuming evaporation steps (which can result in analyte loss) may be required.

Hence the purpose of employing an off-line SPE method may be to remove particulate matter, interferences, salts and proteins and, also to enrich analytes. In this work the C18 SPE cartridge was used to remove inorganic salts, interferences, and to pre-concentrate nicotine and its metabolites from urine. The SPE procedure employed to obtain the recovery data summarized in table 2.1 is described in section 2.2.2.

Table 2.1. Percentage recovery of cotinine from 100 mg and 1 g C18 SPE cartridges (n = 3).

C18 SPE cartridge-type	Pre-concentration	Initial concentration (μg/ mL)	Final concentration (μg/ mL)	% RSD	% Recovery
100 mg	1 times	6.25	6.35	1.67	101.5
100 mg	5 times	6.25	31.22	0.14	99.7
100 mg	10 times	6.25	63.59	0.22	101.7
100 mg	20 times	3.13	28.15	3.59	45.1
1 g	100 times	0.63	63.86	0.07	102.2
1 g	100 times	1.25	118.34	0.07	94.7

HPLC/MS: Electrospray ionisation; 60 % mobile phase (10 mM ammonium acetate-15 % MeCN/5 % methanol/ 80 % deionised water) + 40 % methanol; C18 Phenomenex Luna- 5 μm, 250 mm; flow rate = 0.5 mL/ min, 1/50 split. The relative standard deviations (RSDs) were determined by the migration times of cotinine.

Table 2.1 summarises the data obtained from the LC/MS recovery experiments. For the 100 mg SPE cartridges, percentage recoveries were calculated from a linear calibration plot of six points, recorded in duplicate (before and after analysis), with the R^2 value for the calibration data being 0.9993. The 6.25 μg/ mL cotinine-spiked urine samples, for 1 to 10 times pre-concentration, were analysed in triplicate and an overall mean percentage recovery of 100.99 % was obtained.

For the 1 g SPE cartridges, percentage recoveries were calculated from a linear calibration plot of eight points and recorded in duplicate (before and after analysis). The R^2

value for the calibration data was 0.9993, and the 1.25 and 0.625 μg/ mL cotinine-spiked urine samples were analysed in triplicate and an overall mean percentage recovery of 98.42 % was obtained.

Table 2.1 shows good reproducibility for all SPE pre-concentration steps. However, the 20 times pre-concentration (via the 100 mg C18 cartridge) showed very poor recovery (45 %). This was probably due to overloading the C18 cartridge with interfering components of urine, thereby leading to less cotinine being retained on the stationary phase. Additional evidence for this was that only a 17 % recovery was obtained for 50 times pre-concentration on the 100 mg C18 cartridge (data not shown on table 2.1). It was for this reason that a 1 g C18 cartridge was used for 100 times pre-concentration.

It should be noted that the washing step of SPE (section 2.2.2. - step 4) for 1 and 5 times pre-concentrations required 6 mL of deionised water, whereas the 10 to 100 times pre-concentrations required the equivalent volumes of deionised water (e.g. the introduction of 10 mL of sample is followed by 10 mL deionised water). This particular step was employed for the removal of both inorganic salts and the more polar interfering components of urine.

Prior to SPE, the urine sample was filtered with a 0.45 μm acrodisc filter, the purpose of which was to remove any particulate matter from the urine. After SPE, evaporation, and sample reconstitution, the sample was then subjected to ultrafiltration, for the purpose of removing any proteins over 3000 MW. The removal of proteins is of great importance, as they tend to adhere to the inner surface of the fused silica, thereby disrupting the EOF and hence separation.

CE separations were carried out at low pH (2.5 to 2.8), which meant that there were few charged $Si\text{-}O^-$ groups present and, consequently a lower zeta potential (equation 2.2), and little electroosmotic flow. Thus it was essential to condition the capillary, with 1 M NaOH

followed by the buffer system prior to each run. However, when using the coaxial probe arrangement for electrospray (figure 2.3), it is essential to activate the nebulising gas after NaOH introduction to ensure that no NaOH enters and/or is left in the nebulisation chamber prior to conditioning with the buffer, as NaOH and buffer contents will surely enter the source (where they will form crystalline deposits, which can eventually block the ion source) if this is not carried out.

Buffer replenishment was also carried out prior to each run to improve precision. Replenishment is essential as buffer pH in the inlet and outlet vials can change due to electrolysis of water, where protons are produced at the cathode and hydroxide ions at the anode. Buffer depletion could also occur as a result of electrolysis and migration of buffer ions. [9].

Buffer replenishment was performed by rinsing and refilling the inlet vial, which contained approximately 4 mL of buffer, with fresh buffer. Replenishment is essential as buffer pH in the inlet and outlet vials can change due to electrolysis of water, where protons are produced at the cathode and hydroxide ions at the anode. Buffer depletion could also occur as a result of electrolysis and migration of buffer ions. For MS detection no outlet vial was used and so buffer replenishment was only required for the inlet vial (figure 2.3).

Figure 2.3. The coaxial probe arrangement.

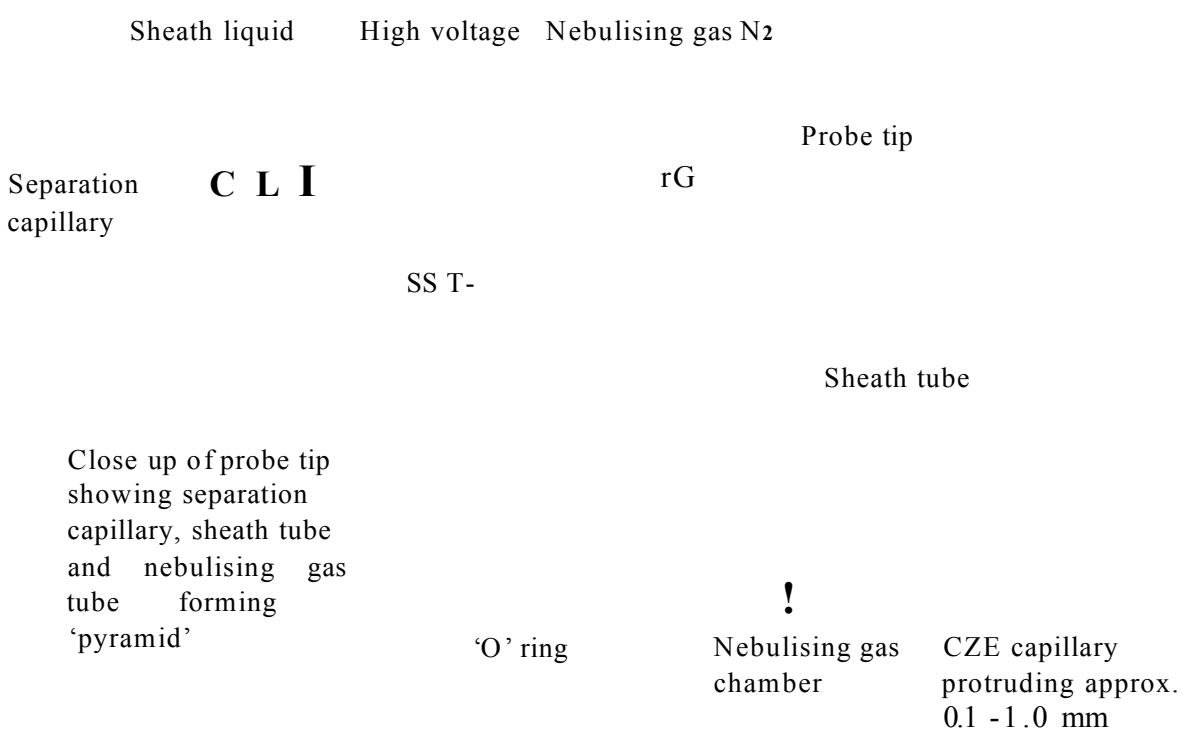


Figure 2.3 was adapted from M. Palmer (2001 PhD thesis, Sheffield Hallam University) [20].

When coupling the CE system to the MS, the capillary outlet was placed directly into the ESI interface via the coaxial probe. There was no requirement of the capillary outlet and cathode to be in a reservoir, since there was sufficient electrical contact with the run buffer flowing out of the fused silica capillary. The electrical contact was made via the sheath liquid, a large proportion of which was the volatile solvent, acetonitrile. Acetonitrile served to aid evaporation, and reduce surface tension for better spray production [12].

The sheath liquid flowed around the capillary, at 5 μL/ min. A voltage was applied to the stainless steel spray capillary to ensure the production of ions during electrospray. Thus upon the application of this voltage (3.5 kV for the VG Quattro triple quadrupole mass spectrometer), it was negative with respect to the anode (which is the fused silica capillary inlet of the CE system), which was in the CZE buffer in the high voltage region (30 kV), a potential difference was set up for CZE separation (based on the size-to-charge ratio of the analytes) [13]. Ionised solutes then exited the capillary through nebulisation into the electrospray ion source. Solute ions proceeded through the MS system where they were separated according to their mass-to-charge (M/Z) ratios and were observed as the protonated molecule, $[M + H]^+$.

Because of the difference in physical size of the CE device and of the ion source (i.e. the height of the CE in relation to the height of the ion source, from the ground or a bench), there was a limit to the smallest length of fused silica capillary that could be used [13,14]. This was important since the longer the fused silica capillary, the longer the analysis time, as there is a voltage drop per centimetre of capillary. For this study a 100 cm capillary was used [13].

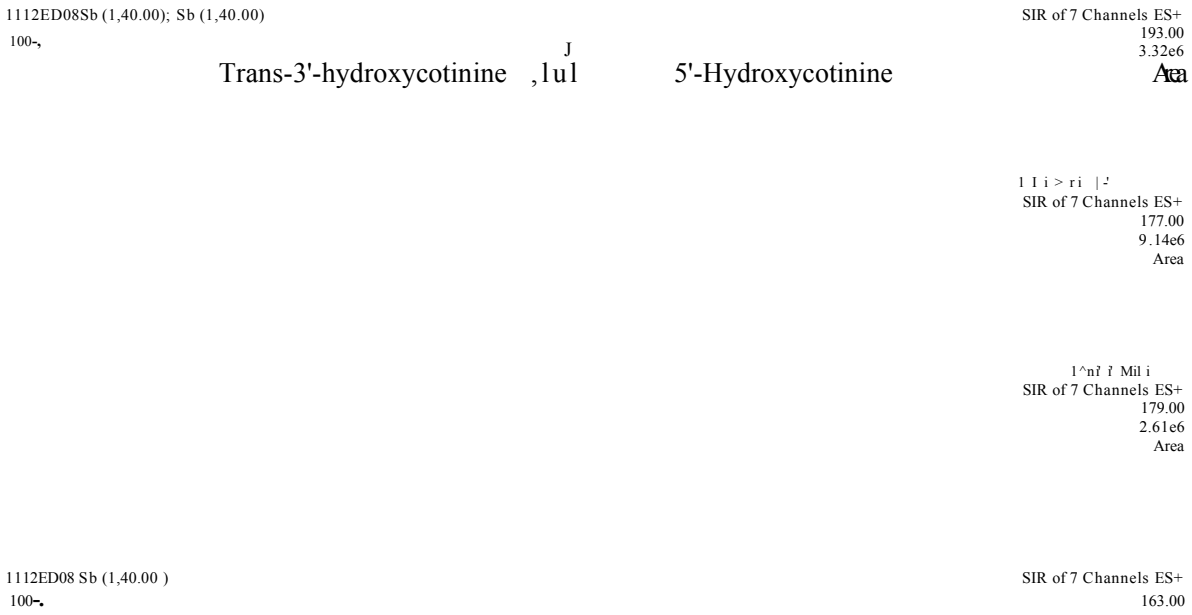
Formation of gas bubbles in the spray capillary (stainless steel) and/or the fused silica capillary may lead to instability in the spray. The former may result in the isolation of the liquid solution from the metal high voltage contact, halting the spraying process [15]. The latter may lead to an unstable current within the capillary. Thus the buffer system and the sheath liquid were degassed by sonication, in order to reduce the occurrence of gas bubbles.

CE-ESI interfacing is further complicated by the need to complete electrical paths for both CE and ESI systems [16]. This predicament may be worsened, since a constant voltage must be applied between the capillary outlet and the MS entrance, during ESI. Ideally the

capillary outlet should be maintained at ground potential (where the electric field applied across the fused silica capillary is zero volts with respect to the outlet end), as is normally the case in CE/UV [16]. Since this could not be achieved with the MS, the CE/MS system was subjected to arcing/ fluctuating currents. A second major issue in obtaining reproducible high quality CE/MS data using the co-axial arrangement was the drying out of the electrophoresis capillary outlet end. One possible approach to solving this was the application of a supplementary pressure to the head of the column. This ensured the presence of fluid at the tip at all times during CE separation.

The sheath liquid functions as the terminal buffer reservoir and is therefore a source of chemical background [16,17]. Thus background from the sheath liquid may interfere with analyte detection and raise detection limits. It may also be possible for counter ions, from the sheath liquid, to enter the fused silica capillary, at the outlet end, and alter the migration of analytes, and facilitate the loss of separation and resolution [16]. The counter ions may do this by forming a moving ionic boundary inside the capillary counter to the flow of analyte [18]. This problem is most noticeable in capillaries with low electroosmotic flows (EOF) [18]. The application of supplementary pressure to the fused silica capillary and/ or the elevation of the CE device (to prevent siphoning) will also ensure that very few to no counter ions enter the capillary [17]. However, the application of a supplementary pressure will result in loss of resolution and peak efficiency as shown in figure 2.4.

Figure 2.4. Selected ion recording (SIR) mass electropherogram of a smokers' urine sample (sample C2).



Two hundred times pre-concentration of urine sample via SPE; the sample was reconstituted in buffer. 25 mM ammonium formate (90% deionised water and 10 % acetonitrile), which was adjusted to pH 2.5 with formic acid; sheath liquid was MeCN/ H_2O 1:1; 100 cm capillary + 50 pm i.d.; sheath flow rate used was 5 pL/ min; sample was introduced at 25 mbar for 0.2 min; CZE separation took place at 30 kV and 50 mBar supplementary pressure. Reproducibility was determined by the RSD of the resolution (mean = 1.25) between nicotine and cotinine (RSD = 3.5 %, where n = 3). Resolution was calculated by R - 2(t2-fi)/(wi+w2) (equation 2.6) [9], where t is the time and w is the peak width.

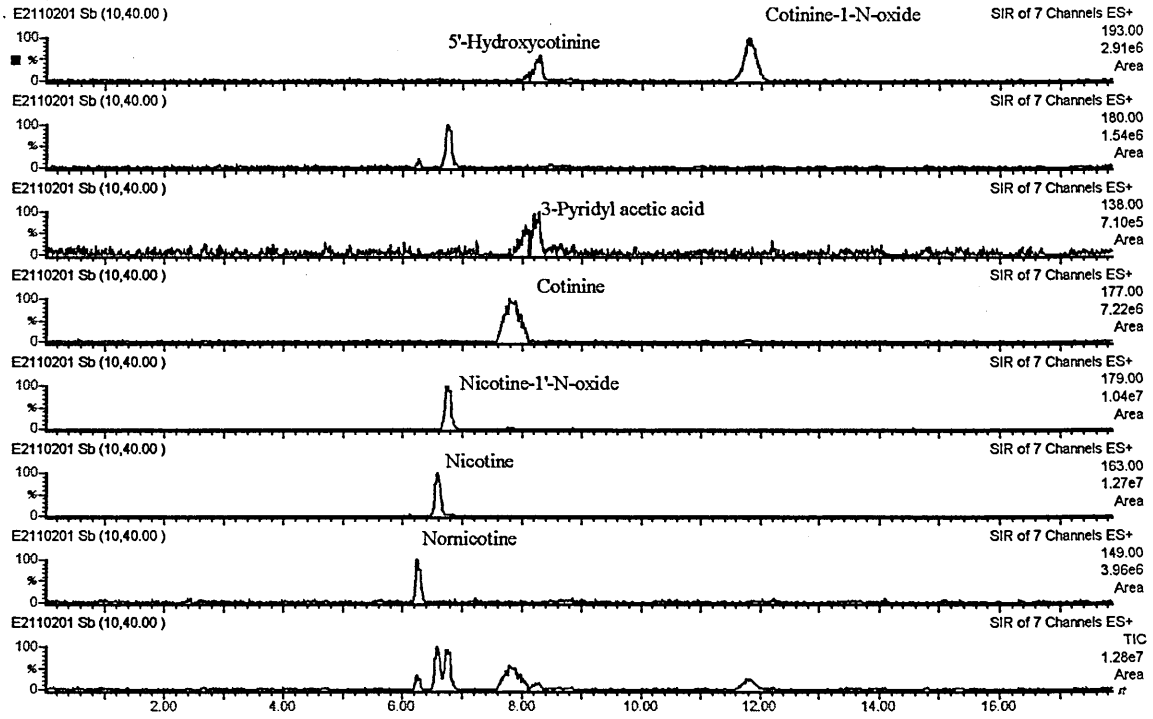
When CZE/MS was applied to a smoker's urine sample, (Figure 2.4) the same pattern of elution was observed for nicotine, nicotine-l'-N-oxide, cotinine, trans-3'-hydroxycotinine and 5'-hydroxycotinine as that seen by Palmer *et al.* [11]. However, it was evident from the above mass electropherogram (figure 2.4) that this separation profile resembled that of a

pressure driven system rather than an electrically driven one. This had a markedly detrimental effect on separation efficiency.

The higher viscosity of the sample plug may have led to an intermittent spray, and/ or the lack of sensitivity generated by this method. Thus Figure 2.4 shows a non-optimised CE/MS mass electropherogram obtained from the analysis of a smoker's urine sample (C2).

When using a coaxial probe from CE the contents of the capillary become diluted by the sheath flow since CE flow rates are in nL/ min whereas the sheath flow rate is in the μL/ min range. It was for the purpose of reducing this dilution effect that a 75 μm i.d. fused silica capillary was used instead of a 50 μm i.d. capillary (which was used by Palmer *et al.* [11]). Palmer *et al.* [11] used 100 μg/mL concentrations for nicotine and nine of its metabolites, whereas 50 μg/mL concentrations were used for this study.

Figure 2.5. SIR mass electropherogram of nicotine and six of its metabolites.



Refer to table 2.2 for peak efficiencies. 50 μg/mL nicotine, nornicotine, cotinine, nicotine-1'-N-oxide, 3-pyridyl acetic acid, 5'-hydroxycotinine, and cotinine-1-N-oxide were made up in buffer. The buffer system consisted of 25 mM ammonium formate (pH adjusted to 2.5 with formic acid) + 10 % MeCN; sheath liquid was MeCN/ H_2O 1:1 + 0.1 % formic acid; 100 cm, 75 μm i.d. fused silica capillary was used; the sheath flow rate used was 5 μL/ min; sample was introduced 25 mbar for 0.2 min; CZE separation took place at 30 kV and 50 mBar supplementary pressure. Reproducibility was determined by the RSD of the resolution (mean = 2.89) between nicotine and cotinine (RSD = 3.41 %, n = 3). Resolution was calculated by $R = 2(t_2-t_1)/(w_1+w_2)$ (2.6) [9], where t is the time and w is the peak width.

Figure 2.5 shows a reasonable separation of nicotine and six of its metabolites. But even though this method achieved an acceptable RSD it was not as robust as one would have liked, since only three out of eight runs were successful. This was probably due to poor electrical contact between the fused silica capillary and the stainless steel sheath tube provided by the sheath liquid, since in CE/UV the outlet vial consists of the same buffer system as the inlet. Because the 50 mbar supplementary pressure applied was there to

ensure no entry of the sheath liquid and/ or air into the fused silica capillary, it was unlikely that the siphoning of either would have affected the robustness of the technique. What was observed was the degradation of the baseline at various points within the mass electropherogram. Unfortunately those periods of degradation tended to coincide with the elution of some analytes. But the migration times and separation order (for those metabolites observed), for the five failed mass electropherograms, were more or less unchanged.

The same pattern of elution was observed by Palmer *et al.* [11], but in our case cotinine-1-N-oxide eluted at a mean time of 12.06 minutes (refer to figure 2.5, and table 2.2) whilst Palmer *et al.* found it to elute at approximately 21.2 minutes. This can be simply explained by the use of different inner-diameter fused silica capillaries. For this study a 75 μm i.d. fused silica capillary was used whilst Palmer *et al.* [11] used a 50 μm i.d. capillary. This meant that the supplementary pressure applied would result in a faster elution of the anlayte in the 75 μm i.d. capillary due to its greater i.d. That is the greater the inner capillary diameter the shorter the length of time it takes to fill a capillary, t_c, and hence to elute. This is shown in equation 2.7 [9].

$$t_c = 6900\, L^2/\Delta P d^2 \qquad (2.7)$$

where L is the total capillary length, ΔP is the pressure in mBar, and d is the inner capillary diameter. This also expressed by electrokinetic injection [9]

$$Q_{inj} = V\pi ctr^2(\mu_{EP} + \mu_{EOF})/L \qquad (2.8)$$

where Q_{inj} is the quantity of sample injected V is the voltage, c is the sample concentration, t is the time duration the voltage is applied, r is the capillary radius, μ_{EP} is the electrophoretic mobility of the solute, and μ_{EOF} is the electroosmotic mobility.

Table 2.2. Peak efficiencies obtained for nicotine and six of its metabolites via CZE/MS (n = 3). Mass electropherograms shown in figure 2.5.

	Retention time t (min)	Peak width w (min)	Number of theoretical plates (N)
Nornicotine	6.28	0.22	13665
Nicotine	6.61	0.23	14168
Nicotine-1'-N-oxide	6.79	0.28	9411
Cotinine	7.89	0.63	2551
3-pyridyl acetic acid	8.27	0.51	4183
5'-Hydroxycotinine	8.35	0.4	6996
Cotinine-N-oxide	12.06	0.53	8450

Refer to figure 2.5 for conditions. Peak efficiency was determined by $N = 16(t/w)^2$ (equation 2.9) [9]; where t is the migration time and w is the peak width at the base of the peak.

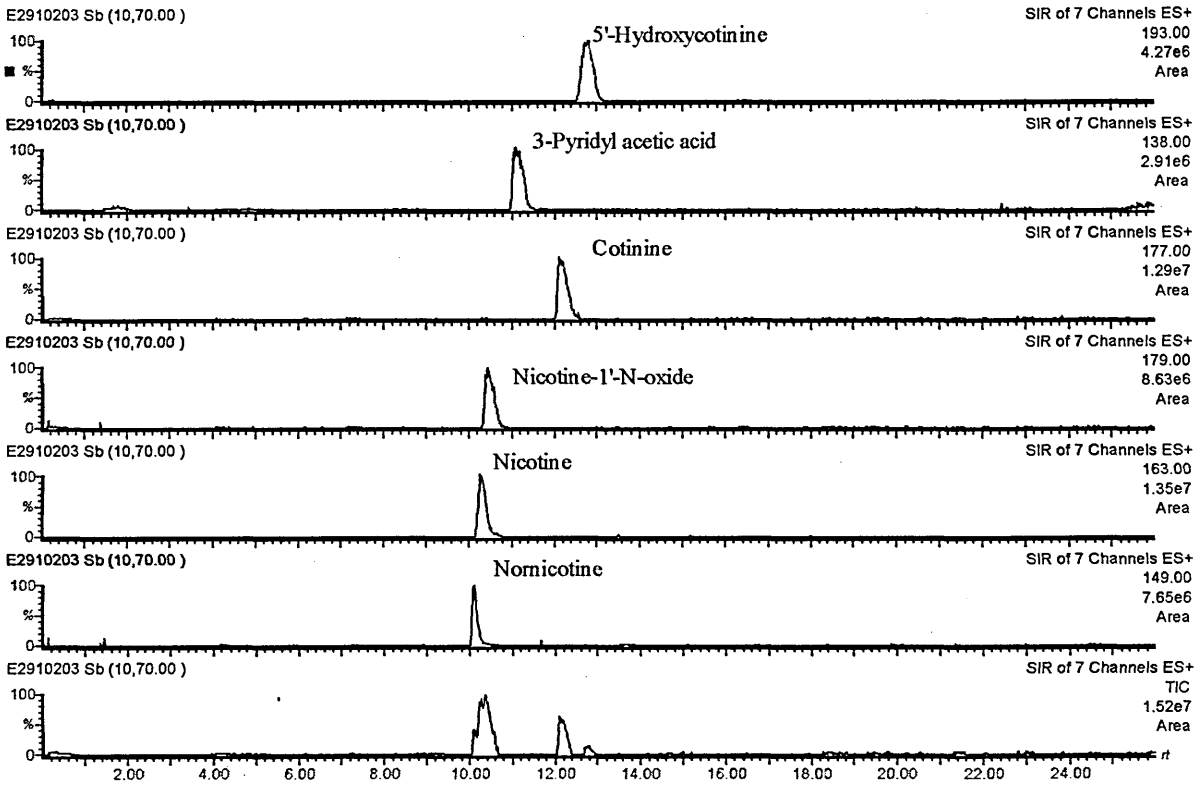
The peak efficiencies obtained (table 2.2) were rather low in comparison to those obtained by Palmer *et al.* [11]. They obtained theoretical plate numbers of 18000 and 22000 for nicotine and cotinine by sample stacking/ MS [11]. But the low theoretical plate numbers could be attributed to the use of CZE rather than sample stacking, which was used by Palmer *et al.* [11], and/ or the shorter retention times, which were evidently due to the use of a greater inner diameter fused silica capillary. The use of a 75 μm i.d. capillary

rather than a 50 μm i.d. capillary would have meant that the faster flows generated within the capillary presumably led to the flow profile having the characteristic parabolic shape of a pressure driven system (when a 50 mbar supplementary pressure was applied) rather than the plug flow profile of an electrically driven system. The flat plug flow profile of CE has the advantage that all of the solute molecules experience the same velocity component caused by EOF regardless of their cross-sectional position in the capillary, thereby causing analytes to elute as narrow bands giving peaks of high efficiency.

2.3.2. Optimisation of electrospray

In order to obtain good signal to noise in CE/MS and hence good detection limits a stable electrospray is required. The stability of the electrospray was improved (figure 2.6) by (a) withdrawing the fused silica capillary into the stainless steel sheath capillary until it was level with the sheath capillary and (b) replacing the previously used sheath liquid i.e. 1:1 acetonitrile/deionised water + 0.1% formic acid, with the buffer system. By withdrawing the fused silica capillary into the stainless steel capillary, the length of the fused silica that can be surrounded by the sheath capillary is maximised. Hence the maximum electrical contact between the sheath capillary and the fused silica capillary is obtained. A buffer system of 10 mM ammonium formate, 75 % acetonitrile/ 25 % deionised water was employed in order to improve spray formation due to its greater organic content. The use of this run buffer as sheath liquid improved beam stability presumably due to the homogeneous nature of the sheath liquid and the buffer reducing mixing effects.

Figure 2.6. SIR mass electropherogram of nicotine and five of its metabolites obtained via CZE.



50 µg/mL nicotine, nornicotine, cotinine, nicotine-1'-N-oxide, 5'-hydroxycotinine, cotinine-1-N-oxide,and 3-pyridyl acetic acid were made up in buffer. The buffer system consisted of 10 mM ammonium formate (pH adjusted to 2.5 with HCl), 75 % MeCN/25 % H_2O; 100 cm, 75 µm i.d. fused silica capillary was used; the sheath flow rate was 5 µL/ min; the sample was introduced at 10 kV + 25 mbar for 0.2 min. Separation took place at 30 kV + 20 mbar. Reproducibility was determined by the RSD of the resolution (mean = 4.48, n = 5) between nicotine and cotinine (RSD = 1.40 %).

The new CZE/MS method (figure 2.6) showed greater robustness, as five out of five runs were successful. The migration times were increased since a lower supplementary pressure of 20 mbar was used. A lower supplementary pressure was utilised primarily because of the reduced viscosity of the 10 mM ammonium formate buffer system due to its greater organic content. The order of elution was comparable to that shown by Palmer *et al.* [11].

Figure 2.6 also showed improved resolution (4.48) between nicotine and cotinine when compared to the previous CZE/MS method (figure 2.5). Theoretical plate numbers for nicotine (11543 for figure 2.6) were comparable with those obtained for figure 2.5 (14168, from table 2.2), whereas those for cotinine were significantly higher for the new method (12331 for figure 2.6). This improvement in theoretical plate numbers and resolution was primarily attributed to the lower supplementary pressure applied, as the system became more electrically driven. However, the theoretical plate numbers obtained were still, on average, lower than those obtained by Palmer *et al.* [11], since they utilised sample stacking.

2.3.3. Sample stacking/MS of nicotine and its metabolites by hydrodynamic-electrokinetic injection

Sample injection in CE is generally performed by hydrodynamic or electrokinetic injection. During hydrodynamic injection the sample vial is pressurised, forcing the sample into the capillary. Thus the volume of sample injected is dependent on the magnitude and duration of the pressure applied, the capillary dimensions, and the sample viscosity. Electrokinetic injection is performed by placing the capillary and the anode into the source inlet vial and applying a voltage over a period of time. Thus ionic solutes are injected as a result of their electrophoretic mobilities, with neutrals being pushed through the capillary as a result of the electroosmotic flow.

Electrokinetic injection provides sensitivity enhancements in comparison to hydrodynamic injection, since the volume of sample solution that can be introduced does

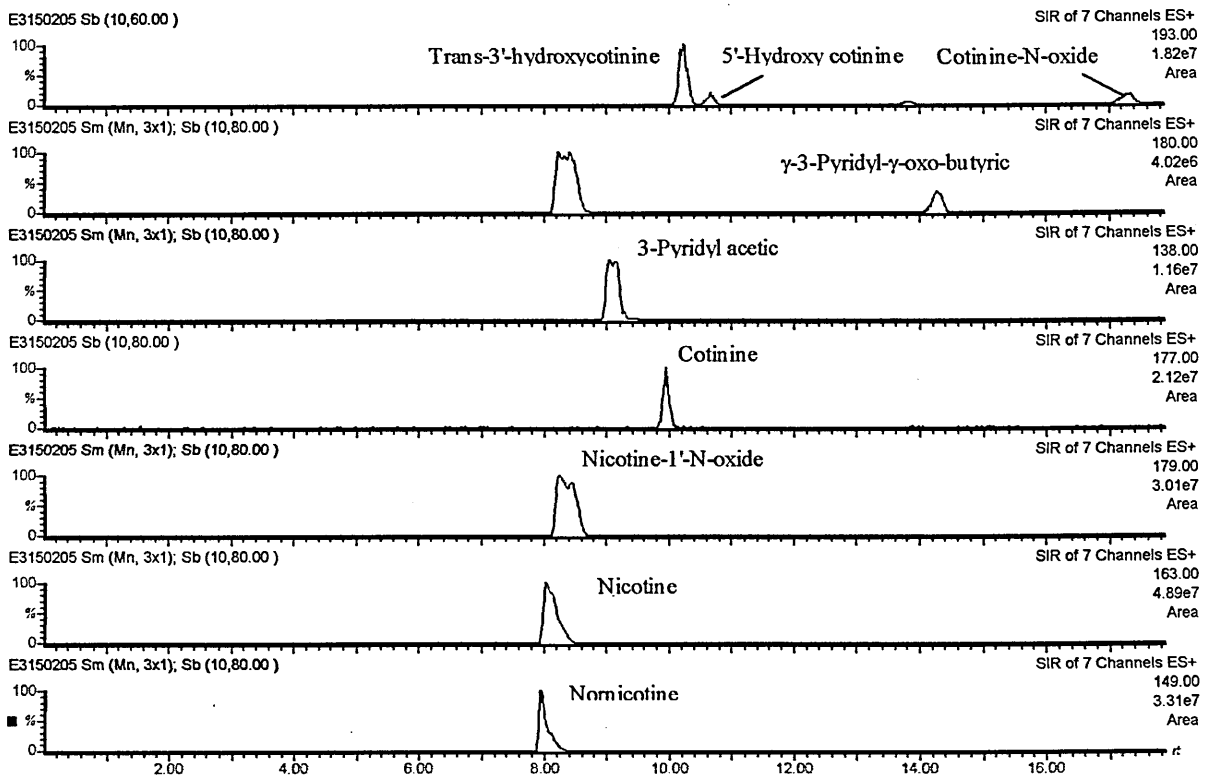
not limit the injected amount of the sample. However, sampling bias is a major drawback of electrokinetic injection, since larger quantities of higher mobility solutes are injected than those of lower mobility. An example of this is the absence of γ-3-pyridyl-γ-oxo-butyric acid and cotinine-N-oxide (table 2.4), where both analytes were clearly not injected in an adequate amount to yield a sufficient response from the mass spectrometer for detection. In order to reduce such a sampling bias a combination of electrokinetic and hydrodynamic (HE) injection was used.

Table 2.3. The measurement of standard and relative standard deviations of peak-to-peak resolutions between cotinine and 5'-hydroxycotinine, for urine samples from five smokers, and cotinine and trans-3'-hydroxycotinine from a standardised mix of nicotine and eight of its metabolites.

	Mean resolution	$_{Res}$SD	$_{Res}$ % RSD
Sample stacking HE-injection/MS (n = 3)			
Sample A (cot-5HC)	2.59	0.12	3.15
Sample B (cot-5HC)	1.41	0.01	4.52
Sample C_1 (cot-5HC)	2.21	0.10	1.02
Sample D (cot-5HC)	2.16	0.08	3.57
Sample E (cot-5HC)	2.18	0.08	3.85
Sample stacking (cot-3HC, n = 5)			
Hydrodynamic injection	0.75	0.02	2.51
Electrokinetic injection	1.26	0.01	0.80
Hydrodynamic + electrokinetic injection	0.77	0.02	3.15

For urine samples A to E n = 3; n = 5 for the three injection methods; where n is the number of repeat analyses. The resolution, R, between two adjacent peaks (cotinine and 5'-Hydroxycotinine for A to E and cotinine and *trans*-3'-hydroxycotinine for the three injection methods) was calculated as follows: $R = 2(t_2 - t_1)/(w_1 + w_2)$ (2.6) [9]. Urine samples A to E were obtained from smokers.

Figure 2.7. SIR mass electropherogram of nicotine and eight of its metabolites obtained via sample stacking and HE injection.



50 μg/mL nicotine, nornicotine, cotinine, nicotine-1'-N-oxide, 5'-hydroxycotinine, and cotinine-1-N-oxide were made up in 25 % H_2O/75 % MeCN. The buffer system consisted of 10 mM ammonium formate (pH adjusted to 2.5 with HCl), 75 % MeCN/25 % H_20; 100 cm, 75 μm i.d. fused silica capillary was used; the sheath flow rate was 5 μL/ min; the sample was introduced at 30 kV + 25 mbar for 0.2 min. Separation took place at 30 kV + 20 mbar. Refer to table 2.3 for RSD.

Sample stacking was employed in conjunction with the combined injection procedure in order to achieve the best possible sensitivity (figure 2.7). The limits of detection, by CE-sample stacking/ MS, of nicotine and cotinine, respectively, for the three injection methods were found to be: 1) 0.11 and 2.25 μg/ mL for the HE injection method, 2) 2.86 and 6.25 μg/ mL for hydrodynamic injection, and 3) 0.18 and 3.27 μg/ mL for electrokinetic injection. Thus it is clear that the HE injection method yields the lowest limits of detection,

followed by electrokinetic, and finally hydrodynamic injection. The lowest detection limits yielded by the HE injection method can be explained by its ability to introduce the greatest quantity of sample to the capillary (see table 3.3). It should also be noted that the higher electrophoretic mobility of nicotine results in greater quantities of this solute being introduced to the capillary than cotinine. This, accompanied with the higher ionising potential of nicotine, ensured its lower limit of detection. However, such a bias can be corrected, when applying this method to biological samples, by taking account of the ratio of peak areas of the analyte and internal standard.

Table 2.4. Mean peak efficiences obtained for the three injection methods (n=5) used in conjunction with sample stacking CE/MS.

	‡Number of Theoretical plates (*N*)		
	Hydrodynamic injection	Electrokinetic injection	Hydrodynamic + electrokinetic injection
Nornicotine	5911	17047	5542
Nicotine	6141	12023	3323
Nicotine-1'-N-oxide	20367	45201	5150
Cotinine	24024	11122	11894
3-Pyridyl acetic acid	19093	13086	15545
γ-3-Pyridyl-γ-oxo-butyric acid	36415		14010
trans-3'-Hydroxycotinine	10622	23189	13125
5'-Hydroxycotinine	45666	162140	23130
Cotinine-N-oxide	14830		13617
Mean total number of theoretical plates	20341	40555	11704

‡ Calculated using $N = 16(t/w)^2$ (equation 2.9). The concentration of each analyte was 50 μg/ mL. Refer to table 2.3 for RSD and figure 2.7 for analysis procedure.

The mean total number of theoretical plates achieved was greatest in electrokinetic injection (table 2.4), since during sample introduction the samples are stacked into narrow,

discrete, zones, whereas for hydrodynamic injection solute ions are dispersed within the sample plug (thus stacking only takes place during separation). The HE injection method has the lowest mean total number of theoretical plates. This was most probably due to sample overload of the capillary, when both injection methods were employed. The highest number of theoretical plates was achieved by 5'-hydroxycotinine with the electrokinetic injection method yielding a theoretical plate number of 162,140 (table 2.4). However, not all solute ions were observed by electrokinetic injection, as the introduction of ions with lower mobility, such as cotinine-N-oxide and γ-3-pyridyl-γ-oxo-butyric acid was compromised via the sampling bias exhibited by electrokinetic injection.

Efficiency, in contrast to chromatography is independent of capillary length, providing all the Joule heating is dissipated. Thus peak efficiency from the 90 cm CZE capillary (as used by Palmer *et al.* [11]) should be comparable to that of the 1 metre sample stacking capillary.

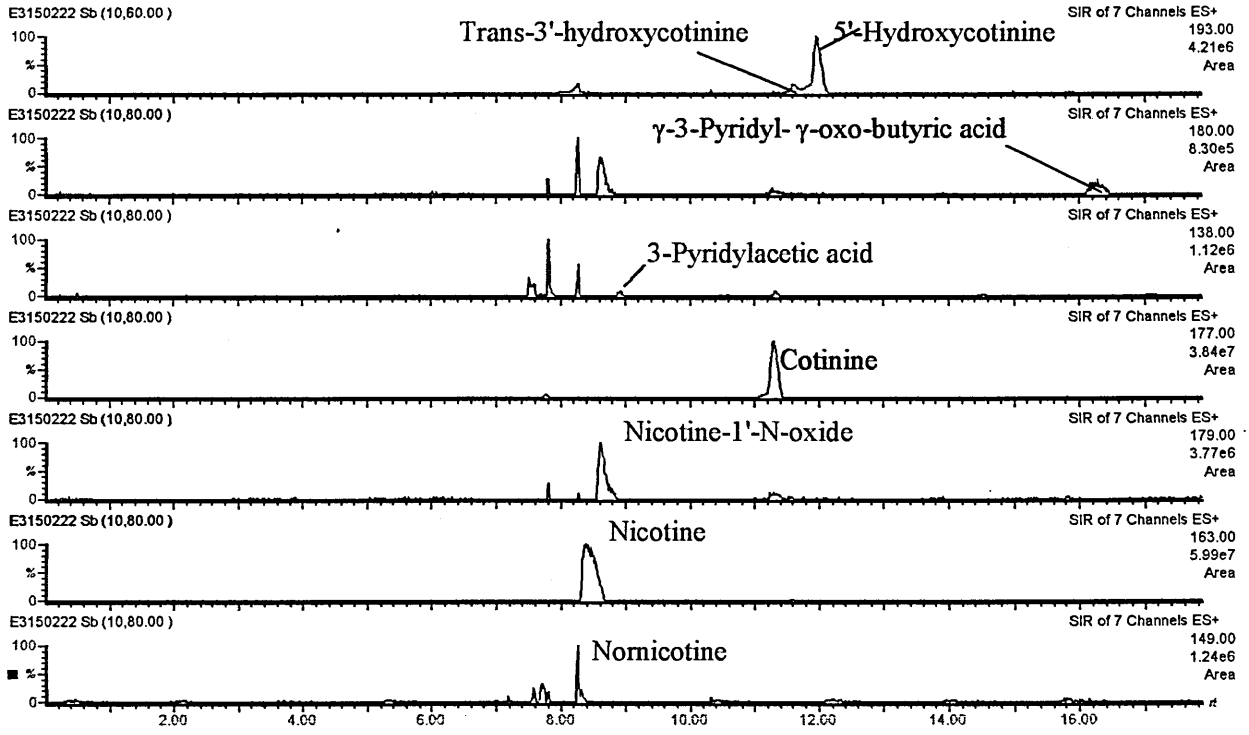
Interestingly, the HE injection method produced theoretical plate numbers of 26287, 36805, and 88953 for cotinine, *trans*-3'-Hydroxycotinine, and nicotine respectively (sample C_1- not shown). These were, on average, 131.6 fold greater than the number of theoretical plates observed by CZE/ MS (sample C_2). The higher number of theoretical plates would be attributed to the lower injected quantities of the analytes in sample C_1 (a more dilute sample). Therefore due to the lower detection limit, and reasonable number of theoretical plates produced by HE injection, this method of sample introduction would be used for sample stacking/ MS.

Optimisation of the electrospray together with the application of sample stacking (via HE injection) has greatly improved peak efficiency, baseline stability, and detector response to

the analytes (figure 2.7), and the added selectivity that SIM provides ensured the identification of nicotine and its metabolites in urine (figure 2.8).

2.3.4 Sample stacking/MS of smokers' urine via HE injection

Figure 2.8. SIR mass electropherogram of a smokers urine sample (E) via sample stacking.



The buffer system consisted of 10 mM ammonium formate (pH adjusted to 2.5 with HCl), 75 % MeCN/25 % H_2O; 100 cm, 75 μm i.d. fused silica capillary was used; the sheath flow rate was 5 μL/ min; the sample was introduced at 10 kV + 25 mbar for 0.2 min. Separation took place at 30 kV + 20 mbar. Refer to table 2.3 for RSD.

When applying the optimised sample stacking/MS HE-injection method to urine samples, sample pre-concentration via solid phase extraction was required (with the 200 fold pre-concentration of sample achieved). Data from a smoker's urine (figure 2.8, sample E)

shows the clear identification of nicotine, cotinine and the other major metabolites of nicotine. Since nicotine has only a half-life of approximately two hours, the relatively high abundance on the mass electropherogram could be attributed to the recent exposure of the smoker to tobacco smoke.

The relative abundance of cotinine in the five mass electropherograms obtained (see figures 2.8-2.10, samples C1 and D are not shown), confirms its status as the major metabolite of nicotine. The total ion chromatograms shown in figures 2.9 and 2.10 demonstrate this observation clearly.

Samples C_1 and D (not shown) exhibited a more extensive metabolism of nicotine, and having a relatively low abundance of this analyte on both mass electropherograms. The high abundance of 5'-hydroxycotinine with respect to *trans*-3'-hydroxycotinine in all the mass electropherograms, with the exception of sample C_2, suggests that the major pathway for cotinine metabolism is via 5'-hydroxycotinine. This is further confirmed by the presence of 3-pyridyl acetic acid. However, it is possible that *trans*-3'-hydroxycotinine could mainly exist in the glucuronide form. More work in this area (with respect to patient studies) is required.

The inter-urine sample migration times of the metabolites, among samples A to E, appeared to differ slightly. It was unlikely that changes in air temperature could have influenced the migration times, since the laboratory that housed the CE and mass spectrometer was climate controlled. Temperature change has been shown to indirectly affect both the current and the electric field strength via the power density, Q. Where [9]

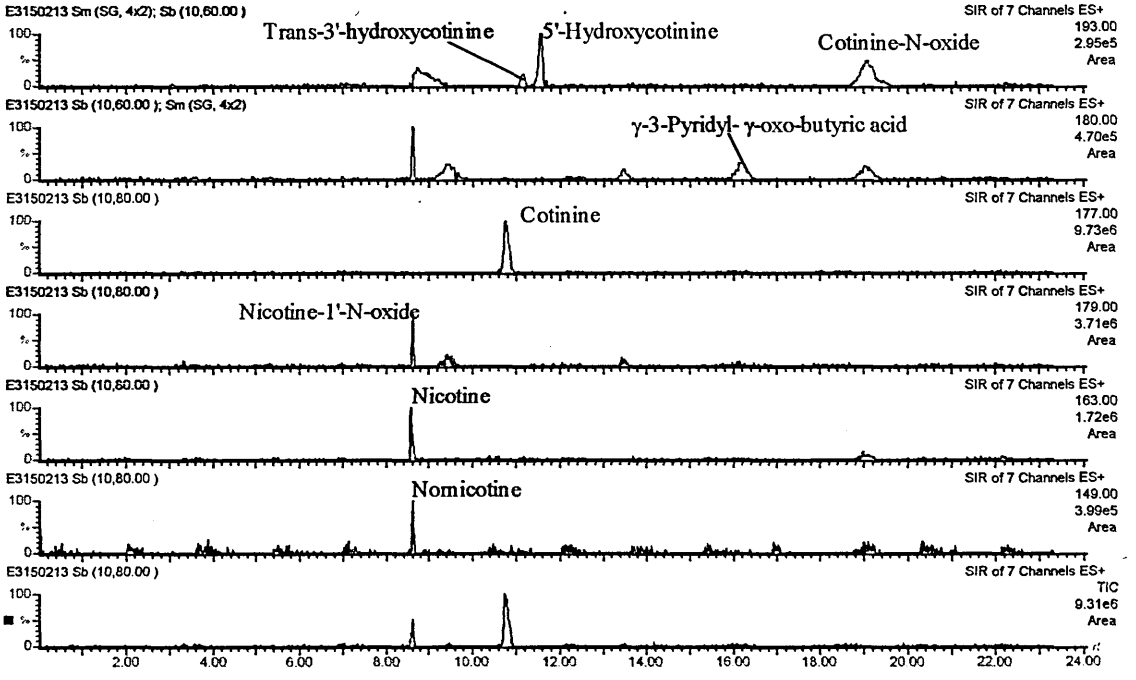
$$\Delta T = (0.239\, Q / 4k) r^2 \qquad (2.4).$$

Thus by changing Q we inevitably change the electric field (E), the current (I), and the voltage (V) all of which can affect the separation since [9]

$$Q = EI/\pi r^2 = VI/\pi r^2 l \qquad (2.10).$$

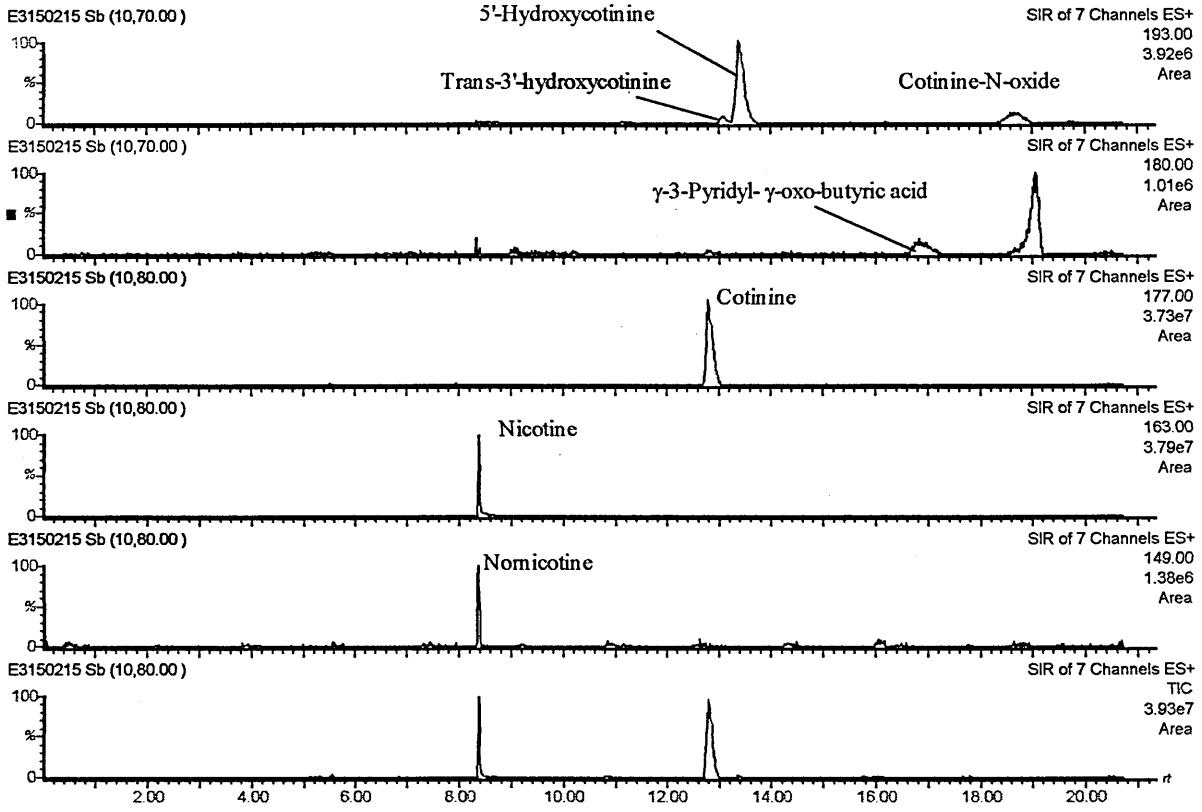
The notion that excessive Joule heating within the capillary (as a result of the high voltage used for separation) was the cause, was also dispelled since the current observed was stable at approximately 12.5 μA (with no significant fluctuations). It should also be noted that the buffer used to replenish the inlet vial was kept in a polystyrene cooler unit at < 5 °C, and so the internal capillary temperature would have been the same at the beginning of each run and the fact that the laboratory used was climate controlled would have prevented any external temperature changes from taking place.

Figure 2.9. SIR electropherogram of a smokers' urine sample (A) obtained via sample stacking.



50 μg/mL nicotine, nornicotine, cotinine, nicotine-1'-N-oxide, 5'-hydroxycotinine, and cotinine-1-N-oxide were made up in 25 % H_2O/75 % MeCN. The buffer system consisted of 10 mM ammonium formate (pH adjusted to 2.5 with HCl), 75 % MeCN/25 % H_2O; 100 cm, 75 μm i.d. fused silica capillary was used; the sheath flow rate was 5 μL/ min; the sample was introduced at 10 kV + 25 mbar for 0.2 min. Separation took place at 30 kV + 20 mbar. Refer to table 2.3 for the RSD.

Figure 2.10. SIR mass electropherogram of a smokers' urine sample (B) obtained via sample stacking.



50 μg/mL nicotine, nornicotine, cotinine, nicotine-1'-N-oxide, 5'-hydroxycotinine, and cotinine-1-N-oxide were made up in 25 % H_2O/75 % MeCN. The buffer system consisted of 10 mM ammonium formate (pH adjusted to 2.5 with HCl), 75 % MeCN/25 % H_2O; 100 cm, 75 μm i.d. fused silica capillary was used; the sheath flow rate was 5 μL/ min; the sample was introduced at 10 kV + 25 mbar for 0.2 min. Separation took place at 30 kV + 20 mbar. Refer to table 2.3 for the RSD.

The probable cause for the differing inter-urine sample migration times of the metabolites (figures 2.8, 2.9 and 2.10) was the presence of urobilins and urobilinogens in urine. The presence of these compounds in urine may be brought about by the derivation of bilirubin from senescent red blood cells and from other haem-containing proteins, such as cytochromes. Bilirubin is poorly water-soluble and so undergoes glucuronidation to form

bilirubin diglucuronide, which is excreted into the bile. The glucronide residues are released in the terminal ileum by intestinal bacterial hydrolases. Free bilirubin is then reduced to colourless urobilinogens, which are oxidised to coloured products known as urobilins. Urobilins are mainly excreted in the faeces, but small proportions of urobilins and urobilinogens are excreted in the urine. Urobilins give urine its yellow pigment [21,22].

Since ultrafilitration of the urine sample only filters molecules of 3000 MW and over, urobilins and urobilinogens (584.65 MW) are still present in the filtered urine. When pre-concentrating urine by 200 fold the concentration of urobilins increase and hence the viscosity increases. An increase in viscosity could cause an increase migration times, but not necessarily the order of elution providing the fused silica capillary is long enough for effective separation. In order to prove this hypothesis practically, both nicotine and cotinine, prepared in solutions of varying urobilinogen concentrations, would have to be run under the same conditions to establish whether migration time, resolution, and peak efficiency are affected by the increasing urobilinogen concentrations. Unfortunately due to time constraints this experiment was not carried out. However, the presence of glucuronides may have also contributed to the varying migration times.

2.4. Conclusion

The 100 fold pre-concentrations of 1.25 μg/ mL and 625 ng/ mL of cotinine, in urine, showed a mean percentage recovery of 98.42 % from a 1g C18 SPE cartridge. Hence the 200 fold pre-concentration of sample could be achieved through drying with nitrogen gas and reconstituting with buffer.

A combination of hydrodynamic and electrokinetic injection was used for sample stacking/ MS due to the lower detection limit, and the reasonable number of theoretical plates achieved. Thus the sample-stacking mode of CE, when compared to CZE, was shown to improve peak efficiency.

LODs of nicotine and cotinine, by CE-sample stacking/ MS (via HE injection), were found to be 0.11 and 2.25 μg/ mL, respectively. Thus the LODs of nicotine and cotinine after 200 fold pre-concentration could be up to 0.55 ng/ mL and 11.25 ng/ mL, respectively. It should also be noted that the higher electrophoretic mobility of nicotine probably resulted in greater quantities of this solute being introduced to the capillary than cotinine during HE injection. This, accompanied with the higher ionising potential of nicotine, ensured its lower limit of detection. However, for quantitative analysis of biological samples, such a bias can be corrected by taking account of the ratio of peak areas of the analyte and internal standard.

The added selectivity that SIM provided ensured the clear identification of nicotine and its metabolites.

The same pattern of elution was observed for nicotine and its metabolites when analysing smokers' urine. However, the inter-urine sample migration times of the metabolites appeared to differ slightly and were attributed to the presence of urobilinogens and urobilins. The extraction of both tetrapyrroles could be performed by acetaldehyde and petroleum ether. An alternative would be to use molecular imprinted polymers as a second phase extraction step complementary to SPE, as their added selectivity might ensure that only the analytes of choice were present in the sample.

2.5. References

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Chapter 3

Optimisation of Hydrodynamic-Electrokinetic Injection and Supplementary Pressure Utilisation.

3.1. Introduction

In chapter 2 the application of a dual injection method, comprised of electrokinetic and hydrodynamic injection was described. The method produced lower limits of detection when compared to hydrodynamic or electrokinetic injection alone, but yielded a lower number of theoretical plates. A sampling bias was also evident in favour of nicotine. Therefore correction of this bias (in terms of the ratio of analyte peak area to that of the internal standard) is required when attempting quantitative analysis of nicotine and its metabolites in urine.

The most noticeable drawback of this injection method was the reduced peak efficiency, and increased peak broadening observed when large quantities of a concentrated sample were introduced. This means that for concentrated samples broader peaks are observed as compared to dilute ones. The relatively low peak efficiencies observed may also have been contributed to by the use of supplementary pressure to prevent column drying.

Aims and objectives

The objective of this study was to optimise the HE injection method (in terms of the voltage used) and to find the optimum supplementary pressure needed for the production of

high quality data. This will allow 'real' samples containing potentially widely varying amounts of analyte(s) to be examined, using this method.

3.2. Methods and Materials

For CE/UV the detection window was burnt into the capillary 15 cm from the end of the capillary and analyses were performed at 205 nm. Inlet and outlet ends of the capillary were housed in run buffer vials during a run. Separations were achieved using +30 kV. Data acquisition was performed by DAx 6.0 software (PrinCE Technologies, Emmen, Netherlands) for CE/UV. Refer to section 2.2 for further methods and materials.

3.3. Results and Discussion

As previously described the degree of stacking and on-column pre-concentration is proportional to the ratio of resistivities of the sample solution and the run buffer, where solute ions are concentrated to an amount equal to λ, the enrichment factor. However, this enrichment may not be fully observed as the difference between sample and buffer zone electric field strengths can generate a laminar flow, which in turn may lead to peak broadening. It was precisely for this reason that the sample solution, used thus far, was one tenth of the run buffer concentration.

According to table 3.1, the dissolution of nicotine in the solvent mixture does not significantly affect peak efficiency and sensitivity when compared to dissolution in 1/10 buffer. In fact the number of theoretical plates for cotinine was increased by approximately 40,000 when the analyte was dissolved in the solvent mixture (table 3.1). The resolution between nicotine and cotinine was also slightly improved when the analytes were dissolved in the solvent mixture and only a minor reduction in peak asymmetry was observed (table 3.1). Since there was no band broadening observed, solute ions analysed from here on were made up in the appropriate solvent mixture.

Table 3.1. Observation of the band broadening effect when the analyte is dissolved in the solvent mixture as compared with that dissolved in 1/10 buffer (n = 5).

	Resolution (nic-cot)	N_{nic}	$Area_{nic}$ (V.min)	$Height_{nic}$ (V)	Asymmetry nic	N_{cot}	$Area_{cot}$ (V.min)	$Height_{cot}$ (V)	Asymmetry cot
75% MeCN 25% H_2O	10.2	66076	$2.9x10^{-4}$	$3.1x10^{-3}$	10.8	138560	$3.0 x10^{-4}$	$3.77 x10^{-3}$	5.96
1/10 buffer	9.98	64282	2.9x10-4	$3.4x10^{-3}$	8.60	95963	$4.6 x10^{-4}$	$5.34 x10^{-3}$	4.82

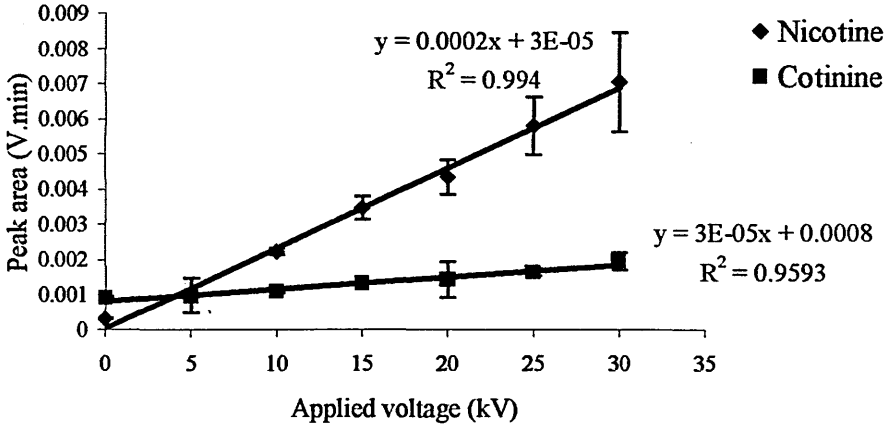
CE-sample stacking/UV was used to determine these measurements (at 205 nm). 50 μg/mL concentrations of nicotine and cotinine were used. The buffer system consisted of 10 mM ammonium formate (pH adjusted to 2.5 with HCl), 75 % MeCN/25 % H_2O; 100 cm, 75 μm i.d. fused silica capillary was used, with effective length 85 cm; the sheath flow rate was 5 μL/ min; the sample was introduced at 25 mbar for 0.2 min. Separation took place at 30 kV only. Subscripts (nic and cot) denote nicotine and cotinine respectively. Reproducibility was determined by the RSD of the retention times for nicotine dissolved in the solvent mixture (1.26 %) and the 1/10 buffer (0.83 %). Resolution was calculated by $R = 2(t_2-t_2)/(w_1+w_2)$ (equation 2.5) [1]. $N = 5.54(t/w_{1/2})^2$ (equation 3.1) [1].

3.3.1 Hydrodynamic-electrokinetic (HE) injection

It is important to gauge how much voltage to use when attempting HE injection as the combination of hydrodynamic and electrokinetic injection could lead to sample overload of the capillary. It is evident from figure 3.1 that there is a linear increase in peak area as a result of increasing the voltage applied during HE injection. This was especially true for nicotine, which had an R^2 value of 0.994 (figure 3.1), and suggesting that increasingly more sample was being introduced to the capillary.

There appeared to be more nicotine loaded onto the capillary than cotinine. This was probably as a result of greater pre-concentration of nicotine as a result of its higher electrophoretic mobility.

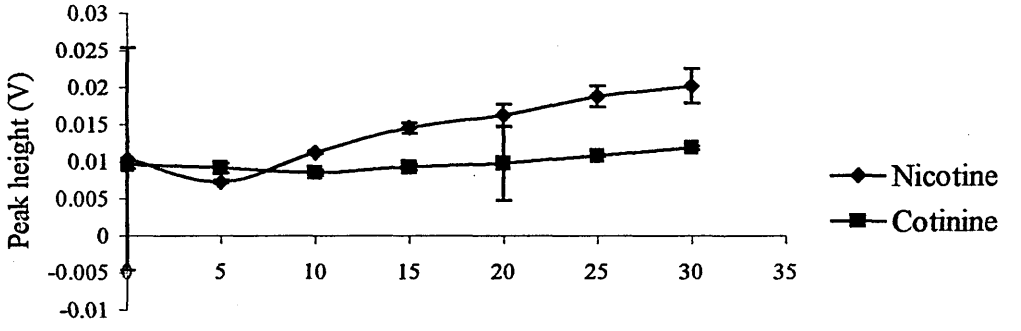
Figure 3.1. Peak area versus applied voltage for HE injection of nicotine and cotinine.



Sample stacking was performed at 205 nm. 50 μg/mL nicotine and 50 μg/mL cotinine were made up in 25 % H_2O/75 % MeCN. The buffer system consisted of 10 mM ammonium formate (pH adjusted to 2.5 with HCl), 75 % MeCN/25 % H_2O; 100 cm, 75 μm i.d. fused silica capillary, with effective length 85 cm; the sheath flow rate was 5 μL/ min; the sample was introduced at a range of voltages + 25 mbar for 0.2 min. Separation took place at 30 kV. Error bars were a product of the standard deviation (n = 7).

Since a fixed amount of sample is introduced into the capillary by hydrodynamic injection, the only other variable is the voltage applied during the electrokinetic part of this injection method. Equation 2.8 ($Q_{inj} = V\pi ctr^2(\mu_{EP} + \mu_{EOF})/L$) [1] clearly illustrates the importance of voltage (V) as the major variable since both the EOF and the electrophoretic mobility of the analyte are dependent upon it. However, this linear relationship was not confirmed by using peak height (figure 3.2).

Figure 3.2. Peak height versus applied voltage for HE injection of nicotine and cotinine.



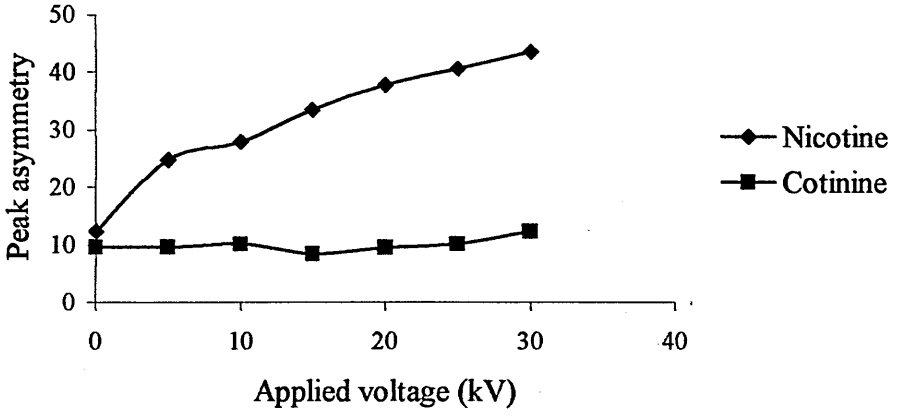
Sample stacking was performed at 205 nm. 50 μg/mL nicotine and 50 μg/mL cotinine were made up in 25 % H_2O/75 % MeCN. The buffer system consisted of 10 mM ammonium formate (pH adjusted to 2.5 with HCl), 75 % MeCN/25 % H_2O; 100 cm, 75 μm i.d. fused silica capillary, with effective length 85 cm; the sheath flow rate was 5 μL/ min; the sample was introduced at a range of voltages + 25 mbar for 0.2 min. Separation took place at 30 kV. Error bars were a product of the standard deviation (n = 7).

A possible reason for this was that at higher sample concentrations, increasing peak broadening and/or distortion occurred, which caused changes in peak height but not area.

Peak broadening/distortion is most evident in figure 2.9 due to HE injection at 30 kV (refer to figure 3.2).

Probably the only way of monitoring peak distortion is by calculating peak asymmetry. In this study peak asymmetry is a serious issue, since the analytes have lower mobilities than buffer cations causing peak tailing. Therefore at higher voltages, peak shape becomes increasingly asymmetric due to increased sample introduction. As a result, the distorted peak shape may have a significant effect on the peak height. That is, if peaks become increasingly broad then they will tend to lose height.

Figure 3.3. Peak asymmetry versus applied voltage for HE injection of nicotine and cotinine.



Sample stacking was performed at 205 nm. 50 μg/mL nicotine and 50 μg/mL cotinine were made up in 25 % H_2O/75 % MeCN. The buffer system consisted of 10 mM ammonium formate (pH adjusted to 2.5 with HCl), 75 % MeCN/25 % H_2O; 100 cm, 75 μm i.d. fused silica capillary, with effective length 85 cm; the sheath flow rate was 5 μL/ min; the sample was introduced at a range of voltages + 25 mbar for 0.2 min. Separation took place at 30 kV. Error bars were a product of the standard deviation (n = 7).

Figure 3.3 shows only a slight increase in peak asymmetry for cotinine from 15 kV to 30 kV and so this increase is not significant. However, there is a more significant increase in peak asymmetry for nicotine from 0 to 30 kV.

As the voltage was increased for HE injection, the resolution between the nicotine and the cotinine peak decreased (table 3.2). This is presumably related to increasing band broadening as there was a progressive increase in peak width from 0 kV to 30 kV, especially in the case of the nicotine peak (table 3.2). From $R = 2(t_2-t_1)/(w_1+w_2)$, where R is resolution, t is time, and w is the peak width, (equation 2.5) [1] it can be seen that an increase in peak width will cause a reduction in resolution.

Table 3.2. Resolution between nicotine and cotinine at various voltages for HE injection (n = 7).

Voltage (kV)	t_1 (mins)	t_2 (mins)	w_1 (mins)	w_2 (mins)	Resolution	t_2 %RSD	t_1 %RSD
0	9.76	12.14	0.22	0.33	8.58	0.79	0.31
5	9.99	12.72	0.37	0.42	6.97	1.17	0.66
10	10.12	12.94	0.49	0.32	7.0	0.77	0.81
15	10.20	13.13	0.70	0.35	5.61	0.49	0.89
20	10.02	13.01	0.63	0.33	6.20	3.03	3.80
25	10.16	13.24	0.79	0.42	5.10	0.50	0.76
30	10.42	13.68	1.0	0.43	4.54	0.55	0.78

Sample stacking was performed at 205 nm. 50 µg/mL nicotine (t_1) and 50 µg/mL cotinine (t_2) were made up in 25 % H_2O/75 % MeCN. The buffer system consisted of 10 mM ammonium formate (pH adjusted to 2.5 with HCl), 75 % MeCN/25 % H_2O; 100 cm, 75 µm i.d. fused silica capillary, with effective length 85 cm; the sheath flow rate was 5 µL/ min; the sample was introduced at a range of voltages + 25 mbar for 0.2 min. Separation took place at 30 kV.

Thus far the use of peak area versus increasing kV, for HE injection, has been a useful way of evaluating the extent of sample introduction. However, in order to confirm this relationship, it becomes essential to know the injected quantities of nicotine and cotinine at increasing voltages for this method of sample introduction.

Table 3.3. Variation in the quantities of nicotine and cotinine injected at a range of voltages for HE injection (n = 3).

Voltage applied (kV)	$t_{m\,nic}$ min	$t_{m\,cot}$ min	$t_{nm\ urea}$ min	v_{nic} mm/min	v_{cot} mm/min	v_{urea} mm/min	$Q_{inj}1$ (ng)	$Q_{inj}2$ (ng)	α
0	10.49	14.56	41.55	60.60	37.92	20.45	2.30	2.30	0.87
6.5	10.52	14.45	41.58	60.36	38.39	20.44	3.17	2.94	0.87
10	10.44	14.44	41.79	61.05	38.79	20.34	3.66	3.28	0.87
20	10.05	14.19	40.05	63.35	38.68	21.22	5.12	4.30	0.86
26.5	10.33	14.74	44.18	63.05	38.42	19.24	5.93	4.85	0.86
30	10.40	14.63	42.19	61.60	37.94	20.15	6.39	5.20	0.87

Sample stacking was performed at 205 nm. Laboratory temperature = 20 °C. Subcripts (nic, cot, and urea) denote nicotine, cotinine, and thiourea respectively. HE injection was accomplished by introducing the sample at 25 mbar + at a range of voltages, for 0.2 min. Separation was carried out at 26.5 kV. Results were obtained from CE-sample stacking/ UV. The viscosity of the buffer system was calculated as 0.51 cP. $Q_{inj} = (V_i / 1 \times 10^6) \times c \times 1000$ (equation 3.2); $L_p = (\Delta P \times t \times d^2) / (53.3 \times \eta \times L) + (v + v_{EOF}) \times t_m \times (V_{inj} / V_{sep})$ (equation 3.3); $V_i = (r \times 1 \times 10^6)^2 \times (L_p \times 1 \times 10^3) \times \pi \times 1 \times 10^{12}$ (equation 3.4). $Q_{inj}1$ and 2 are nicotine and cotinine respectively, where Q_{inj} is the injected quantity; V_i is the injected volume (nL); c is the concentration in µg/ mL; L_p is the sample plug length; ΔP is the pressure across the capillary; t is the injection time (min); d is the capillary inner diameter (75 µm); η is the viscosity of the sample solution; L is the total length of the capillary (100cm); t_m is the migration time; v is the velocity of the analyte or the EOF in the case of v_{urea}; V_{inj} is the injection voltage; V_{sep} is the separation voltage. α = selectivity, i.e. $\alpha = (t_2 - t_{nm})/(t_1 - t_{nm})$ (equation 3.5) [1], where t_2 and t_1 are migration times of adjacent peaks and t_{nm} is the migration time of a neutral marker.

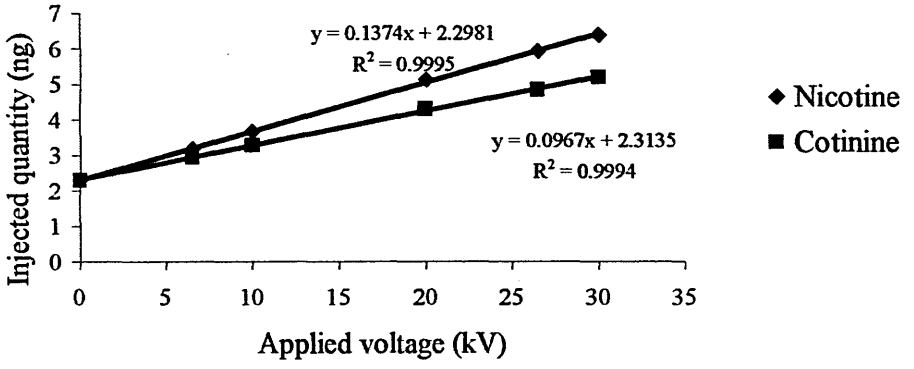
Since a UV detector was used for the above study, it was necessary to try and reproduce the same conditions as experienced by the mass spectrometer. It should be remembered that the voltage used in the MS experiments was 3.5 kV at the capillary outlet. This creates a potential difference along the fused silica capillary of 26.5 kV, when 30 kV was applied at the inlet end. Thus in order to determine the correct amounts of sample injected, separation would have to be conducted under this potential difference.

When considering electrokinetic injection it is important to note that solutes are carried into the capillary by the electroosmotic flow and the electrophoretic mobility. Therefore both electroosmotic velocity and the electrophoretic velocity of the solute ion will have to be calculated, since both are dependent upon the voltage applied. Measurement of the former requires the introduction of a neutral marker (as neutral solutes are pulled into the capillary by just the EOF), such as thiourea, for measurement. Hydrodynamic injection, however, is mainly dependent on the length of the capillary, the capillary diameter, the pressure applied, and the viscosity (table 3.3).

The charge states of nicotine and cotinine within the solvent mixture at pH 7 would be +1 and 0 respectively. This was probably due to pyrrolidine N of nicotine having a pKa between 9 and 10, whilst the pyrrolidine N of cotinine has a pKa of –0.41. Therefore in theory there should be no increase in the amount of cotinine introduced to the capillary. This was observed to some extent in figure 3.1 where there was a significantly greater linear increase in peak area for nicotine, at increasing voltage, when compared to that of cotinine. In theory if cotinine was charged then it too would show a linear increase in amount introduced to the capillary (as shown in figure 3.4 and table 3.3). Thus the calculated amounts (ng) shown in figure 3.4 and table 3.3 rely on the assumption that

cotinine is charged since the electrokinetic part of the technique is dependent on the voltage applied. But in practice this was not the case (refer to figures 3.1).

Figure 3.4. Injected quantity versus applied voltage for HE injection of nicotine and cotinine.

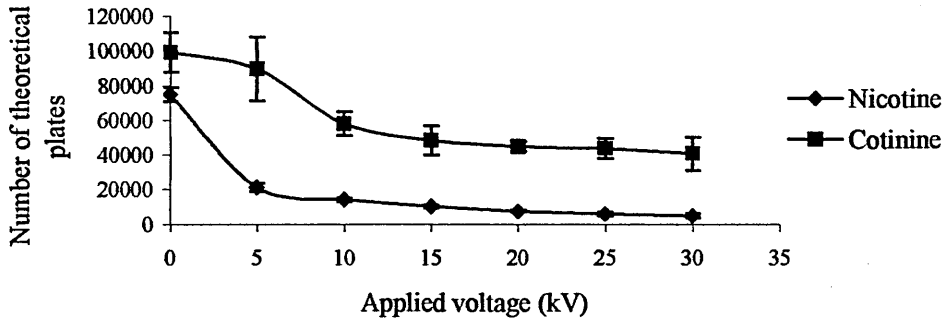


Sample stacking was performed at 205 nm. 50 µg/mL nicotine and 50 µg/mL cotinine were made up in 25 % H_2O/75 % MeCN. The buffer system consisted of 10 mM ammonium formate (pH adjusted to 2.5 with HCl), 75 % MeCN/25 % H_2O; 100 cm, 75 µm i.d. fused silica capillary was used; the sheath flow rate was 5 µL/ min; the sample was introduced at a range of voltages + 25 mbar for 0.2 min. Separation took place at 30 kV only. Error bars were a product of the standard deviation (n = 7).

Figure 3.4 shows a linear increase in the injected quantities of both nicotine and cotinine relative to increased voltage for HE injection. Figure 3.4 also shows the clear sampling bias for nicotine with respect to cotinine for this injection method at increasing voltages. This confirms that greater quantities of nicotine were being introduced into the capillary than cotinine. The greater quantities of sample introduced into the capillary at higher voltages for HE injection corresponds with the decline in peak efficiency (figure 3.5) and resolution (table 3.2).

It is interesting to note that selectivity between nicotine and cotinine remained constant at all the voltages applied for HE injection (table 3.3). However this is perhaps not surprising as selectivity is only a measure of the distance between two solute peaks, whereas resolution, which is a measure of separation between two adjacent peaks, was most likely to succumb to the effects of increasing peak broadening at higher voltages for HE injection, since resolution is partly dependent upon the peak width. Thus the constant selectivity observed between nicotine and cotinine peaks at all the applied voltages for this injection method further confirmed the significance of peak broadening at higher voltages, as only peak-to-peak resolution was compromised at higher kV for HE injection (table 3.2).

Figure 3.5. Peak efficiency versus applied voltage (kV) for HE injection of nicotine and cotinine.



Sample stacking was performed at 205 nm. 50 μg/mL nicotine and 50 μg/mL cotinine were made up in 25 % H_2O/75 % MeCN. The buffer system consisted of 10 mM ammonium formate (pH adjusted to 2.5 with HCl), 75 % MeCN/25 % H_2O; 100 cm, 75 μm i.d. fused silica capillary, with effective length 85 cm; the sheath flow rate was 5 μL/ min; the sample was introduced at a range of voltages + 25 mbar for 0.2 min. Separation took place at 30 kV only. $N = 5.54(t/w_{1/2})^2$ (equation 3.1) [1]. Error bars were a product of the standard deviation (n = 7).

As expected peak efficiency was at its highest when only hydrodynamic injection was applied (i.e. at 0 kV on figure 3.5) and declined at higher voltages for HE injection.

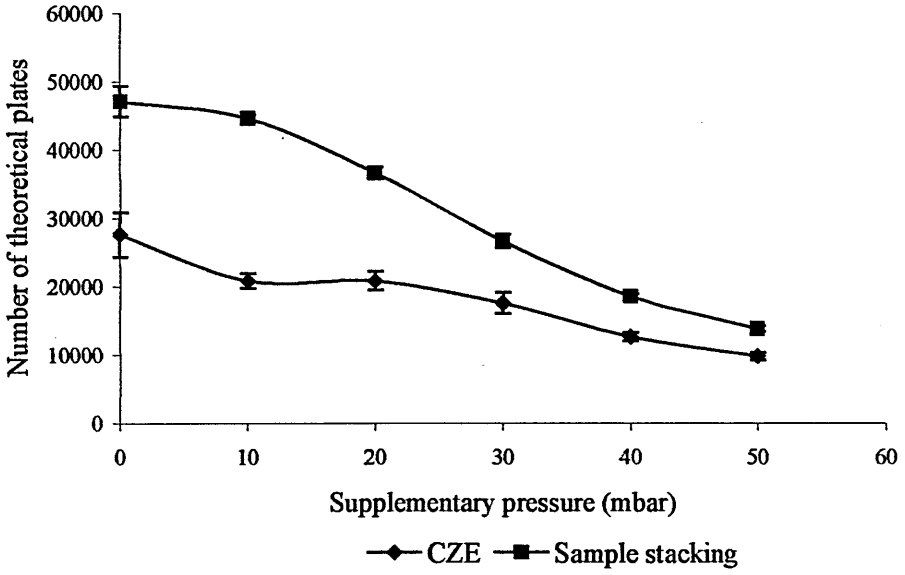
Cotinine exhibited the greatest number of theoretical plates, with the greater decline between 5 and 10 kV for HE injection. The number of theoretical plates declined to a lesser extent between 10 and 15 kV, and then began to plateau. Nicotine, on the other hand, had the greatest decline in theoretical plate numbers from 0 to 5 kV. However, from 5 to 30 kV, theoretical plate numbers for the nicotine were only diminished by approximately 15,000 theoretical plates. Values obtained for hydrodynamic injection were in keeping with the theoretical plate numbers of those shown in table 2.5.

When considering the optimum peak efficiency, voltages of 10 kV and below were desirable for cotinine, whereas those of 5 kV and below were desirable for nicotine (figure 3.5). However, in terms of separation, the greatest resolutions were obtained from voltages of 10 kV and below (figure 3.2).

3.3.2 Supplementary pressure optimisation

As already mentioned, supplementary pressure was used to prevent the outlet-end of the fused silica capillary from getting blocked, drying out (which can result from an influx of air), and to prevent siphoning effects when the CE system was coupled to the mass spectrometer [2,3]. Thus it was necessary for some supplementary pressure to be applied to the inlet end during separation.

Figure 3.6. The effect of supplementary pressure on peak efficiency.



Sample stacking was performed at 205 nm. 50 μg/mL cotinine was made up in 25 % H_2O/75 % MeCN. The buffer system consisted of 10 mM ammonium formate (pH adjusted to 2.5 with HCl), 75 % MeCN/25 % H_2O; 100 cm, 75 μm i.d. fused silica capillary, whose effective length was 85 cm; the sheath flow rate was 5 μL/ min; the sample was introduced at 25 mbar for 0.2 min. Separation took place at 30 kV + at a range of mbars. $N = 5.54(t/w_{1/2})^2$ (equation 3.1). Error bars were a product of the standard deviation (n = 7).

Figure 3.6 shows clearly the higher theoretical plate numbers exhibited by sample stacking with respect to CZE. This further emphasises the justification for using sample stacking rather than CZE. Figure 3.6 shows a steady decline in theoretical plate numbers for both sample stacking and CZE at increasing increments of supplementary pressure. For sample stacking there is a slight decrease in theoretical plate numbers between 0 and 10 mbar of applied supplementary pressure. However, it could also be said that acceptable numbers of theoretical plates are achieved at 20 mbar for sample stacking. CZE exhibits no significant change in theoretical plate numbers between 10 and 20 mbar. But there is a minor increase of approximately 8000 theoretical plates from 10 to 0 mbar for CZE. This

suggests that acceptable numbers of theoretical plates are achieved from 0 to 20 mbar supplementary pressure.

It should be noted that the lower theoretical plate numbers achieved in figure 3.6 may also have been the result of using a different capillary, which might also account for the lower peak-to-peak resolution observed (refer to tables 2.8 and 2.9).

The small variations in migration times observed was probably in this case accounted for by changes in ambient temperature, since the laboratory was not climate controlled (tables 2.8 and 2.9)

Table 3.4. The effect of varying supplementary pressure on the resolution of nicotine and cotinine for CZE (n = 7).

mbar	t_{nic} (min)	t_{cot} (min)	w_{nic} (min)	w_{cot} (min)	Resolution	t_{nic} %RSD	t_{cot} %RSD
0	9.15	10.98	0.24	0.35	6.21	1.4	1.38
10	7.39	8.55	0.23	0.32	4.25	0.97	0.74
20	6.45	7.29	0.28	0.33	2.76	0.79	0.62
30	5.86	6.50	0.28	0.30	2.21	0.92	0.99
40	5.12	5.61	0.33	0.40	1.34	0.54	0.52
50	4.572	4.96	0.27	0.35	1.25	0.27	0.26

Sample stacking was performed at 205 nm. Subcripts denote nicotine and cotinine respectively; t and w are the migration time and peak width respectively. 50 μg/mL nicotine and 50 μg/mL cotinine were made up in 25 % H_2O/75 % MeCN. The buffer system consisted of 10 mM ammonium formate (pH adjusted to 2.5 with HCl), 75 % MeCN/25 % H_2O; 100 cm, 75 μm i.d. fused silica capillary, with effective length 85 cm; the sheath flow rate was 5 μL/ min; the sample was introduced at 10 kV + 25 mbar for 0.2 min. Separation took place at 30 kV + at a range of mbars..

Table 3.5. The effect of varying supplementary pressure on the resolution of nicotine and cotinine sample stacking (n = 7).

mbar	t_{nic} (min)	t_{cot} (min)	w_{nic} (min)	w_{cot} (min)	Resolution	t_{nic} %RSD	t_{cot} %RSD
0	9.61	11.41	0.20	0.32	6.97	0.52	0.51
10	7.68	8.80	0.17	0.29	4.86	0.34	0.39
20	6.18	6.89	0.20	0.27	3.00	0.32	0.43
30	5.32	5.84	0.19	0.27	2.28	0.56	0.61
40	4.69	5.10	0.22	0.26	1.70	0.94	0.92
50	4.22	4.54	0.21	0.24	1.42	1.70	1.77

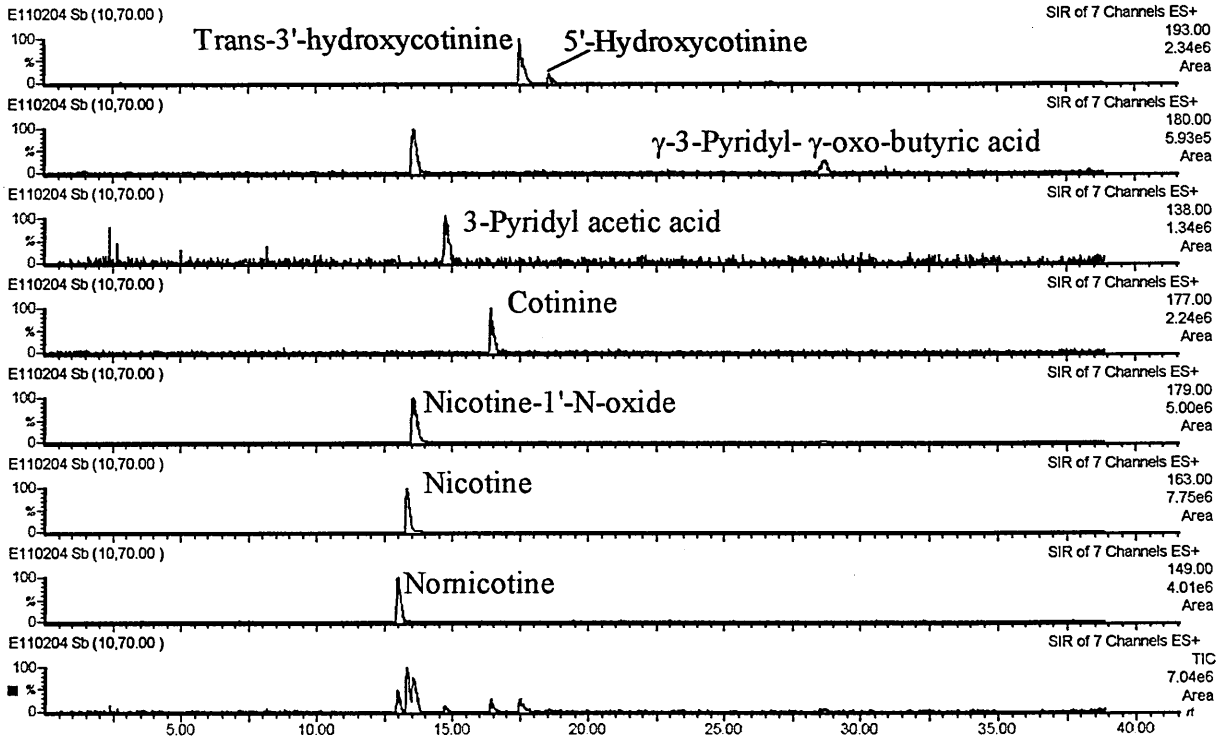
Sample stacking was performed at 205 nm. Subcripts denote (nic and cot) nicotine and cotinine respectively; *t* and *w* are the migration time and peak width respectively. 50 µg/mL nicotine and 50 µg/mL cotinine were made up in 25 % H_2O/75 % MeCN. The buffer system consisted of 10 mM ammonium formate (pH adjusted to 2.5 with HCl), 75 % MeCN/25 % H_2O; 100 cm, 75 µm i.d. fused silica capillary, with effective length 85 cm; the sheath flow rate was 5 µL/ min; the sample was introduced at 10 kV + 25 mbar for 0.2 min. Separation took place at 30 kV + at a range of mbars.

Tables 3.4 and 3.5 both show a reduction in resolution as a result of increasing supplementary pressure, due to the flow profile becoming increasingly more laminar. That is, the flow profile increasingly resembles that of a pressure driven system. The resolutions exhibited by both CZE and sample stacking were very similar, with the latter exhibiting only a slight improvement. However, the initially recorded resolution between nicotine and cotinine i.e. that recorded without supplementary pressure was lower than that previously reported (table 3.2). This may have been due to the elevated external temperature causing a rise in internal capillary temperature, which probably lead to some zone spreading and hence a reduction in resolution.

3.3.3 Optimised HE injection and supplementary pressure for the analysis of nicotine and its metabolites.

Ideally the best separations are achieved with low voltage applied for HE injection (i.e. 10 kV) and without supplementary pressure. These conditions were then transferred to the mass spectrometer and the optimised coaxial probe arrangement was used throughout (refer to section 2.3.1).

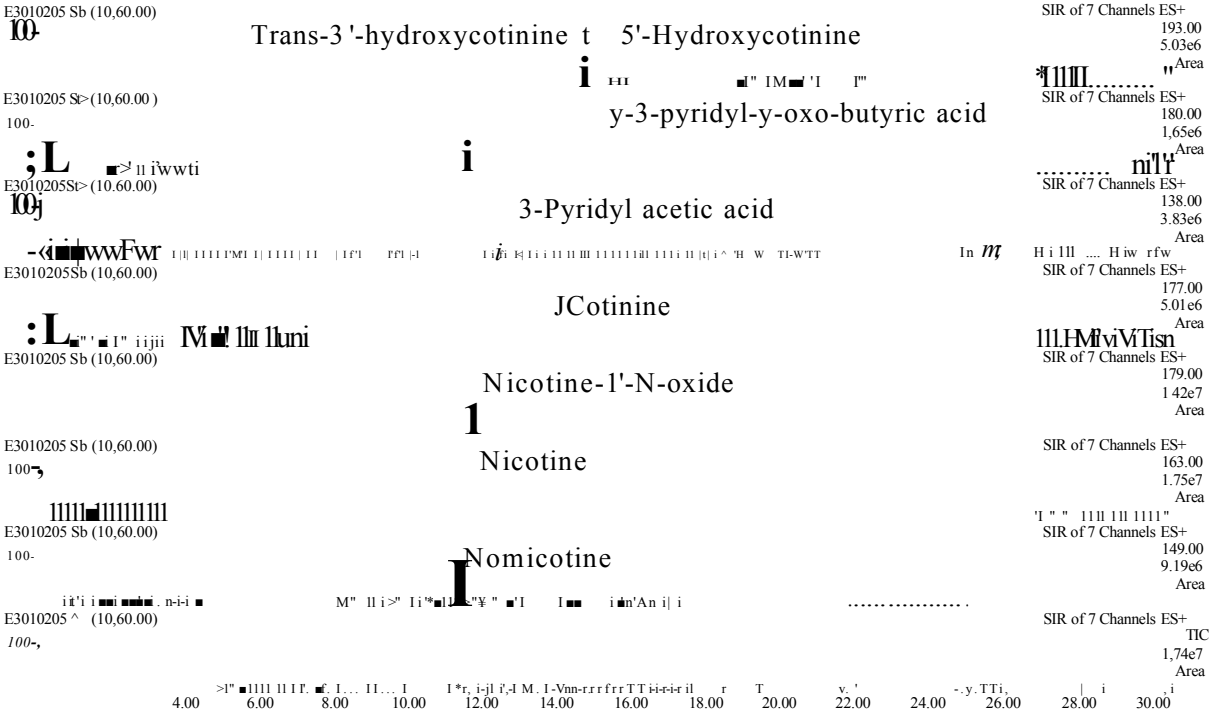
Figure 3.7. SIR mass electropherogram of nicotine and seven of its metabolites, obtained via HE-sample stacking/MS with no supplementary pressure during separation.



50 µg/mL nicotine, .nornicotine, cotinine, nicotine-1'-N-oxide, 5'-hydroxycotinine, and cotinine-1-N-oxide were made up in 25 % H_2O/75 % MeCN. The buffer system consisted of 10 mM ammonium formate (pH adjusted to 2.5 with HCl), 75 % MeCN/25 % H_2O; 100 cm, 75 µm i.d. fused silica capillary was used; the sheath flow rate was 5 µL/ min; the sample was introduced at 10 kV + 25 mbar for 0.2 min. Separation took place at 30 kV. Reproducibility was determined by the RSD of the resolution (mean = 10.45; n = 3) between nicotine and cotinine (RSD = 3.99 %).

The advantages of using no supplementary pressure and a lower voltage (10 kV) for HE injection were that extremely good separation and highly efficient peaks (with theoretical plate numbers of 31164 and 51066 obtained for nicotine and cotinine respectively) were obtained (figure 3.7). The most notable drawback of this method, however, was the relatively long analysis time of almost 30 minutes. Another major disadvantage of not using supplementary pressure was that, on numerous occasions, air entered the capillary outlet (as a result of siphoning) causing considerable fluctuations in the current. Thus only three out of eight runs were successful. Therefore some supplementary pressure was required to improve the robustness of this method. The lowest supplementary pressure considered was 10 mbar (figure 3.8).

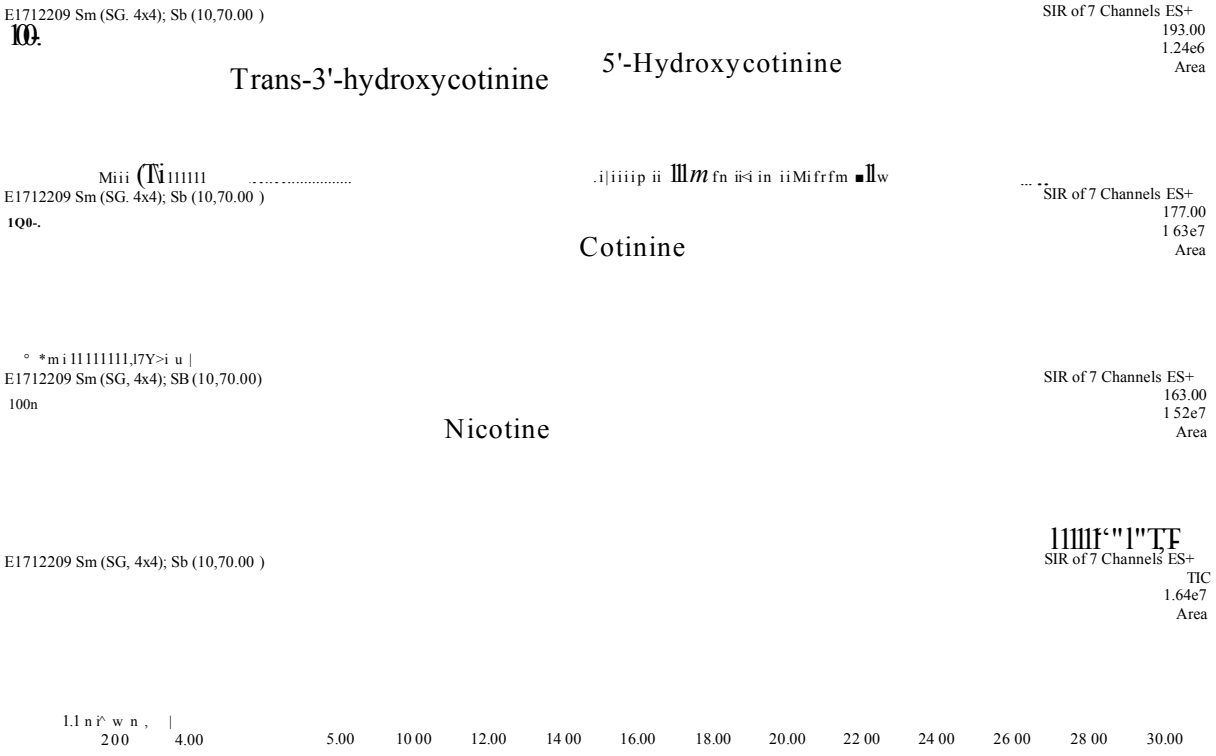
3.8 SIR mass electropherogram of nicotine and seven of its metabolites obtained via HE-sample stacking/MS and the application of 10 mbar supplementary pressure during separation.



50 pg/mL nicotine, nomicotine, cotinine, nicotine-1'-N-oxide, 5'-hydroxycotinine, and cotinine-1-N-oxide were made up in 25 % H_2O/75 % MeCN. The buffer system consisted of 10 mM ammonium formate (pH adjusted to 2.5 with HC1), 75 % MeCN/25 % H20; 100 cm, 75 pm i.d. fused silica capillary was used; the sheath flow rate was 5 pL/ min; the sample was introduced at 10 kV + 25 mbar for 0.2 min. Separation took place at 30 kV + 10 mbar supplementary pressure. Reproducibility was determined by the RSD of the resolution (mean = 9.64; n = 5) between nicotine and cotinine (RSD = 4.83 %).

Figure 3.8 shows good separation, highly efficient peaks (with theoretical plate numbers of 33614 and 50589 obtained for nicotine and cotinine respectively), and a reduction in the analysis time. This method was then considered for the analysis of a smoker's urine sample (after SPE pre-concentration).

Figure 3.9. SIR mass electropherogram of a smoker's urine sample obtained via HE-sample stacking/MS.

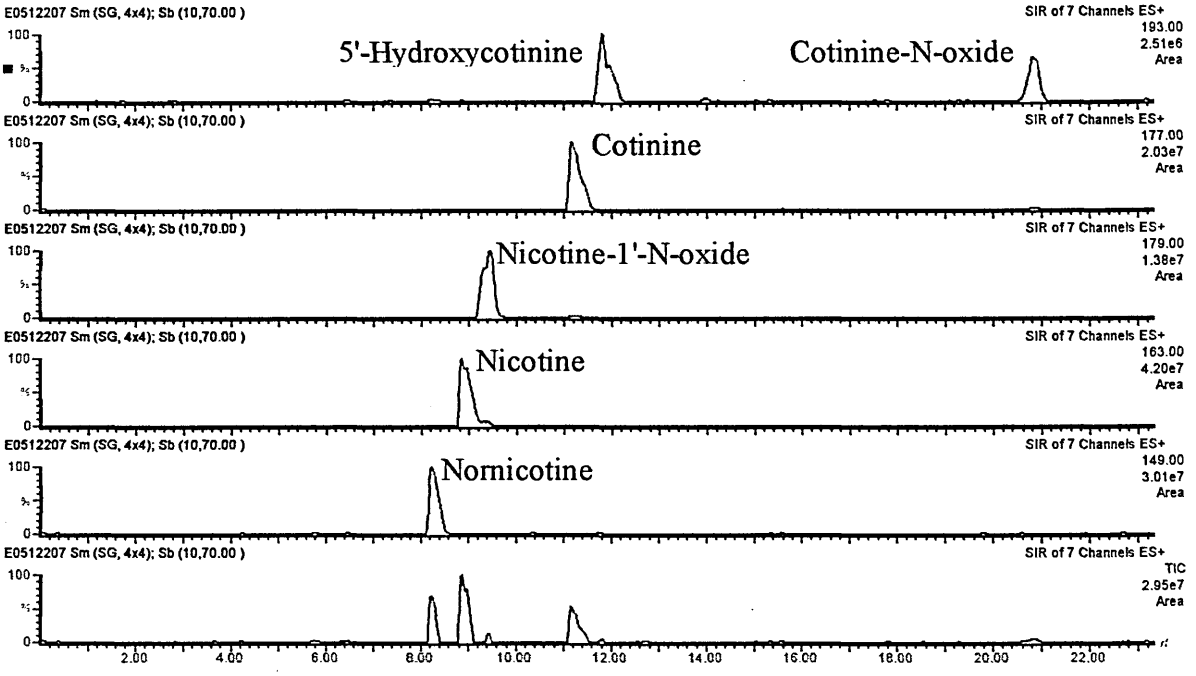


Two hundred fold pre-concentration of a smoker's urine via SPE (made up in 25 % H20/75 % MeCN). The buffer system consisted of 10 mM ammonium formate (pH adjusted to 2.5 with HC1), 75 % MeCN/25 % H20; 100 cm, 75 pm i.d. fused silica capillary was used; the sheath flow rate was 5 pL/ min; the sample was introduced at 10 kV + 25 mbar for 0.2 min. Separation took place at 30 kV + 10 mbar supplementary pressure. Reproducibility was determined by the RSD of the resolution (mean = 10.02) between nicotine and cotinine (RSD = 4.01 %, n = 3).

Figure 3.9 shows the clear identification of nicotine, cotinine, trans-3'-hydroxycotinine, and 5'-hydroxycotinine from a smoker's urine. This mass electropherogram proved that the applied 10 kV, for HE injection, was sufficient to provide the sensitivity needed to identify nicotine and its metabolites. The 10 mbar supplementary pressure ensured good separation as well as high peak efficiency.

An optimised voltage for HE injection was also achieved together with an optimised supplementary pressure for a predominantly aqueous system (figure 3.10).

Figure 3.10. SIR mass electropherogram of nicotine and five of its metabolites obtained via HE-sample stacking/MS.



50 µg/mL nicotine, normicotine, cotinine, nicotine-1'-N-oxide, 3-pyridyl acetic acid, 5'-hydroxycotinine, and cotinine-1-N-oxide were made up in buffer. The buffer system consisted of 25 mM ammonium formate (pH adjusted to 2.8 with formic acid) + 10 % MeCN; sheath liquid was MeCN/ H_2O 1:1 + 0.1 % formic acid; 100 cm, 75 µm i.d. fused silica capillary was used; the sheath flow rate used was 5 µL/ min; sample was introduced at 25 mbar +10 kV for 0.2 min; CZE separation took place at 30 kV and 30 mBar supplementary pressure. Reproducibility was determined by the RSD of the resolution (mean Res = 4.95) between nicotine and cotinine (RSD = 4.14, n = 3).

Figure 3.10 shows a good separation of nicotine and five of its metabolites. The migration times resemble those observed by Palmer *et al.* [4]. However, the electrical contact established was not maintained for long periods and only three successive runs were

achieved. The non-homogenous nature of the sheath liquid, with respect to the composition of the run buffer, appears to have weakened the electrical contact. However, there was a slight improvement in spray stability, which was probably due to the withdrawal of the fused silica capillary into the sheath tube (until they were at the same level), as compared with the previous arrangement (refer to section 2.3.1). Potentially the aqueous systems offer greater separation efficiency and sensitivity, due to their greater conductive nature.

3.4. Conclusion

Optimum peak efficiency and separation is obtained if a sample is hydrodynamically injected but this causes a reduction in sensitivity, since less sample is introduced into the capillary compared to HE-injection. This is especially true for use with the mass spectrometer, since this detector is concentration dependent. It is also important to note that the highest peak efficiencies and separations were only obtained when no supplementary pressure was applied during separation, but severe fluctuations in current (due to the influx of air at the outlet end of the fused silica capillary) were also noticed.

Daily changes in laboratory temperature did not affect reproducibility, but appeared to affect peak efficiency and resolution. Thus climate-controlled laboratories should be welcomed for this type of analysis.

Sensitivity and throughput are also important parameters when considering the analysis of multiple compounds within a sample. In the case of the former the optimal voltage (e.g. 10 kV), which is one that achieves great sensitivity enhancements without significantly compromising resolution and peak efficiency, is required. But the effect of HE injection on resolution and peak efficiency, at higher voltages, is dependent upon analyte concentration,

prior to injection. Thus if the analyte concentration is relatively high then lower voltages may be applied for HE injection, but if it is low, as in the case of most biological samples, then higher voltages can be used.

The application of supplementary pressure during separation may increase the throughput of analyses, but cause a reduction in resolution. There has to be a careful balance between obtaining high quality data and generating fast analysis times. The optimum appears to be 10 mbar and below, where acceptable separations and theoretical plate numbers are achieved. However, since separation and peak efficiency are also dependent upon analyte concentration, both variables should be taken into account when considering the supplementary pressure applied.

Higher throughput analyses could be achieved by raising the concentration of the run buffer (i.e. between 25 and 50 mM), which would improve separation and analyte pre-concentration, as well as peak efficiency, and would allow for higher supplementary pressures to be applied.

When applying the optimised method to a real sample (e.g. from SPE pre-concentration of urine), initially that sample would be spiked with a known quantity of internal standard, from which it would be possible to determine the relative concentrations of nicotine and its metabolites.

We have recently developed a counterflow transient isotachophoresis (tITP) method, with MS detection, for the analysis of even lower analyte concentrations. A major advantage of this method was that less sample pre-concentration by SPE was required, due to the higher pre-concentrating capability of tITP with respect to sample stacking. Thus by using less sample pre-concentration via SPE lower levels of urobilinogens/urobilins will be present in

the sample, which would mean that separation would be less hampered by changes in sample viscosity. This is reported in the next section.

3.5. References

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Chapter 4

The Determination of Nicotine and its Metabolites, in Urine, by Transient Isotachophoresis/Mass Spectrometry.

4.1. Introduction

The major limitation of capillary zone electrophoresis (CZE) is low concentration sensitivity [1]. This is due primarily to the extremely small dimensions of the fused silica capillary (25-150 i.d. and 40 cm - 1 m length), which only allows for small sample volumes (usually less than 50 nL) to be loaded onto the column, as well as a drastically reduced path-length for the most common optical detection techniques, including UV detection [2-4]. As already mentioned, the dilution effect of the sheath liquid also reduces sensitivity during mass spectrometric analysis.

To greatly improve the sensitivity of CE, larger volumes of sample need to be introduced into the capillary [5,6]. But the introduction of a large dilute sample plug ensures that there is a high field strength is in the solute zone, with the field strength in the run buffer at times becoming extremely low. Therefore when solute ions reach the run buffer their electrophoretic velocities can drop to near zero. In such circumstances solute ions are not very well separated. In order to minimise the problems associated with injection of large sample volumes isotachophoresis can be employed [7].

The German chemist Kohlrausch laid down laws governing isotachophoretic separation in 1897, but isotachophoresis was only established as a full working method during the

1970s. Much of the work reported since then has been on devising a theoretical description of the dynamics of the separation, and calculating the composition of the electrophoretic zones [8].

Isotachophoresis

Electrophoretic pre-concentration can be brought about through the use of continuous or discontinuous buffers. However, the former is mainly suitable for samples in non-ionic matrices. This technique does not work well for trace components present in complex ionic matrices because of CZE overloading, leading to poor resolution [9]. For isotachophoresis (ITP) the electroosmotic flow (EOF) is usually kept to a minimum (by using very low pH buffers) or removed entirely (by coating the capillary with a hydrophilic polymer) as the presence of the EOF would otherwise limit the focusing effect.

In capillary ITP the sample plug is placed between the leading and terminating buffers, in the capillary, and a voltage at a constant current is applied. Leading electrolyte ions have greater mobilities than the mobilities of ions present in the sample, whilst terminating electrolyte ions have the lowest mobilities [7,10,11]. For cations, the application of an electric field will cause cations in the leading buffer to move the fastest. At the point of focusing, cations in the sample arrange themselves in order of mobility, with those of the highest mobility next to the leading electrolyte, whilst those of the lowest mobility are next to the terminating electrolyte [12]. After solutes distribute themselves in the capillary, an equilibrium is reached and hence a steady state. In this situation all buffer and solute cations migrate at the same velocity, v_L, the velocity of the leading cations. Hence the term isotacho, which means constant velocity [7]

$$v_L = \mu_{EP} E \qquad (4.1)$$

where μ_{EP} is the electrophoretic mobility, and E is the electric field (where a different electric field develops in each zone) [7] E is highest in the zone with the lowest mobility since

$$t = l/\mu_{OBS} E \qquad (4.2)$$

and therefore

$$E = l/t\mu_{OBS}$$

where t is the migration time and μ_{OBS} is the observed mobility. The boundaries between each zone are extremely sharp since a solute that migrates into a different zone experiences a different electric field, which inevitably causes it to diffuse back into the zone with the appropriate electric field for its mobility [2,7]. When a steady state is reached each analyte moves as a discrete band according to its mobility. Bands with the highest mobility will elute before those of lowest mobility. During the steady state, all zones migrate with equal velocity, have distinct borders, and the discreteness in field strengths between each zone corrects diffusional band broadening. The steady state concentrations of the compound zones are proportional to the concentration of the leading buffer. From the Kohlrausch regulating function an equation linking the concentration of solute ions to those ions of the leading buffer has been derived [7]. The concentration of solute ions, C_S, is related to the concentration of the leading ions, C_L, in the following way

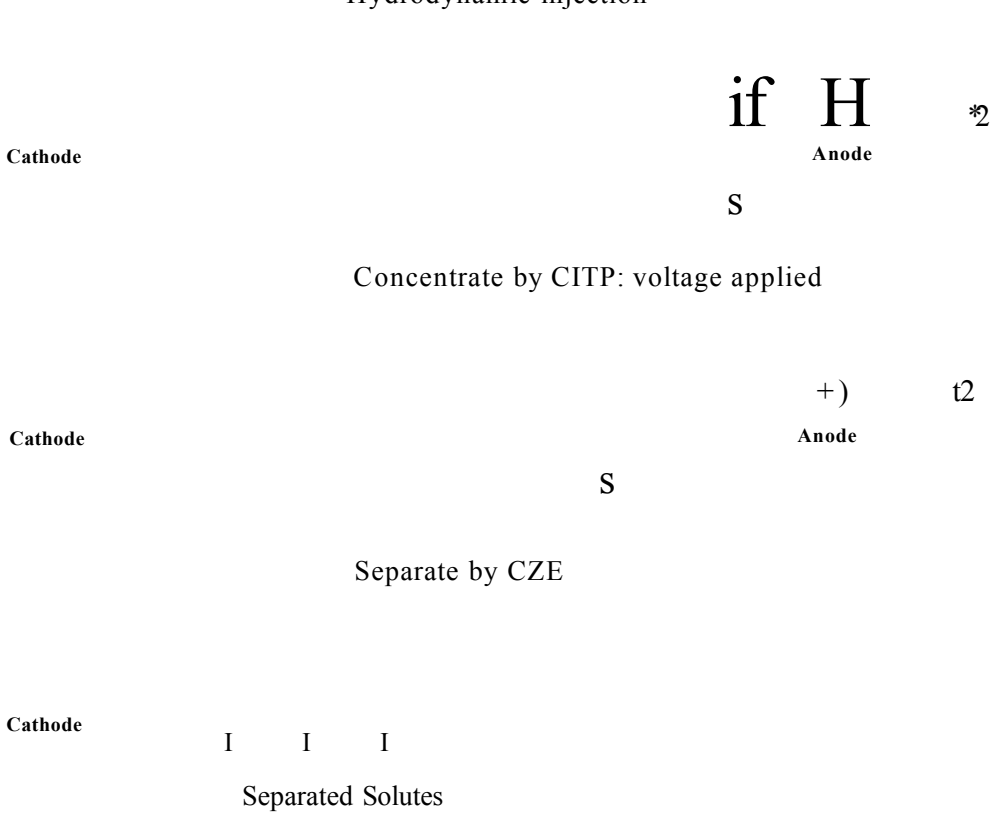
$$C_L/C_S = [\mu_L/(\mu_L + \mu_C)][(\mu_S + \mu_C)/\mu_S] \qquad (4.3)$$

where μ is the electrophoretic mobility, the subscript L represents the leading ion, S represents the solute co-ion, and C the counter-ion. The final concentration may be given by [7]

$$C_S = C_L k \qquad (4.4)$$

where A is a proportionality factor (between 0.2 and 1 for common electrolytes). This is equal to 1 when the concentration of solute ions becomes the same as the concentration of ions in the leading buffer [7].

Figure 4.1. A schematic representation of the transient isotachophoresis procedure for the separation of cations (adapted from Baker, 1995) [7].



C is the co-ion (leading electrolyte), T is the terminating ion, and S is the sample plug.

Continuation of the separation in the CZE mode may be performed by placing the capillary inlet in the leading electrolyte. When the leading electrolyte catches up with the sample zone the field gradient will be lost and CZE separation will begin. Since ITP only takes place for a short period of time the technique is termed “transient” ITP [7].

On-line ITP can be either on-column or coupled-column in arrangement. Two disadvantages of the coupled column procedure are that the apparatus has to be modified and it is difficult to automate these procedures [13]. Since the on-column procedure gradually changes from ITP to CZE it is referred to as on-column transient isotachophoresis (tITP). In this arrangement the analysis is conducted such that both ITP pre-concentration and CZE separation proceed in the same capillary [14,15].

Interfacing a CE-tITP system to a triple quadrupole mass spectrometer would ensure the provision of structural information on compounds after separation and enables the clear identification of selected analytes within complex mixtures [16].

Aims and Objectives

The aim of this study was to produce a highly sensitive transient isotachophoretic/MS method that was complementary to the CE-sample stacking/MS method used for the analysis of nicotine and its metabolites in urine (chapters 2 and 3). The optimised method should yield limits of detection comparable to that observed by HPLC/MS, and with a separation efficiency to match that of CE-sample stacking/MS.

4.2 Methods and Materials

4.2.1 Samples and Sample Preparation

Standards of nicotine and its major metabolites were prepared in the appropriate solvent mixtures (made up to 1 mM acetic acid) for tITP experiments.

Urine was obtained from five male smokers (aged 21 to 39 years), 1a to 5a, and stored at –20 °C in tissue culture flasks. Urine samples were also obtained from eleven subjects (1b to 11b), five of whom were non-smokers (7b to 11b). Prior to extraction urine samples were thawed overnight at 4 °C, thoroughly mixed and filtered with a 0.45 μm Supor acrodisc filter (Pall Corporation, Portsmouth, UK).

4.2.2 Solid Phase Extraction

Solid phase extraction (SPE) was carried out using a 100 mg C18 cartridge (Varian, Ltd, Surrey, UK). For conditioning purposes 3 mL of methanol followed by 3 mL of deionised water (Milli- Q, UK) were passed through the SPE cartridge sequentially. Then 1 mL of sample was introduced into the cartridge followed by 6 mL of deionised water. It should be noted that for 2 and 10 fold pre-concentrations, 5 and 10 mL of sample were introduced respectively. The sample was then eluted with 1 mL of methanol. The eluted product was dried with nitrogen gas. The dried product was then reconstituted with 1 mL of mobile phase for recovery experiment or 1 mL of solvent mixture for tITP.

4.2.3 Buffer and Mobile Phase Preparation

A suitable quantity of ammonium salt (Sigma-Aldrich, Dorset, UK) was dissolved in the solvent mixture (acetonitrile [Fischer Scientific, Manchester, UK]/ deionised water [Milli-

Q, UK]) as the leading buffer; L-alanine was dissolved the appropriate solvent mixture (acetonitrile/ deionised water [Milli-Q, UK]) as the terminating buffer; buffer pH's were adjusted with formic, acetic acid or HCl (Sigma-Aldrich, Dorset, UK), as appropriate, and were freshly prepared. Buffers were degassed prior to analysis using a Branson 1210 ultrasonic bath (Branson Ultrasonics, Danbury, CT, USA) and filtered through a 0.2 μm Supor acrodisk syringe filter (Pall Corporation, Portsmouth). The buffers prepared were 10 mM ammonium formate (10 % acetonitrile, 90 % deionised water), which was adjusted to pH 2.5 with formic acid; 10 mM ammonium acetate (in deionised water) adjusted to pH 2.5 with acetic acid; 10 mM ammonium formate (75 % acetonitrile, 25 % deionised water) adjusted to pH 2.5 with HCl; 10 mM L-alanine (dissolved in the appropriate solvent mixture) adjusted to pH 2.5 with either acetic acid or HCl depending on the composition of the leading buffer system.

4.2.4 TITP Separation

TITP was carried out in either a 65 (50 or 75 μm i.d. x 365 μm o.d) or a 100 cm (75 μm i.d. x 365) μm o.d., untreated fused silica capillary (Composite Metal Services Ltd., Hallow, UK). The CE/UV system used (Crystal CE System, PrinCE Technologies, Lauerlabs, Emmen, Netherlands) utilizes programmable injection with pressure and voltage control. The detection window was burnt into the fused silica capillary 15 cm from the end for CE/UV and analyses were performed at 205 nm. Separations were achieved using +30 kV. Data acquisition was performed by DAx 6.0 software (PrinCE Technologies) for CE/UV. Injections were performed either hydrodynamically (25 mbar/ 0.2 min) or hydrodynamically + electrokinetically (HE: 25 mbar + x kV/ 0.2 min). Initial conditioning of the capillary took place with 1 M NaOH (5 min / 2000 mbar [Sigma-Aldrich, Dorset,

UK]) followed by the buffer system (7 min/ 2000 mbar). The capillary was conditioned prior to each run with 1 M NaOH (2000 mbar/ 1min) followed by buffer system (2000 mbar/ 1.5 min). The buffer system was replenished after each run cycle.

4.2.4a TITP separation steps for a 65 cm, 50 µm i.d. capillary using 10 mM ammonium acetate (100 % deionised water) as the leading buffer; 10 mM L-alanine (100 % deionised water) as the terminating buffer

1) Leading buffer (capillary inlet): + 1000 mbar and 30 kV for 0.3 min.
2) Sample (capillary inlet): + 75 mbar and 0 kV for 1.85 min (approximately 10 cm injection plug).
3) Terminating buffer (capillary inlet): + 75 mbar for 0.185 min (approximately 1 cm injection plug).
4) Terminating buffer (capillary inlet), tITP step: -36.0 mbar and 30 kV, for 0.2 min.
5) Terminating buffer (capillary inlet), counterflow (counterflow step 5 [CF_5]): - 75 mbar for 0.90 min.
6) Leading buffer (capillary inlet): CZE separation –30 kV only (unless otherwise stated) for x min (where x is the number of minutes).

4.2.4b TITP separation steps for a 65 cm, 75 µm i.d. capillary using 10 mM ammonium acetate (100 % deionised water) as the leading buffer; 10 mM L-alanine (100 % deionised water) as the terminating buffer

7) Leading buffer (capillary inlet): + 1000 mbar and 30 kV for 0.3 min.
8) Sample (capillary inlet): + 75 mbar and 0 kV for 0.82 min (approximately 10 cm injection plug).

9) Terminating buffer (capillary inlet): + 75 mbar for 0.08 min (approximately 1 cm injection plug).

10) Terminating buffer (capillary inlet), tITP step: -18.0 mbar and 30 kV, for 0.2 min.

11) Terminating buffer (capillary inlet), counterflow (CF_5): - 75 mbar for 0.64 min.

12) Leading buffer (capillary inlet), CZE separation: -30 kV only (unless other wise stated) for x min (where x is the number of minutes).

4.2.4c TITP separation steps for a 100 cm, 75 µm i.d. capillary using 10 mM ammonium formate (90 % acetonitrile/ 10 % deionised water) as the leading buffer; 10 mM L-alanine (10 % ACN/ 90 % deionised water) as the terminating buffer

1) Leading buffer (capillary inlet): + 1000 mbar and 30 kV for 0.3 min.

2) Sample (capillary inlet): + 75 mbar and x kV for 1.26 min (approximately 10 cm injection plug).

3) Terminating buffer (capillary inlet): + 75 mbar for 0.126 min (approximately 1 cm injection plug).

4) Terminating buffer (capillary inlet): tITP - -28 mbar and 30 kV, for 0.2 min.

5) Terminating buffer (capillary inlet), counter-flow (CF_5): - 75 mbar for 0.64 min.

6) Leading buffer (capillary inlet): +75 mbar for 0.64 min.

7) Leading buffer (capillary inlet), CZE separation: + 50 mbar and 30 kV (unless otherwise stated) for x min (where x is the number of minutes).

4.2.4d TITP separation steps for a 100 cm, 75 μm i.d. capillary using 10 mM ammonium formate (75 % acetonitrile / 25 % deionised water) as the leading buffer; 10 mM L-alanine (75 % ACN/ 25 % deionised water) as the terminating buffer

1) Leading buffer (capillary inlet): + 1000 mbar and 30 kV for 0.3 min.
2) Sample (capillary inlet): + 75 mbar and x kV for 0.64 min (approximately 10 cm injection plug).
3) Terminating buffer (capillary inlet): + 75 mbar for 0.06 min (approximately 1 cm injection plug).
4) Terminating buffer (capillary inlet), tITP step: -14.0 mbar and 30 kV, for 0.2 min.
5) Terminating buffer (capillary inlet), counterflow (CF_5): - 75 mbar for 0.32 min.
6) Leading buffer (capillary inlet), CZE separation: + 30 mbar and 30 kV (unless otherwise stated) for x min (where x is the number of minutes).

4.2.5 CE/ MS

CE/ESI/MS coupling was achieved using the coaxial sheath-flow interface via a VG Quattro I triple quadrupole mass spectrometer (Micromass, Manchester, UK). The sheath liquid comprised either; 1:1 MeCN/H_2O + 0.1% formic acid, or run buffer and was delivered by a Harvard Model 11 syringe pump (Harvard Apparatus, Edenbridge, UK) at a flow rate of 3-5 μl/min. Refer to section 2.2.6 for a full description of experimental parameters.

4.3 Results and discussion

On column tITP allows injection of larger volumes of dilute sample solution than can be achieved with sample stacking or CZE [7]. Recently Toussaint *et al.* [3] described the application of a counter-flow pressure during ITP to keep the focused sample stationary near the inlet end of the capillary, prior to CZE separation. Counter-flow pressure increases the amount of sample that can be loaded onto the system and may also improve the CZE separation path length (i.e. a further counter flow may be applied in order to bring the sample plug back to the inlet end of the capillary). A counter-flow pressure can be automatically applied by commercial equipment, such as the Crystal CE system, which enables pressure fine-tuning. There is no significant loss of efficiency upon the generation of an optimised counter-flow within the capillary due to the self-correction of diffusion band broadening during ITP [3].

This approach was adopted rather than pre-concentration through a hydrophilic polymer coated capillary, since counter-flow tITP offered ease of method development and was relatively inexpensive to utilise. TITP is easily automated, with the instrumentation required being relatively simple. However, the volume of sample injected will influence the resolution. Thus the larger the sample volume, the shorter the effective length for CZE separation [17].

Counter-flow tITP is based on the measurements of the electrophoretic and hydrodynamic migration rates of the analyte during ITP-CZE. Thus by knowing the migration of the sample in the capillary during ITP, a counter-flow pressure can be applied to keep the sample stationary at the capillary inlet prior to the CZE separation [3].

A strong EOF requires a high counter-flow balance. But if this counter flow is too high then it may result in the loss of analytes. EOF velocity (v_{EOF}) is given by [17]

$$v_{EOF} = \mu_{EOF} E \qquad (4.5)$$

where μ_{EOF} is the electroosmotic mobility and E is the electric field strength. A high counter-flow pressure may also disturb the focusing process, causing loss of analytes. Thus a lower counterflow pressure provides greater sensitivity [17].

In order to reduce v_{EOF} both the electric field strength and the electroosmotic mobility may be reduced [17]. One way of doing this is to use a relatively low pH for both the leading and terminating buffers.

Since the EOF is brought about through the force exerted on any charged particle in an electric field, this force may be balanced by the frictional counterforce of the fluid surrounding the particle. Because these two forces are equal, a steady state velocity, V_{ss} achieved where [2]

$$V_{ss} = qE/f \qquad (4.6)$$

where q is the charge, E is the electric field strength, and f is the frictional coefficient.

Figure 4.2 shows a schematic representation of the counter-flow tITP procedure used for all analyses. Prior to sample injection the capillary was filled with the leading buffer (figure 4.2).

Figure 4.2. Schematic representation of the counter-flow tITP-CZE procedure for the separation of cations.

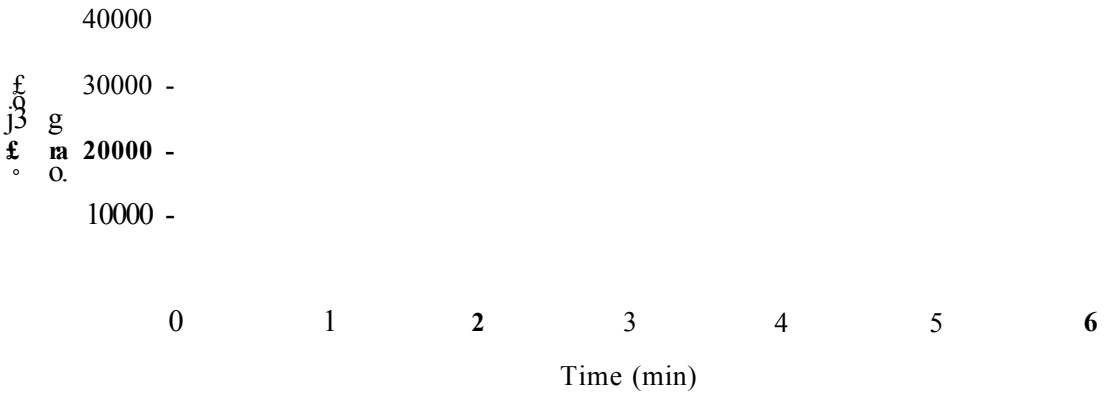
Figure 4.2 was adapted from Toussaint *et al.* [3]. Where L is the leading electrolyte, T is the terminating electrolyte, and S is the sample plug. A is the conditioning step; B is the introduction of the sample followed by the terminating buffer; C is the counter-flow tITP step; D is a further counter-flow step; E is CZE separation. Refer to section 4.2.4 for the conditions of each step.

A 10 cm sample plug was introduced to the column, followed by a 1 cm plug of terminating buffer. During the tITP step (C) the higher mobility solute ions initially move towards the boundary with the leading electrolyte, until a steady state is reach, whereas lower mobility solute ions lag behind. Thus it is essential for the leading electrolyte co-ion (ammonium) to have a higher electrophoretic mobility than the sample ions, and for the terminating electrolyte co-ion to have the lowest mobility of all [3].

Toussaint *et al.* [3] used this approach for the enantiomeric separation of clenbuterol. They incorporated 40 mM dimethyl-p-cyclodextrin into their leading electrolyte, which was 10 mM ammonium acetate, with a terminating electrolyte of 10 mM p-alanine (both at pH 2.5). This was the method initially attempted for the separation of nicotine and its metabolites. However, no cyclodextrins were used since chiral separations were not required and the p-alanine was replaced with L-alanine.

Instead of finding the optimum counter flow pressure through the use of migration studies, a slightly different approach was utilised. The only variable considered was the time for tITP to take place. By varying the time for tITP it was possible to gauge the optimum time by monitoring the quality of the data produced. Thus the number of theoretical plates for cotinine was used as a marker for the optimisation of this variable.

Figure 4.3. Number of theoretical plates obtained for cotinine in tITP as a function of tITP time.



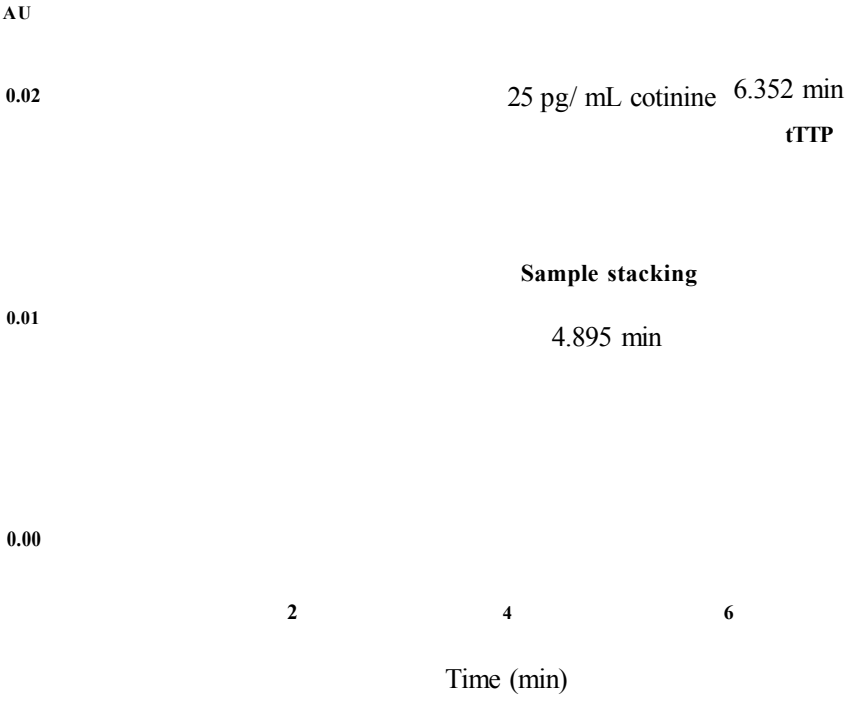
Error bars were a product of the standard deviation (n = 5). Refer to section 4.2.4a for procedure. A 65 cm capillary + 50 pm i.d. was used, with a detection window at 50 cm and a wavelength of 205 nm. $N = 5.54(//wi/2)2$ (equation 3.1) [1], where N is the number of theoretical plates, t is the migration time and $w\backslash/2$ is the peak width measured at half the peak height.

Figure 4.3 shows that the optimum time for tITP to take place was 0.25 minutes and below, since the highest numbers of theoretical plates were observed at these lower times. As there was no significant difference in theoretical plate numbers between 0.25 and 0.05 minutes, a time of 0.2 minutes was considered suitable for tITP. When tITP was employed for longer periods there was a rapid decline in the numbers of theoretical plates from 0.5 to 5 minutes.

However, these results were extremely surprising as it is generally considered that the longer the tITP process the greater the stacking effect. Therefore what was observed may have been the result of the breakdown of the tITP process, which would inevitably have led to considerable band broadening, and hence a reduction in peak efficiency. This could only have occurred if the counter flow pressure applied was not sufficient to keep the solute zone stationary during tITP focusing. In other words the counter-flow pressure applied was not adequate for the maintenance of the steady state for longer periods. Thus only for short periods of time (e.g. 0.2 mins) would this counter-flow provide the necessary steady state for tITP focusing. This is understandable as the original method was designed for clenbuterol, which when particularly complexed to a cyclodextrin has a lower charge-to-size ratio than cotinine, and hence a lower mobility through the capillary. However, what seemed to have been a problem, in the end, proved to be an advantage as the short time for tITP would inevitably lead to shorter analysis times.

A switch was made from using a 50 μm i.d. to a 75 μm i.d. fused silica capillary, as the latter produced peak heights and areas 19.3 (n =5; RSD [based on the retention time of cotinine] = 1.52 %) and 2.16 times (n =5; RSD [based on the retention time of cotinine] = 1.22 %) greater than the former. The reason for this signal enhancement was the increase in path-length for UV detection. The 75 μm i.d. capillary was used for the remaining studies.

Figure 4.4. An electropherogram showing the signal enhancement of 25 pg/ mL cotinine by tITP/UV as compared with sample stacking/UV.



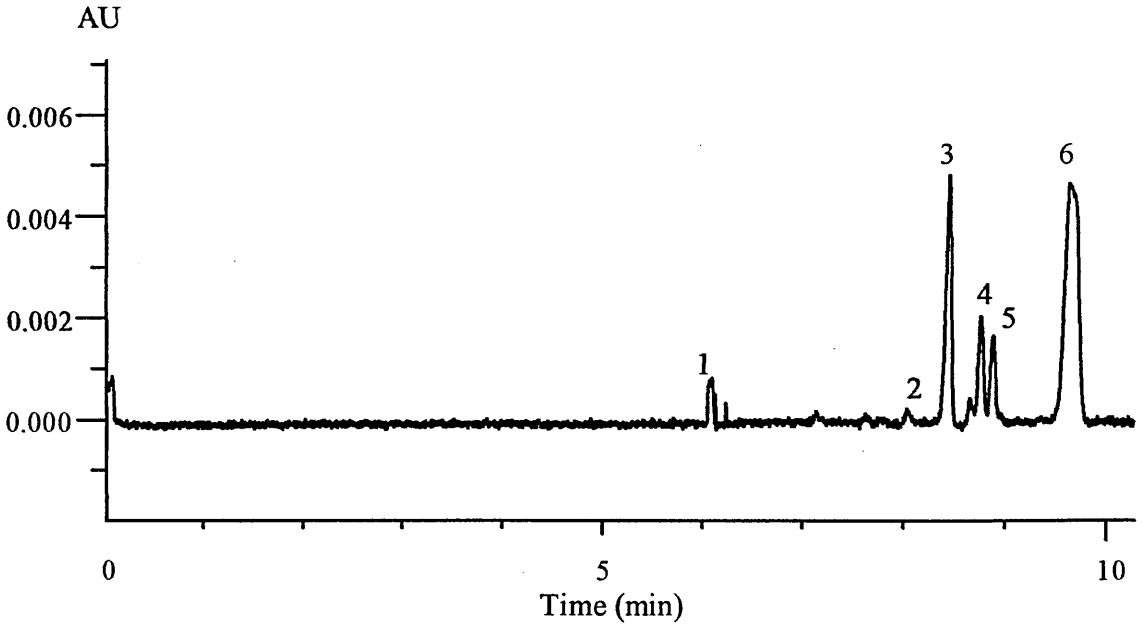
Refer to section 4.2.4b for tITP procedure. For sample stacking (refer to section 2.2 for procedure) the sample was injected for 0.2 min. at 25 mbar (8 mm plug); for tITP the sample was injected for 0.82 min at 75 mbar (10 cm plug). Wavelength = 205 nm; 65 cm capillary + 75 pm i.d.; detection window at 50 cm. Reproducibility was determined by the migration time for sample stacking (RSD = 2.46 %, where n = 5) and tITP (RSD = 2.89, where n = 5).

When tITP was compared to sample stacking for the analysis of 25 pg/mL of cotinine, a ten-fold increase in peak area was observed (figure 4.4). The introduction of a greater quantity of sample (10 cm sample plug), for tITP, brought about this increase. That is the greater mass of analyte in the capillary produced a greater response at the detector [2].

The focusing of analyte bands in the capillary caused this enhancement via stacking. Thus a decrease in the peak width will result in an increase in the peak height, greater signal-to-noise ratio, and hence lower detection limits. Since the stacking process significantly reduces peak width, large sample volumes, such as that used to produce figure 4.4, can be injected without a significant loss of separation efficiency [11]. This results in a greater mass of analyte that can be introduced to the capillary and therefore a greater response at the detector [2].

In order to test the effect of tITP on separation efficiency, the method was used for the analysis of five nicotine metabolites and the internal standard, NN-diethylnicotinamide.

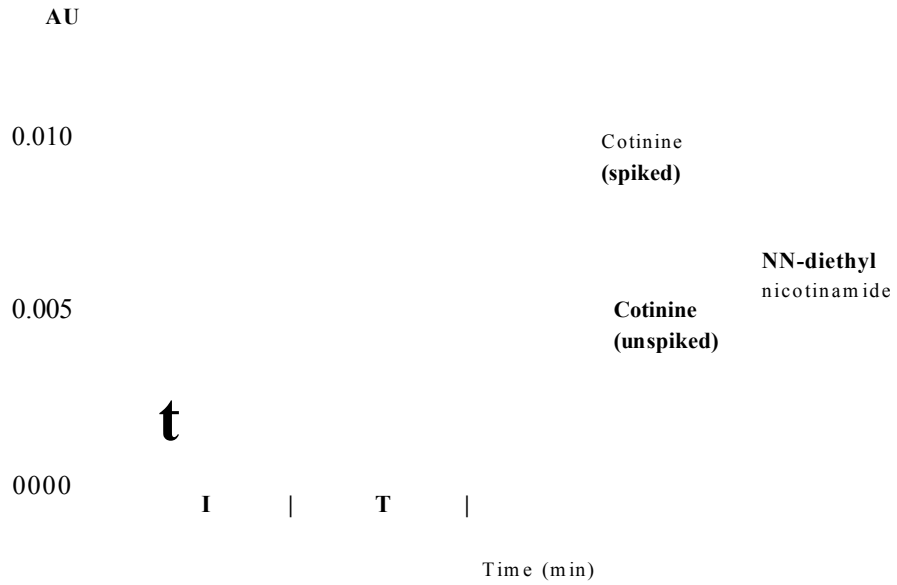
Figure 4.5. TITP/ UV of five nicotine metabolites and NN-diethylnicotinamide.



Refer to section 4.2.4b for tITP procedure. 1 = Nornicotine 2 μg/mL; 2 = Norcotinine 2 μg/mL; 3 = Cotinine 1.25 μg/mL; 4 = 3HC 2 μg/mL; 5 = 5HC 2 μg/mL; 6 = NN-diethylnicotinamide 5 μg/mL. The sample was injected for 0.82 min at 75 mbar (10 cm plug). Wavelength = 205 nm; 65 cm capillary + 75 μm i.d.; detection window at 50 cm. Reproducibility was determined by the migration time of cotinine (RSD = 1.12, where n = 5).

Figure 4.5 shows the clear separation of five nicotine metabolites with no significant loss in separation efficiency, clearly establishing the success of counter-flow tITP. Hence the analysis of real samples could now be attempted. The sensitivity enhancements provided by tITP meant that pre-concentration, by SPE, could be kept to a minimum (i.e. two times pre-concentration via a 100 mg C18 cartridge- refer to table 2.1). This, it was anticipated, would also have the advantage that urobilinogen/ urobilin concentrations would not be high enough to influence viscosity and hence the separation.

Figure 4.6. TITP/UV electropherograms of spiked and unspiked smoker's urine.



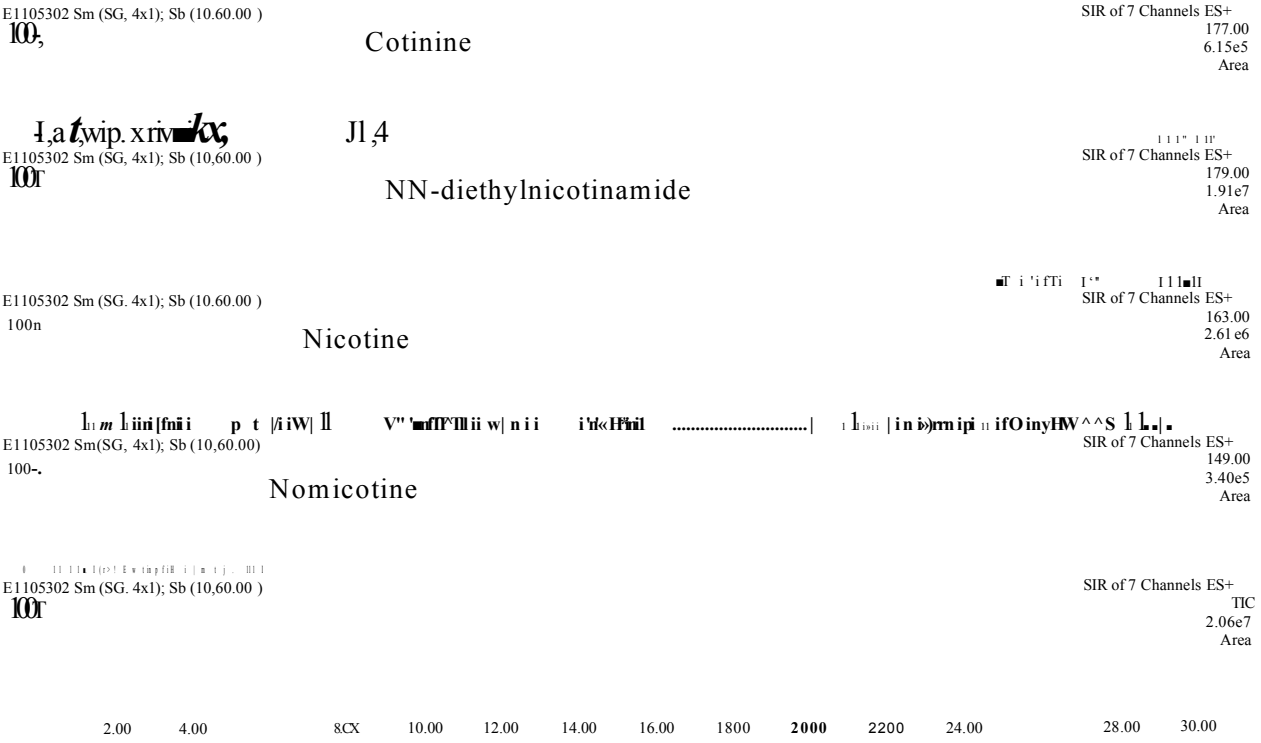
Refer to section 4.2.4b for tITP procedure. Wavelength = 205 nm; 65 cm capillary + 75 pm i.d.; detection window at 50 cm. Reproducibility was determined by the migration time of cotinine for spiked (RSD = 0.78, where n = 5) and unspiked (RSD = 0.56, where n = 5) samples.

Figure 4.6 shows the clear identification of cotinine from a smoker's urine, which had been subjected to only two times pre-concentration by SPE. This extraction procedure was significantly less time consuming than the two hundred fold pre-concentration of urine as less volume was required. A further five samples collected from five different smokers were also analysed (not shown). It was therefore evident that this method could provide the sensitivity required for use with the coaxial sheath flow interface for ESI-MS.

When coupling tITP to the MS, the fused silica capillary was withdrawn into the stainless steel sheath capillary until it was level with the sheath capillary, as this approach was successful in stabilising the electrospray when the predominantly aqueous buffer mixture was used (refer to figure 3.10). The buffer systems used were 10 mM ammonium formate and 10 mM L-alanine in 90 % MeCN and 10 % H_2O, at pH 2.5 (HCl) (ammonium formate was used because it tended to produce a more stable spray than ammonium acetate).

Initially coupling tITP to the MS proved to be extremely difficult. The main problem encountered was an influx of sheath liquid (MeCN/ H_2O 1:1 + 0.1 % formic acid) in the outlet end of the fused silica capillary during the further counter-flow step (figure 4.2- step D). This was probably due to components of the sheath liquid disrupting CE separations by setting up transient moving boundaries migrating towards the injection side of the capillary. The solution to this problem was to push the sample plug approximately 5 cm forward, from the inlet, in order to remove the 5 cm sheath liquid plug at the outlet. It should also be noted that during the tITP step the sheath flow rate was increase to 25 μL/ min to ensure that no air entered the capillary. Once these practical issues were addressed tITP/MS was used to analyse nicotine and its metabolites in the urine of smokers (figures 4.7 and 4.8).

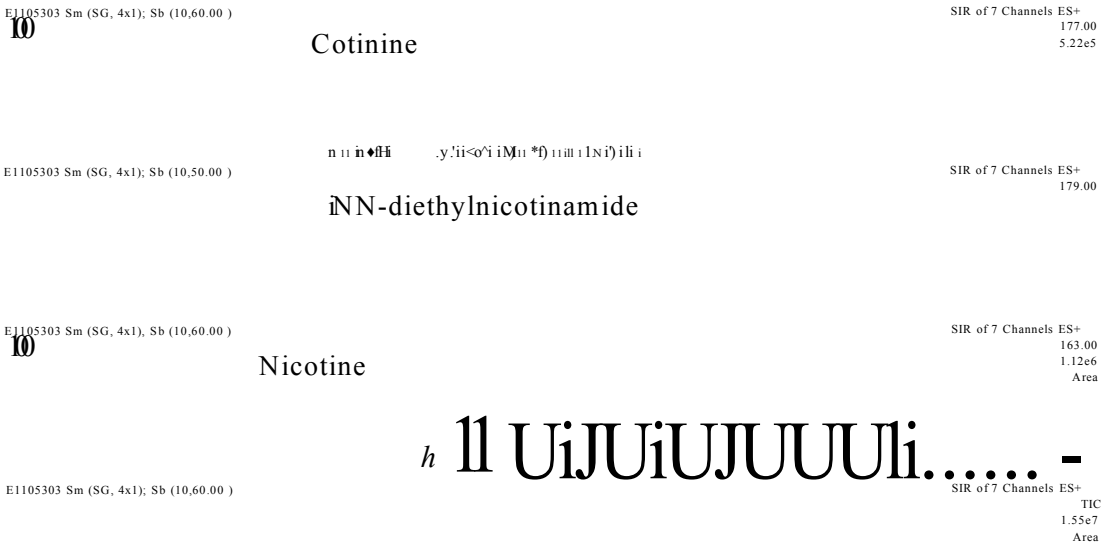
Figure 4.7. SIR mass electropherogram of a smoker's urine sample (1) via tUP.



Refer to section 4.2.4c for tITP procedure. Two times pre-concentration of urine sample via SPE; the sample was reconstituted in 90 % deionised H_2O/IO % MeCN. 10 mM ammonium formate or 10 mM L-alanine (90% deionised water and 10 % acetonitrile), which were adjusted to pH 2.5 with HC1; sheath liquid was MeCN/ H_2O 1:1; 100 cm capillary + 75 pm i.d.; sheath flow rate used for separation was 5 pL/ min.

In figure 4.7 nicotine, nomicotine and cotinine are clearly observed. From the above electropherogram it is clear that good separation and peak efficiency were achieved. However, under the conditions used baseline stability was still an issue.

Figure 4.8. SIR mass electropherogram of a smoker's urine sample (2) via tITP.



Refer to section 4.2.4c for tITP procedure. Two times pre-concentration of urine sample via SPE; the sample was reconstituted in 90 % deionised H_2O/IO % MeCN. 10 mM ammonium formate or 10 mM L-alanine (90% deionised water and 10 % acetonitrile), which were adjusted to pH 2.5 with HC1; sheath liquid was MeCN/ H_2O 1:1; 100 cm capillary + 75 pm i.d.; sheath flow rate used for separation was 5 pL/ min.

For smoker's urine (2) (figure 4.8) nicotine and cotinine were clearly observable. Once again good separation and peak efficiency were achieved despite the application of 50 mbar supplementary pressure. However, spray stability could not be maintained for long periods.

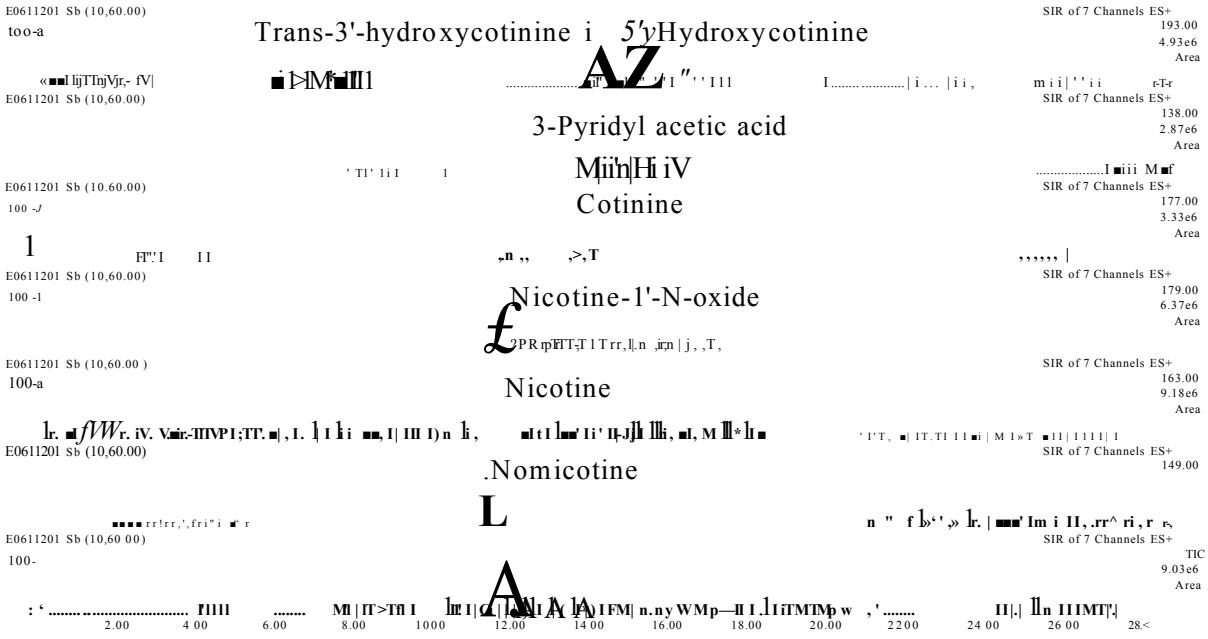
In order to improve the stability of the electrospray, it was essential to raise the organic content of the buffer systems used. Thus a buffer composition of 75 % MeCN and 25 % deionised H_2O was employed. The previously used sheath liquid, i.e. 1:1 acetonitrile/deionised water + 0.1% formic acid, was also replaced with the buffer system.

The use of this run buffer as sheath liquid significantly improved beam stability (figure 4.9). The sheath flow rate was also raised to 25 μL/ min during tITP to ensure that no influx of air occurred.

HE injection was now incorporated into the sample introduction step for tITP/MS (refer to section 4.2.4b for method). This ensured that even greater sensitivity enhancements were achieved. The optimum voltage used was 7.5 kV at the inlet, with a potential difference of 4 kV when the capillary voltage is taken into account of (figure 4.9). A low voltage was used to prevent overloading. The limits of detection (which was a linear representation of $y_B + 3s_B$, i.e. three times the signal/noise ratio) for nicotine and cotinine obtained by this method were 30 ng/ mL and 330 ng/ mL respectively; whereas the limits of quantitation (which was a linear representation of $y_B + 10s_B$) for nicotine and cotinine were 90 ng/ mL and 1.14 μg/mL respectively. Limits of detection for both nicotine and cotinine were considerably lower than those obtained by HE injection-sample stacking/MS. It should also be noted that greater sensitivity enhancements might also be achieved, for HE injection-tITP/MS, by increasing buffer concentrations, as this would amplify the focusing effect during tITP. The injection of larger volumes of sample could also be considered.

The HE injection-tITP/MS method was employed for the analysis of nicotine and six of its metabolites (figure 4.9).

Figure 4.9. SIR mass electropherogram of nicotine and six of its metabolites via HE injection-tlTP.



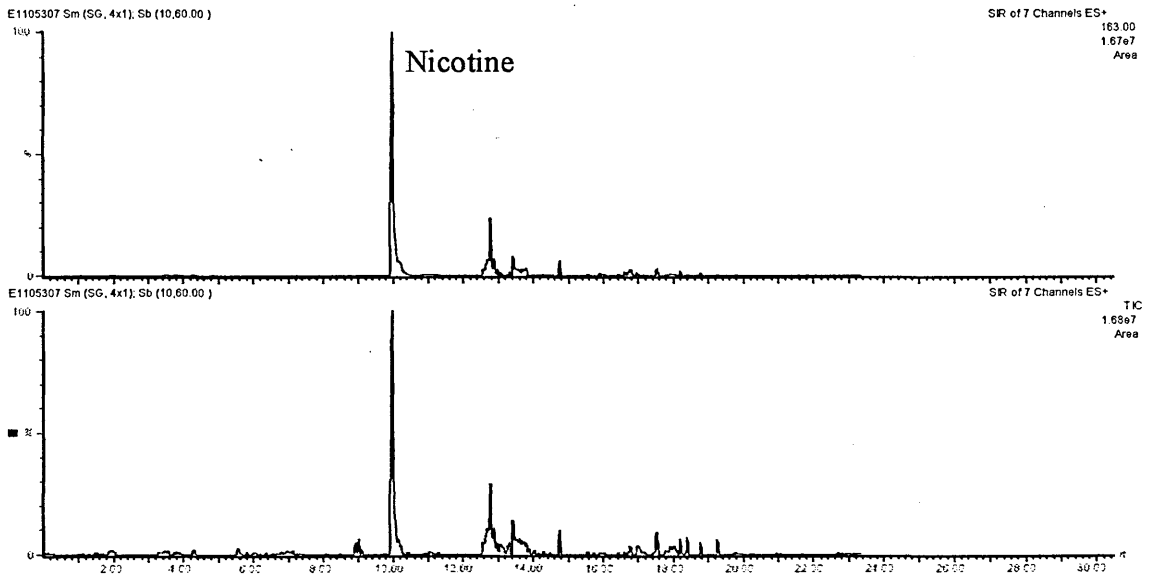
Refer to section 4.2.4d for tITP procedure. Two times pre-concentration of urine sample via SPE; the sample was reconstituted in 25 % deionised H_2O/75 % MeCN. 10 mM ammonium formate or 10 mM L-alanine (25% deionised H_2O and 75 % acetonitrile), which were adjusted to pH 2.5 with HC1; sheath liquid was the leading buffer; 100 cm capillary + 75 pm i.d.; sheath flow rate used for separation was 5 pL/ min. Reproducibility was determined by the resolution (mean = 7.30) between nicotine and cotinine (RSD = 4.19 %, where n = 5).

Figure 4.9 shows good separation and peak efficiency (theoretical plate numbers of 36290 and 36100 were achieved for nicotine and cotinine respectively) by HE injection-tlTP/MS. This was further emphasised by the separation of trans-3'-hydroxycotinine from 5'-hydroxycotinine. The low supplementary pressure applied ensured that this system was more electrically driven as opposed to pressure driven. Five repeat analyses were carried out, confirming the stable nature of the electrospray. Good reproducibility was also obtained. Since no problems were experienced with the further counter-flow step (figure

4.2- step D) there was no requirement for the application of additional pressure to the inlet prior to analysis.

Unfortunately, however, there was only enough time to utilise this method for the analysis of a single smoker's urine sample (figure 4.10).

Figure 4.10. SIR mass electropherogram of a urine sample from a smoker via HE injection-tITP.



Refer to section 4.2.4d for tITP procedure. Ten times pre-concentration of urine sample via SPE; the sample was reconstituted in 25 % deionised H_2O/75 % MeCN. 10 mM ammonium formate or 10 mM L-alanine (25% deionised H_2O and 75 % acetonitrile), which were adjusted to pH 2.5 with HCl; sheath liquid was the leading buffer; 100 cm capillary + 75 μm i.d.; sheath flow rate used for separation was 5 μL/ min. Reproducibility was determined by the migration time (RSD = 3.22 %, where n = 3).

Figure 4.10 shows the clear identification of nicotine, but not the presence of any of its metabolites. With only 10-fold pre-concentration via a 100 mg C18 SPE cartridge, it might have been better to pre-concentrate the urine up to say 50-fold via a 1 g C18 SPE and dilute

down if sample overload of the capillary was experienced. Presumably more metabolites could have been identified this way. But again this would have been more time consuming.

Initially problems were experienced, with an influx of air at the outlet end of the fused silica capillary, during the further counter-flow step (figure 4.2- step D). But only when the sample plug was pushed approximately 5 cm forward, from the inlet, after the further counter-flow step, could the analysis proceed. This was surprising as there was no requirement for this step when nicotine and six of its metabolites were analysed (figure 4.9).

However, the above mass electropherograms suggest that HE injection-tITP/MS could be used as a complementary technique to HE injection-sample stacking/MS. That is, if urine concentrations are too low to be detected by HE injection-sample stacking/MS, then HE injection-tITP/MS might be employed.

4.4. Conclusion

When coupling tITP to the MS, the fused silica capillary was withdrawn into the stainless steal sheath capillary until it was level with the sheath capillary, as this approach was successful in stabilising the electrospray to some extent. Leading and terminating electrolytes, in 90 % MeCN and 10 % H_2O, produced a spray that was stable for only short periods when a sheath liquid consisting of 1:1 MeCN/H_2O + 0.1% formic acid was used.

However, the use of leading and terminating electrolytes, in 75 % MeCN and 25 % H_2O, with the former as the sheath liquid produced a more stable electrospray. Reproducible high quality data was obtained when HE injection was coupled to tITP/MS. Limits of detection for both nicotine and cotinine were considerably lower than those obtained by HE

injection-sample stacking/MS. This suggests that HE injection-tITP/MS could be used to complement HE injection-sample stacking/MS.

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Chapter 5

In-vitro Study of Placental Metabolism of Nicotine via BeWo Cell Lines

5.1 Introduction

Glycogen stores in the endometrium (uterine wall) are only sufficient to nourish the embryo during its first weeks. Thus in order to sustain the growing embryo/foetus for the duration of its intrauterine life, the "placenta", a specialised organ of exchange between the maternal and foetal blood, is rapidly developed [1].

The development of placental tissue

The placenta is derived from both trophoblastic (foetal) and decidual (maternal) tissue. By day 12 of implantation, the trophoblastic layer becomes two cell layers thick and is called the chorion. The chorion forms an extensive network of cavities within the decidua, and as decidual capillary walls are eroded by chorionic tissue, these cavities become filled with maternal blood. Anticoagulants present in the chorion prevent the clotting of this maternal blood. Finger-like projections of chorion extend further into the maternal blood after which the developing embryo sends out capillaries into these chorionic projections to form placental villi. The foetal portion of the placenta is then anchored to the endometrium via the extension of some villi across the blood filled spaces [1]. The thin layer of chorionic tissue that surrounds the embryonic capillaries of the placental villi ensure that the

embryonic/foetal blood is kept separate from the maternal blood in the intervillus spaces. The placenta can therefore be visualised as an entire system of interlocking maternal (decidual) and foetal (chorionic) structures [1].

Placental function

During intrauterine life, the placenta may perform the functions of the digestive system, the respiratory system and kidneys. The syncytiotrophoblast layer of the placenta may also be involved in the synthesis of steroid and peptide hormones, which are required for normal foetal growth and development [2]. The trophoblastic layer facilitates the transfer of nutrients (from the mother's digestive system) and oxygen (from the mother's respiratory system) from the maternal blood into the foetal blood, and carbon dioxide (eliminated via the mother's lungs) and other waste material from the foetal blood into the maternal blood (eliminated via the mother's kidneys) [1].

Substances may traverse the placental barrier by special mediated transport systems in the placental membranes or by simple diffusion. However, not all substances that traverse the placental barrier are beneficial as nutrients, as the many drugs, environmental pollutants, other chemical agents, and microorganisms, that may be present in the mother's blood stream could potentially all pass through the placental barrier. These may have devastating effects on the growing foetus or an infant's development after birth. Similarly newborns can become addicted during gestation by their mothers' use of an abusive drug and could therefore suffer withdrawal symptoms after birth [1].

The presence of cytochrome p450 in the placenta cell lines

Xenobiotics entering the body reach the placenta via the circulation together with nutrients and endogenous substrates. Physio-chemical properties of compounds, such as lipid solubility, molecular weight, and charge state, influence transport across membranes such as the placenta. The rate of transport of chemical compounds across the placenta may also depend on the potential for metabolism within the placenta [3]. Metabolism of the parent compound could produce metabolites, which are potentially more permeable through the placental barrier.

As previously described (section 1.6.1), the cytochrome p450 enzyme system plays an important role in nicotine metabolism. The CYP2A6, 2B6, and 2D6 isoenzymes have all been shown to play active roles in the conversion of nicotine to cotinine, with the 2A6 form predominating in this activity. Hakkola *et al.* [4] described the various CYPs present in placenta that had been identified via RT-PCR: at first trimester placentae expressed at mRNA level were CYP1A1, 1A2, 2C, 2D6, 2E1, 2F1, 3A4, 3A5, 3A7, and 4B1 and at full term CYP1A1, 2E1, 2F1, 3A3/4, 3A5, and 3A7. Of these, of note is CYP2D6, which is reported to convert nicotine to cotinine. However, there was no detection of this CYP2D6 mRNA "at term" [3,4], and in fact Glover *et al.* [5] reported no CYP2D6 activity in human placentae at all. Koskela *et al.* [6] were only able to detect significant amounts of CYP2A6 mRNAs in liver tissues and nasal mucosa but not the placenta. These data suggest that both CYP2A6 and 2B6 are not present in the placenta, and that CYP2D6 may only exist in extremely small amounts.

The BeWo cell line

Problems with culturing human cytotrophoblasts include the spontaneous differentiation to syncytiotrophoblasts in primary culture, and the inability of the syncytialised trophoblasts to form a confluent and consistent monolayer. Thus an alternative culture system for studying transplacental transport and metabolism in vitro is generally required [7]. The BeWo cell line is one such alternative.

The BeWo cell line is a human choriocarcinoma- derived cell line that is used as a model to study the mechanisms of transplacental passage of nutrients and metabolites [8], and is therefore used as a model for trophoblasts [9]. The BeWo cell line is particularly attractive for this purpose since it is relatively stable, easy to maintain by passage, and grows to a confluent monolayer in a relatively short period of time [10,11]. More importantly the BeWo cell displays many of the morphological and biochemical properties common to normal trophoblasts and has been shown to exhibit the various relevant transport mechanisms for transcelluar transport [2,8-14]. Furthermore these cells can be induced to differentiate into cells resembling syncytiotrophoblasts [15] in vitro by pharmacological agents such as forskolin [2,15-19] or methotrexate [16,20].

The presence of cytochrome p450 in BeWo cell lines

Recently Avery *et al.* [21] described the presence of inducible CYP1A1 and 1A2 in the BeWo cell line. Results indicated high activity associated with liver microsomes, sometimes comparable activities in human placenta microsomes prepared from smokers, and relatively low activities in human placenta microsomes from non-smokers and in the primary cultures of cytotrophoblasts isolated from non-smokers, with the BeWo cell lines exhibiting the lowest activities relative to all other microsomes. However, compared to

primary cultures of normal trophoblasts the CYP activity of the BeWo was far more sensitive to typical inducers (e.g. 3-methylcholanthrene). These results suggest major cytochrome p450 forms present in human placenta are present and inducible in BeWo cells, and that this cell line is a potential model of drug metabolism in the human trophoblast [21].

Aims and objectives

The objective of this study was to characterise the metabolic activity of CYPs (e.g. CYP2D6) in the placenta, with respect to nicotine, from a representative in-vitro human trophoblast-like cell line, BeWo. In order to study nicotine metabolism within the BeWo cell line, the monolayer was exposed to nicotine and the relevant metabolites were identified by HPLC/MS and CE/UV.

5.2 Methods and Materials

5.2.1 Cell culture

Media type 1 consisted of 500 mL HAM F12 nutrient mixture (Sigma-Aldrich, Dorset, UK), 60 mL foetal calf serum (Gibco, Invitrogen Corporation, Paisely, Scotland, UK), 5 mL glutamine (Gibco, Invitrogen Corporation, Paisely, Scotland, UK), and 5 mL penicillin/streptocillin (Gibco, Invitrogen Corporation, Paisely, Scotland, UK) (American Type Culture Collection [ATCC] requirements). Media type 2 consisted of 40 mL HAM F12 nutrient mixture, 10 mL foetal calf serum, 1 mL glutamine, and 1 mL penicillin/streptocillin.

BeWo cells (in 10 % media type 1/ 1 % dimethylsulfoxide) were stored in liquid nitrogen, in 1 mL aliquots. The 1 mL aliquots were allowed to thaw at room temperature prior to use. 5 mL media (type 1 or 2) was added to an aliquot of BeWo cells, in a 50 mL Falcon tube (BD Biosciences, Labware Europe, Pont De Claix, France), and centrifuged at 1000 g for 5 minutes. A pellet was observed and the supernatent removed. The Falcon tube was tapped vigorously until the pellet was broken up. 9 mL of media (type 1 or 2) was then introduced to the Falcon tube and mixed thoroughly, after which the 9 mL BeWo mixture was split into three 250 mL Falcon flasks (BD Biosciences, Labware Europe, Pont De Claix, France). To each flask 27 mL of media (type 1 or 2) was added. BeWo cells were incubated at 37 °C and in 5 % carbon dioxide (Hera Cell, Heraeus Instruments – Kendo laboratory products, Germany). The media (type 1 or 2) was replaced every other weekday (with 30 mL media) until the monolayer was 60 to 80 % confluent. It should be noted that the BeWo cell lines were epithelial human choriocarcinoma and obtained from the ATCC (Manassas, VA, USA); and the dimethylsulfoxide (DMSO) was from Sigma-Aldrich (Dorset, UK).

BeWo cells were split as follows: the media (type 1 or 2) was first removed from the flask, and the cells were washed three times with 20 mL PBS (Dubelco's, UK) (removing 20 mL each time); 2 to 3 mL trypsin/EDTA (Gibco, Invitrogen Corporation, Paisely, Scotland, UK) solution was added to the flask and swirled, in order to break up the monolayer; 20 mL of media (type 1 or 2) was then added to the flask and swirled to remove any remaining adherent cells; the contents of the flask were then poured off into a Falcon tube and centrifuged at 1000 g for 5 minutes; after the removal of the supernatant, the Falcon tube was tapped in order to break up the pellet; 10 mL of media (type 1 or 2) was then added to the Falcon tube and the contents were thoroughly mixed; after which the 10 mL BeWo mixture was split into three 250 mL Falcon flasks, followed by the addition of media (type 1 or 2) to give a final volume of 30 mL; the flasks were then incubated as normal.

When the monolayer was 60 to 80 % confluent, treatment with trypsin/EDTA and re-suspension of BeWo cells (as previously described) in 26 mL media (type 1 or 2) was carried out. 1 mL of the BeWo mixture was then added to each well of a 24 well plate. The cells were incubated overnight under normal conditions. The following day the media was replaced with one containing 10 μM forskolin (Sigma-Aldrich, Dorset, UK) (made up in ethanol) and incubated overnight. Timed experiments or concentration experiments or inhibition experiments with nicotine and/ or cotinine were then carried out (see section 4.3). Reactions were stopped when transferring 0.5 mL of BeWo supernatant (from each well) into Ependorff tubes containing 0.5 mL methanol (Fischer Scientific, Manchester, UK). The samples were then centrifuged at 1000 to 2000 g for 5 minutes and the supernatant removed (Tc-6 centrifuge, Sorval Instruments - Dupont, CT, USA). The plates were then discarded in biohazard waste bins.

Haemocytometry requires a precision-made glass counting chamber consisting of a thick glass slide with a depressed central area that is converted into a volumetric chamber when overlaid by a glass cover slip. To facilitate counting the chamber has a ruled grid, with four corner squares and a central square each 1 mm^2. At × 10-magnification, the numbers of cells within the 5 squares were counted. That is, the number of cells × 5 = x × 10^4 cells/ mL.

5.2.2 HPLC/UV and HPLC/MS

Sample preparation was as described in section 4.2.1. These samples were analysed by HPLC/UV and HPLC-MS using a VG Quattro I (Micromass, Manchester, UK) triple quadrupole mass spectrometer (in the selected-ion monitoring (SIM) mode) and an LCQ iontrap (ThermoFinnigan, Hemel Hempstead, UK) mass spectrometer (full-scan) both of which were operating in electrospray ionisation (ESI).

The HPLC/MS conditions were as follows; column Phenomenex Luna (Phenomenex, UK) 5μ C18 column [250 x 4.6 mm], mobile phase 15 % acetonitrile, 5 % methanol and 80 % buffer in deionised water (10 mM ammonium acetate- pH adjusted to 4.8 with HCl) + 40 % methanol; flow rate 1 mL/ min. A 50:1 split post column was employed with a flow of 20 μL/ min being introduced into the mass spectrometer. The injection volume was 5 μL. HPLC/UV used the above mobile phase for separation with detection at 260 nm.

5.2.3. CE/UV and CE/MS

For CE/UV the detection window was burnt into the capillary 15 cm from the end of the capillary and analyses were performed at 205 nm. Inlet and outlet ends of the capillary

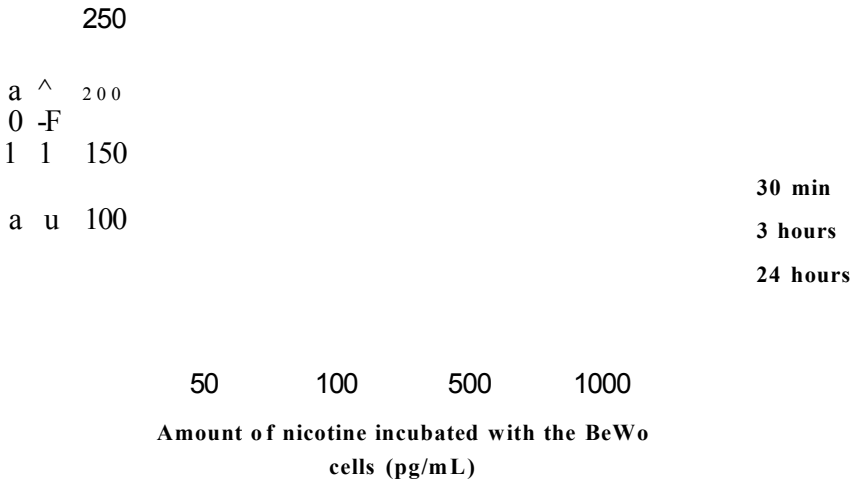
were housed in run buffer vials during a run. Separations were achieved using +30 kV. Data acquisition was performed by DAx 6.0 software (PrinCE Technologies, Emmen, Netherlands) for CE/UV. Samples obtained from cell culture were dried via nitrogen gas and reconstituted in the solvent mixture for both sample stacking and tITP. Refer to sections 2.2, 3.2, and 4.2 for the relevant sample stacking, and tITP/MS methodologies.

5.3 Results and Discussion

5.3.1. *In-vitro* Study of Placental Metabolism of Nicotine via BeWo Cell Lines

Nicotine was introduced to the BeWo cell line via media type 1 and would presumably, be taken up by the cells via passive diffusion and/or the various other cellular transport systems. Once inside the cell, nicotine would be expected to undergo metabolism depending on the availability of the relevant CYPs (refer to figures 5.1 - 5.4).

Figure 5.1. A graph showing the amount of nicotine recovered versus the amount of nicotine incubated with BeWo cells at various incubation times.



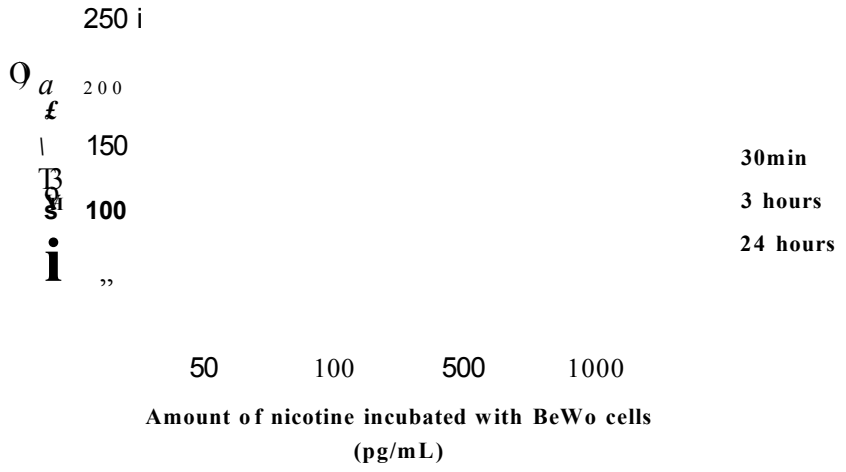
Analyses were performed on a LCQ ion trap mass spectrometer. HPLC/MS: Electrospray ionisation; 10 mM ammonium acetate (15 % MeCN/5 % MeOH/ 80 % H20) + 40 % MeOH; C18 Phenomenex Luna- 5mm, 250 mm, 1/50 split. Linear regression was used to determine the slope and the intercept, which in turn was used to determine analyte concentration.

Figure 5.1 shows the reduction in nicotine levels within the media over time. The recovered nicotine concentrations should have been approximately half of their injected amounts since 500 pL of methanol was added to 500 pL of supernatant from each well. This was more or less true for 50 and 100 pg/mL concentrations of nicotine, after 30 minutes incubation, since the recovered amounts were 23.44 and 51.96 pg/mL respectively. Presumably this was due to a short incubation time for nicotine uptake by the cells. However, the 500 and 1000 pg/mL concentrations of nicotine showed reductions of 36.74 % and 53.67 % respectively. Therefore after 30 minutes incubation there was a mean 76.82 % recovery among all injected concentrations of nicotine. Thus higher concentrations of

nicotine may be required to 'initially' facilitate the uptake and/or metabolism of this compound.

After three hours incubation a mean recovery of 64.4 % was observed among all nicotine concentrations. But only after 24 hours did we observe a near-complete reduction in nicotine levels. This suggested that nicotine uptake by the differentiated BeWo cells was near completion and/or that it was being extensively metabolised. The latter was further emphasized by the production of cotinine after 24 hours for all injected amounts of nicotine (Figure 5.2). However, the near complete reduction of nicotine could also be a result of nicotine-induced expression of the relevant CYP gene, between 3 and 24 hours incubation, since the greatest reduction occurred within this time period. The expression of this gene would lead to the production of the necessary CYP required for metabolism.

Figure 5.2. A graph showing the amount of cotinine recovered as a result of the amount of nicotine incubated with BeWo cells at various incubation times.

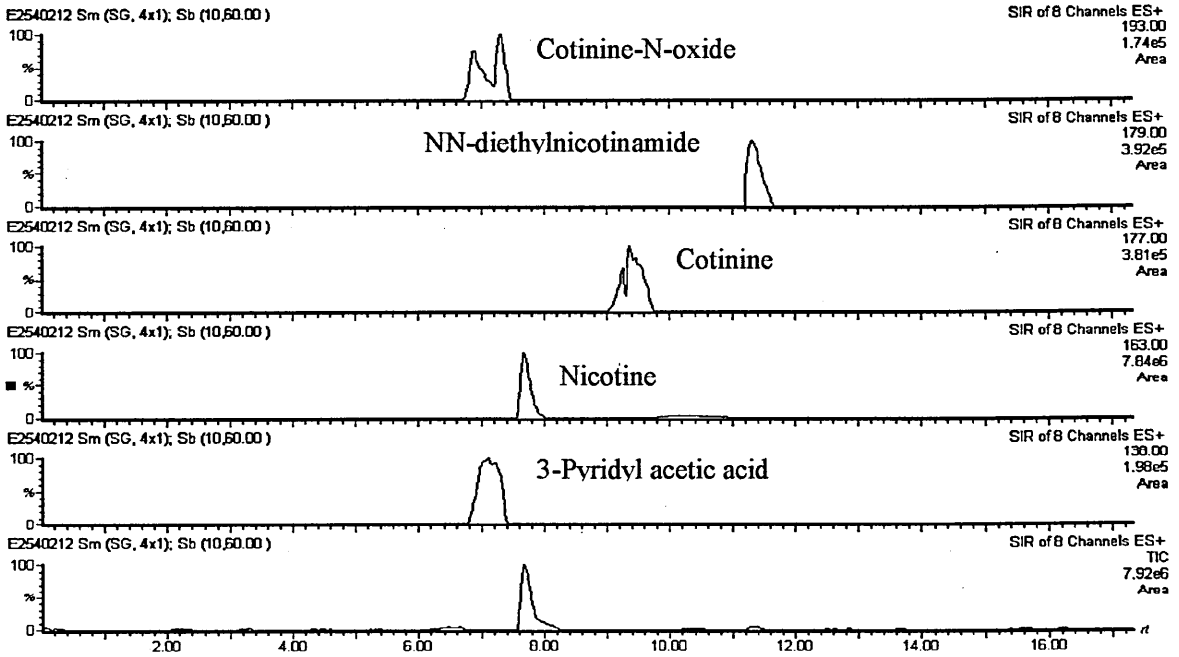


Analyses were performed on a LCQ ion trap mass spectrometer. HPLC/MS: Electrospray ionisation; 10 mM ammonium acetate (15 % MeCN/5 % MeOH/ 80 % H20) + 40 % MeOH; C18 Phenomenex Luna- 5mm, 250 mm, 1/50 split. Linear regression was used to determine the slope and the intercept, which in turn was used to determine analyte concentration.

The 1000 μg/mL concentrations of nicotine, at all incubation times, failed to yield any cotinine (figure 5.2). A possible reason for this was that the cells may have lost their functions due to nicotine toxicity at such a high concentration. In order to test this hypothesis, a further study on cell viability versus nicotine concentration should have been carried out. Unfortunately time did not permit us to do so.

The occurrence of nicotine metabolism suggests the presence of the appropriate CYPs. Thus it is most probable that the appropriate mechanisms for nicotine metabolism were present. If other relevant machinery for metabolism were present, then the various metabolites produced would be excreted from the cells by those same cellular uptake mechanisms. The excreted metabolites would then permeate the media, which would in turn be sampled for analysis. For this reason a second set of samples (media type 1) were analysed for a wider range of nicotine metabolites (figure 5.3).

Figure 5.3. SIR mass chromatogram of 500 μg/mL nicotine incubated with BeWo cells for 24 hours.



It should be noted that the sample was spiked with 10 μg/mL NN-diethylnicotinamide, which was the internal standard. Analyses were performed on a VG Quattro quadrupole mass spectrometer. HPLC/MS: Electrospray ionisation; 10 mM ammonium acetate (15 % MeCN/5 % MeOH/ 80 % H2O) + 40 % MeOH; C18 Phenomenex Luna- 5mm, 250 mm, 1/50 split. Reproducibility was determined by the RSD of the resolution (mean = 2.8, n = 3) between nicotine and cotinine (RSD = 1.22 %).

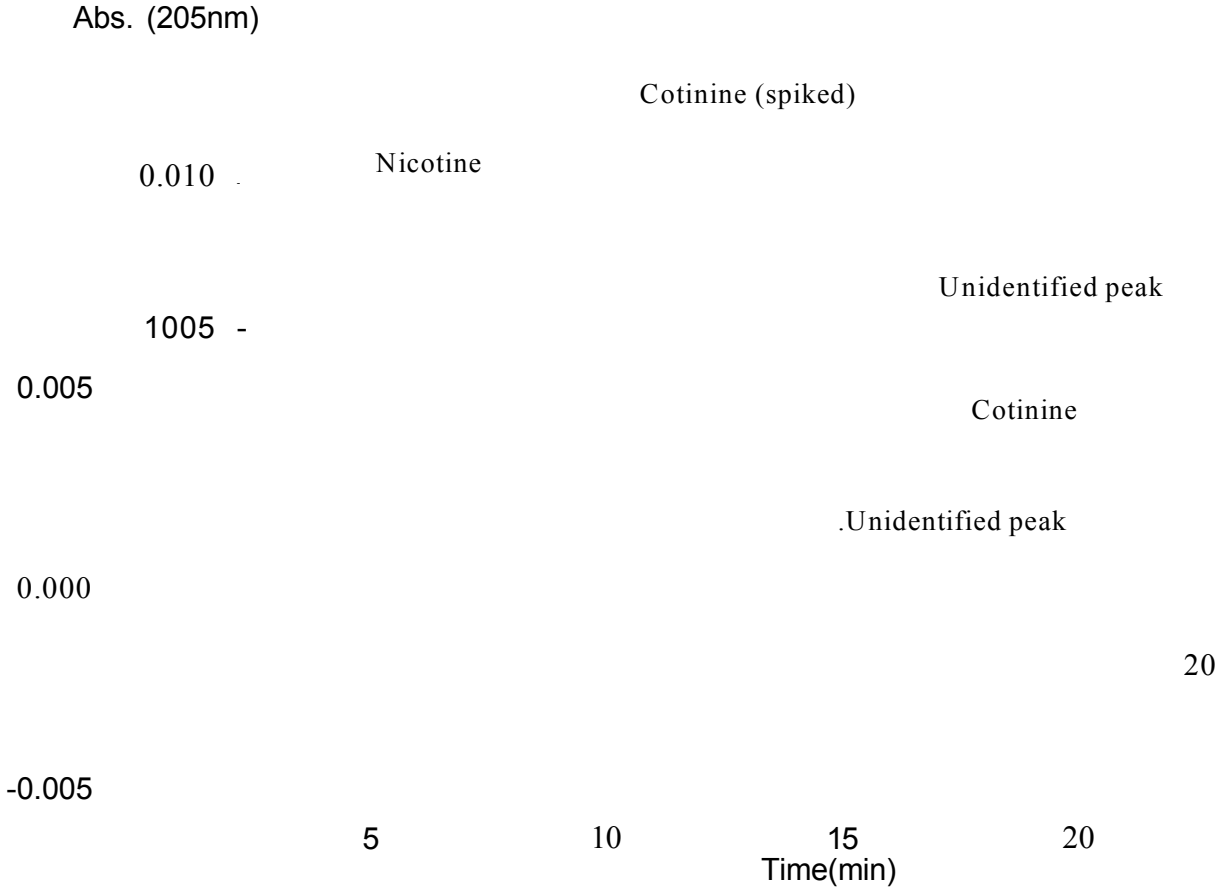
The amount of nicotine recovered (via linear regression) was 4.3 μg/mL. Thus up to 98.28 %, of nicotine, had been taken up by the BeWo cells (figure 5.3). However, only 0.89 μg/mL of cotinine was detected, indicating that either a substantial amount was present in the cells, or that it was extensively metabolised. In parallel to this study, the incubation of 500 μg/mL cotinine for 24 hours, with the BeWo cells, also took place. In this case 42.86 μg/mL of cotinine was recovered (via regression analysis), which meant that 82.86 % was taken up by the cells.

It would therefore have been best to sample the monolayer as well as the supernatant, since many metabolites could still have been trapped within the cells. This would have given a better picture of the extent to which metabolism had progressed. The main reason for sampling the supernatant was to provide an easy way of determining nicotine metabolism. Sampling the monolayer would have required the use of trypsin/EDTA solution to break up the monolayer, which would have been very time consuming if manually done. But it appears that this is probably the only way of ascertaining the true extent of metabolism.

The number of metabolites detected suggest that the required mechanisms were in place for both nicotine and cotinine metabolism to occur in the BeWo cells (figure 5.3).

A third set of samples (media type 2) were analysed by CE/UV (figure 5.4). For the purpose of CE analysis the sample was dried with nitrogen gas and reconstituted with buffer. However, it might have been better to remove the supernatant from each well and extract the contents by SPE (as previously described in section 2.3.1). This would have aided sample clean-up, but would also have been more time consuming.

Figure 5.4. CE-sample stacking/UV analysis of 250 pg/mL nicotine incubated with BeWo cells for 24 hours.



The sample was reconstituted in 1 mL of 25 % H_2O/75 % MeCN. The sample was spiked with 16 pg/mL of cotinine.The buffer system consisted of 10 mM ammonium formate (pH adjusted to 2.5 with HC1), 75 % MeCN/25 % H20; 100 cm, 75 pm i.d. fused silica capillary was used; the sample was introduced at 10 kV + 25 mbar for 0.2 min. Separation took place at 30 kV. Reproducibility was determined by the retention time (n = 3) between of cotinine (RSD was 2.40 and 1.7 for non-spiked and spiked electropherograms respectively).

Figure 5.4 shows the distortion/ broadening of the nicotine peak due to sample overload on the capillary. Nicotine concentrations would in fact be 125 pg/mL after dilution of supernatant collected from BeWo cells with methanol (refer to section 3.3.1). Unfortunately no quantitative analysis was carried out to ascertain the extent of nicotine metabolism. But

in any case figure 5.4 shows that not all of the nicotine was metabolised, as a substantial amount was still present. This could have been due to the fact that the third set of samples were not as well differentiated as the first and second sets and hence did not metabolise nicotine to the same extent. Unfortunately we were not able to carry out fluorescent staining to capture (by photo) the extent of differentiation.

The high concentrating power of HE injection appears to have brought about the considerable overload of nicotine as shown by the distortion of the nicotine peak. But even though, the presence of cotinine was still observed. Hence the application of CE/MS for this study should be considered.

5.3.2 CE/MS versus HPLC/MS

Table 5.1. HPLC/MS versus CE/MS.

	Resolution nic-cot (RSD)	LOD µg/ mL	LOQ µg/ mL
Sample stacking -HE injection-MS			
Nicotine	3.74[a] (4.17) to 9.64[b] (4.83)	0.11	0.37
Cotinine		2.25	7.5
TITP-HE injection -MS			
Nicotine	7.30[c] (4.19)	0.03	0.09
Cotinine		0.34	1.14
HPLC/MS			
Nicotine	2.23 (3.06)	0.04	0.14
Cotinine		0.07	0.22

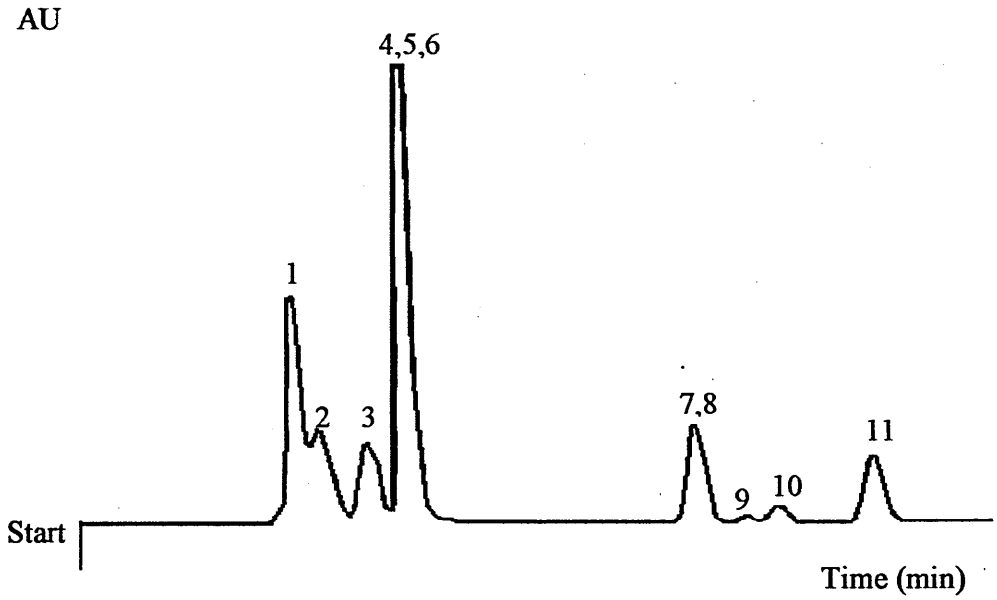
1) 'a' denotes the use of 30 kV + 25 mbar (0.2 min) for HE injection, and separation at 30 kV + 20 mbar; 2) 'b' denotes the use of 10 kV + 25 mbar (0.2 min) for the optimised HE injection, and separation at 30 kV + 10 mbar; 3) 'c' denotes the use of 7.5 kV + 75 mbar (0.64 min) for HE injection, and separation at 30 kV + 10 mbar.

Table 5.1 shows the lowest limits of detection (which was a linear representation of $y_B + 3s_B$) and consequently the limits of quantitation (which was a linear representation of $y_B + 10s_B$) to be found in all cases for nicotine. Transient isotachophoresis exhibited the lowest

limits of detection and quantitation for nicotine, but not for cotinine. This was due to the higher mobility of nicotine, which ensured its greater pre-concentration with respect to cotinine especially as the sample was introduced by HE injection, as in the case of sample stacking. But, HPLC/MS exhibited the lowest detection and quantitation limits for cotinine. Thus overall HPLC/MS demonstrated the lowest limits of detection and quantitation.

Of the three methods described, HPLC showed the poorest resolution. One of the main problems with HPLC method development, for this particular analysis, was trying to separate 5'-hydroxycotinine from trans-3'-hydroxycotinine. The structural isomeric nature of these compounds made them extremely difficult to separate, even in the presence of cyclodextrins (12.5 mM α,β or γ - from the authors previous work, not shown). Further more, 5'-hydroxycotinine was shown to co-elute with 3-pyridyl-γ-oxomethylbutyramide (which shares the same molecular weight), and was not separated by cyclodetrins (figure 5.5). The transfer of this technique, from UV to MS analysis, weakened the data qualitatively, as transfer to the MS required the mobile phase to be more volatile and hence less polar. This speeded up analysis significantly and reduced the time for effective separation.

Figure 5.5. A chromatogram of nicotine and nine of its metabolites by HPLC/UV.



HPLC/UV was carried out at 260 nm. A 5 μL injection volume was used throughout. C18 Phenomenex Luna- 5mm, 250 mm, was used.. 1= solvent front (3.84 min); 2 = 3-pyridyl acetic acid (4.18 min); 3 = nornicotine (4.79 min); 4 = cotinine-N-oxide (5.32 min); 5 = nicotine; 6 = nicotine 1'-N-oxide; 7 = 5'hydroxycotinine (9.16 min); 8 = 3-pyridyl-γ-oxo-N-methylbutyramide; 9 = 3-pyridyl-γ-oxo-butyric acid (9.82 min); 10 = cotinine (10.26 min); 11 = NN-diethylnicotinamide (11.51 min). 10 mM ammonium acetate (15 % MeCN/5 % MeOH/ 80 % H_2O), adjusted to pH 4.8 with HCl. Reproducibility was determined by the migration time of the NN-diethylnicotinamide (RSD = 1.02, n = 5).

Clearly the structures of these compounds could have been elucidated by MS/MS, but the intention was to provide a simple way of identifying these metabolites through separation.

Resolution was shown to be greatest in the CE methods (table 5.1), since both sample stacking/MS and tITP/MS were previously shown to have separated 5'-hydroxycotinine from trans-3'-hydroxycotinine (sections 2.3.1, 3.3.1 and 4.3.1). The numbers of theoretical plates observed via the mass spectrometer for cotinine were highest in HE-sample

stacking/MS (50589), followed by HE-tITP/MS (36100), and finally HPLC/MS (8000). However, the greater robustness of HPLC/MS made this technique very easy to couple to the MS. The fact that two separate electrical systems were used for CE/MS meant that the coupling of the two devices did not always provide us with a robust technique. Since subsequent fluctuations in the current (brought on by a variety of factors- refer to section 2), arcing, and base line dropouts, at times made both CE/MS techniques difficult to operate. The large volumes of dilute sample introduced by tITP/MS made this technique even more difficult to implement than sample stacking/MS. However, changes to interface characteristics, such as the use of the buffer system as the sheath liquid and the withdrawal of the capillary into the sheath tube (until they were both level), greatly improved spray stability. Overall HPLC/MS was the most reliable technique used for this analysis, but the major improvements to the coaxial interface performance, has made CE/MS an attractive alternative.

5.4 Conclusion

It is clear from our data that nicotine metabolism was observed. It is therefore probable that CYP mediated processes play an important role in nicotine metabolism in the placenta. However, there was much CYP activity unaccounted for, since in the second and third sets of BeWo cells the amount of cotinine and hence other major metabolites recovered, was still relatively low. Thus in order to ascertain the true extent of metabolism, the monolayer together with the supernatant should have been sampled.

The consequences of CYP activity in human placenta, with respect to nicotine, could lead to the transport of pharmacologically active metabolites (such as nornicotine) to the developing foetus. It may also lead to the transport of teratogenic byproducts (which may result in reduced placental oxidative capacity and birth defects). However levels of CYP in the placenta are currently unknown and so despite these processes a large proportion of nicotine may pass to the foetus unchanged. This in effect may cause other problems, such as nicotine addiction in the newborn.

Overall HPLC/MS was the most reliable technique used for this analysis, but the major improvements to the coaxial interface performance, has made CE/MS an attractive alternative.

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Chapter 6

Conclusion

The overall aims of this work were to produce a sensitive, highly selective, and simple CE-sample stacking/MS assay for the measurement of nicotine and its metabolites in urine, to develop a highly sensitive transient isotachophoretic/MS method, that yields detection limits comparable to that observed by HPLC/MS and with a separation efficiency to match that of CE-sample stacking/MS, and finally to characterise the metabolic activity of cytochrome P450 (e.g. CYP2D6) in the placenta, with respect to nicotine, from a representative in-vitro human trophoblast-like cell line, BeWo, via HPLC/MS and CE/UV.

Analyses of urine samples were accompanied by sample clean up via SPE to ensure the appropriate removal of inorganic salts. Consequently a two hundred fold pre-concentration of urine, via SPE, was achieved through drying with nitrogen gas and reconstituting with buffer (for CZE) or the solvent mixture (for sample stacking). The removal of inorganic salts enabled the transfer of this sample medium to the mass spectrometer.

The stability of the electrospray was greatly improved by (a) withdrawing the fused silica capillary into the stainless steel sheath capillary until it was level with the sheath capillary and (b) replacing the previously used sheath liquid i.e. 1:1 acetonitrile/deionised water + 0.1% formic acid, with the buffer system (10 mM ammonium acetate, 75 % MeCN/ 25% H2O, adjusted to pH 2.5 with HCl).

Sensitivity and throughput are important parameters when considering the analysis of multiple compounds within a sample. The former was brought about by the use of HE-sample stacking and is especially important for use with the mass spectrometer, since this detector is concentration dependent. It should also be noted that the dilution effect, brought about by the sheath liquid, requires the use of a sensitive method for MS analysis.

The HE injection method used for CE-sample stacking/ MS brought about lower detection limits (LODs of nicotine and cotinine, by CE-sample stacking/ MS (via HE injection), were found to be 0.11 and 2.25 μg/ mL, respectively) when compared to sample stacking/MS via hydrodynamic or electrokinetic injection alone. The hydrodynamic part of HE injection also ensured that lower mobility analytes would always be injected, thus reducing the sampling bias observed by electrokinetic injection.

The added selectivity that SIM provided ensured the clear identification of nicotine and its metabolites. The same pattern of elution was observed for nicotine and its metabolites from smokers' urine. However, the inter-urine sample migration times of the metabolites appeared to differ slightly and were possibly attributed to the presence of urobilinogens and urobilins.

When high voltages (e.g. 20 to 30 kV) were used for HE injection, lower numbers of theoretical plates and reduced separation efficiency were observed. Thus the optimal voltage (e.g. 10 kV) was one that achieved great sensitivity enhancements without significantly compromising resolution and peak efficiency. However, the effect of HE injection on resolution and peak efficiency, at higher voltages, is dependent upon analyte concentration, prior to injection. The greater quantities of sample introduced into the capillary at higher voltages for HE injection correspond with the decline in peak efficiency

and resolution. Thus an optimal voltage for HE injection can be selected to suit analyte composition.

It is also important to note that the highest peak efficiencies and separations were only obtained when no supplementary pressure was applied during separation, but severe fluctuations in current (due to the influx of air at the outlet end of the fused silica capillary) were also noticed.

The application of supplementary pressure during separation appeared to increase the throughput of analyses, but caused a reduction in resolution. Therefore there had to be a careful balance between obtaining high quality data and generating fast analysis times. The optimum appeared to be 10 mbar and below, where acceptable separations and theoretical plate numbers were achieved.

Daily changes in laboratory temperature did not affect reproducibility, but appeared to affect peak efficiency and resolution. Thus climate-controlled laboratories should be welcomed for this type of analysis.

A counterflow transient isotachophoresis (tITP) method was developed, with MS detection, for the analysis of even lower analyte concentrations. A major advantage of this method was that less sample pre-concentration by SPE was required, ensuring that separation would be less hampered by changes in sample viscosity.

Limits of detection for both nicotine (0.03 μg/ mL) and cotinine (0.34 μg/ mL), via HE-tITP/MS, were considerably lower than those obtained by HE injection-sample stacking/MS, suggesting that HE-tITP/MS could be used complement to HE injection-sample stacking/MS.

When HPLC/MS and CE/UV were used to analyse cytochrome p450 activity in the human trophoblast-like BeWo cell line, it was clear from our data that nicotine metabolism

was observed. Thus it is probable that CYP mediated processes play an important role in nicotine metabolism in the placenta. However, there was much CYP activity unaccounted for as only the supernatant and not the monolayer was sampled. Hence the other major metabolites recovered were still in relatively low amounts. A better representation of metabolism could be obtained by sampling the monolayer together with the supernatant.

When compared to HPLC/MS both HE-sample stacking/MS and HE-tITP/MS exhibited higher resolutions and peak efficiencies; with HE-tITP/MS exhibiting comparable limits of detection and quantitation to those obtained by HPLC/MS with respect to nicotine, but not cotinine. Overall HPLC/MS was the most reliable technique used for this analysis, but the major improvements to the coaxial interface performance and sensitivity enhancements, has made CE/MS an attractive alternative.

Chapter 7

Future work

- For the removal of urobilinogens and urobilins from urine, extraction by petroleum ether or acetaldehyde may be explored. A simpler way may be to pass urine through a C_2 or C_3 SPE cartridge prior to performing SPE through a C_{18} cartridge.
- If problems associated with molecular imprinted polymers could be overcome (such as the leaching of template from the sorbent), then this approach could also be explored, in combination with SPE, for the extraction of nicotine and its metabolites.
- Improve throughput of HE-sample stacking/MS and HE-tITP/MS analysis. In the case of the former the buffer pH could be raised to increase the EOF, which would result in faster analysis times. High throughput could also be achieved by increasing the concentration of the run buffer, which would improve separation, thus allowing for an increase in supplementary pressure applied.
- Improve sensitivity of HE-sample stacking/MS and HE-tITP/MS. By increasing the concentration of the run buffers an enhanced stacking effect should result, and hence lower limits of detection be achieved. In terms of the latter, injection of larger volumes of sample injected could be explored.
- Transfer HE-sample stacking/MS and HE-tITP/MS of urine to a patient study.
- Explore the use of capillary LC/MS, micro-LC/MS, and CE/MS for the BeWo study.

- To sample both the supernatant and the monolayer to obtain a better representation of nicotine metabolism within the BeWo cell.
- To carry out inducer/promotor and inhibitor studies for further characterisation of CYP activity in BeWo cells.
- Finally to use MS/MS for the structural elucidation of the protein in question.

Appendix

- Royal Society of Chemistry (University of East Anglia)- Poster presentation. July 2001
- BMSS LCMS Symposium (Robinson College, Cambridge)- Oral presentation: Analysis of metabolites of nicotine by CE/MS. December 2001.
- Royal Society of Chemistry (Southampton University)- Poster presentation. September 2001.
- Royal Society of Chemistry (Kingston University)- Poster presentation. July 2002.
- 26th Annual BMSS Meeting (Loughborough)- Poster presentation. September 2002.
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Determination of nicotine and its metabolites in urine by solid-phase extraction and sample stacking capillary electrophoresis-mass spectrometry

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Abstract

The combination of capillary electrophoresis (CE) and mass spectrometry (MS) with solid-phase extraction (SPE) has been used for the identification of nicotine and eight of its metabolites in urine. The recovery7of cotinine from cotinine-spiked urine, by C18 SPE, was found to be 98%. Smokers urine (200 ml) was preconcentrated 200-fold via SPE prior to analysis. The sample stacking mode of CE, when compared to capillary zone electrophoresis, was shown to improve peak efficiency by 132-fold. Tire combination of hydrodynamic and electrokinetic injection was studied with sample stacking/CE/MS. The on-column limits of detection (LOD) of nicotine and cotinine, by this technique, were found to be 0.11 and 2.25 jxg/ml, respectively. Hence, LODs of nicotine and cotinine in urine after 200-fold preconcentration were 0.55 and 11.25 ng/ml, respectively.

Keywords: Sample stacking; Nicotine, metabolites

1. Introduction

Smoking is generally accepted as the major preventable cause of mortality and morbidity in the western world. It is the major cause of cancer mortality in the UK, and may contribute to the causation of many other disease states including atherosclerosis. Thus, smoking cessation may be the most effective strategy for reducing the incidence of cancer, cardiovascular disease and strokes. It appears that although smoking prevalence is declining within the developed world, it is on the increase in most parts of the developing world [1J.

The tobacco alkaloid, nicotine, is mainly responsible for its addictive properties [2,3J. Nicotine accounts for approximately 90% of the alkaloid fraction in commercial tobacco, *Nicotiana labaewn*, and is a major component of the particulate phase of tobacco smoke. The presence of nicotine in biological fluids, in humans, is primarily due to exposure to tobacco smoke, the consumption of certain nicotine-containing fruits, such as aubergines, making a negligible contribution |4J. In humans approximately 86% of systemically absorbed nicotine is metabolised to cotinine. The half-life of cotinine is approximately 10-fold greater than that of nicotine [5] and hence cotinine can be found in greater concentrations than the more rapidly metabolised nicotine [4]. Cotinine has therefore been widely used as the marker of choice for tobacco consumption (Fig. 1).

Currently, the most common analytical techniques employed for the determination of nicotine and its related metabolites in biological fluids are gas chromatography-mass spectrometry (GC-MS) [5,6], high-pressure liquid chromatography (HPLC), radio immunoassy (RIA), enzyme-linked immunosorbent assay (ELISA) [4,7,8] and colorimetric techniques [8J. Of these, GC-MS is the most sensitive and specific method, but is labour-intensive [9]. HPLC methods (including LC-MS) have been used, but require extensive method development due to the low separation efficiencies obtained. Immunoassay techniques are relatively sensitive but cannot discriminate between several active analytes because of specificity limitations due to cross reactivity with similar molecules [4,8].

In this area of research the application of capillary electrophoresis (CE) is still in its infancy. However, CE offers

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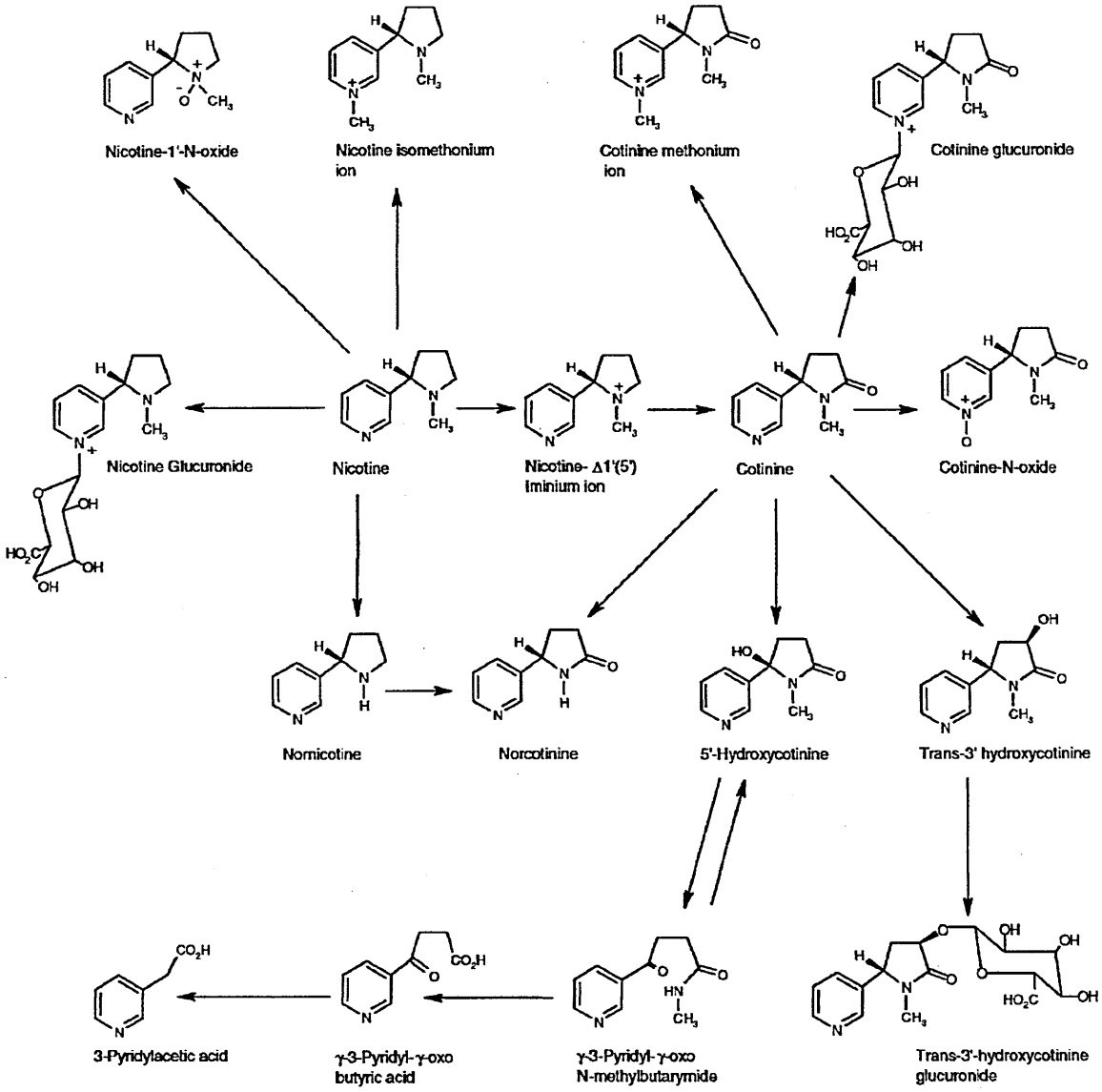

Fig. 1. The major mammalian pathways of nicotine metabolism.

several potential advantages over GC and HPLC for the analysis of complex mixtures of metabolites; including high separation efficiencies, extremely small injection volumes, short analysis times, rapid method development and low reagent costs [10]. CE may be used to perform highly efficient separations of a wide range of sample types, and the number of theoretical plates in CE may reach several hundred thousand [11].

The main limitation of CE is its lack of sensitivity [11–18]. Detection limits in CE are generally 10–100 times higher than in HPLC [13,14]. The relatively higher limit of detection observed in CE comes from its restricted injection volume (where typical injection volumes are in the nanolitre range) and the short path length for on-capillary detection (when coupled to a UV detector) [10,12]. Thus, a major area of interest in CE is to improve its low concentration sensitivity without sacrificing resolution.

Several techniques have been shown to yield on-column sample concentration and to lower detection limits in CE. These include isotachophoresis [10], sample stacking [15], and filed amplified injection [17]. The most practical way to concentrate a sample is in fact the on-line sample stacking approach. The sample stacking phenomenon was first introduced by Tisclius; and can be achieved through the manipulation of sample and buffer solutions with injection procedures common to any CE instrumentation (Fig. 2).

Palmer et al. [4] recently reported the separation and detection of standards of nicotine and 10 of its metabolites using CE/MS. To date, there are no reported studies, to our knowledge, on the use of CE for the determination of nicotine and its metabolites in clinical samples. Its use has been

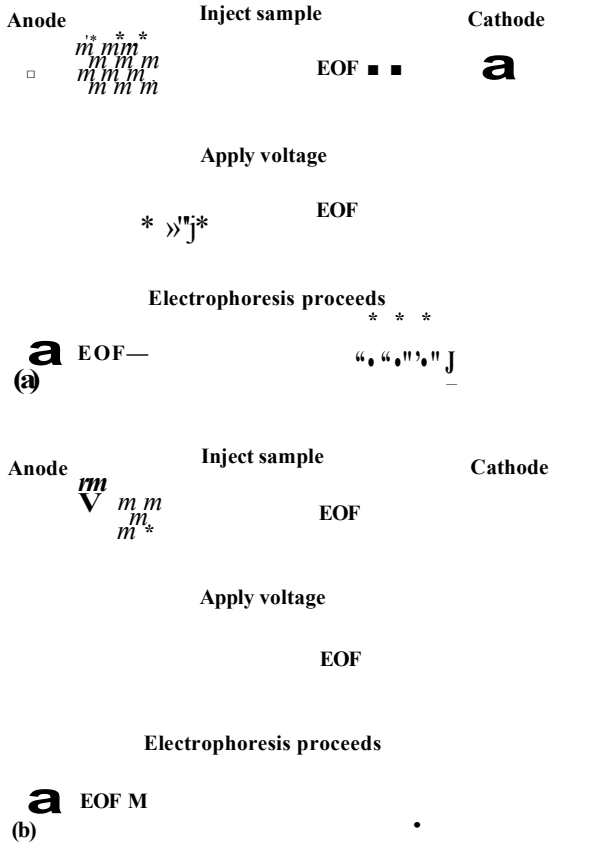

Fig. 2. A comparison of (a) capillaiy zone electrophoresis and (b) sample stacking. In sample stacking the use of an injection solvent of lower ionic strength than the run buffer results in solute ions forming a narrow stacked zone on application of a voltage. This improves both sensitivity and peak shape.

reported, however, for the quantitative determination of both nicotine in tobacco [19J and tobacco alkaloids (including nictotine) in single plant cells [20] The limits of detection (LOD) reported were, for nicotine in tobacco 286 fg (using electrochemical detection) [19] and for nicotine in plant cells 0.013 pmol (i.e. 2.1 pg) (using ultraviolet detection) [20].

By combining CE with mass spectrometry (MS) selective identification and also structural elucidation, can be achieved. In this study, we demonstrate the use of solid-phase extraction (SPE) combined with sample stacking CE/MS for the determination of nicotine and its metabolites in urine.

2. Experimental

2.1. Samples and sample preparation

Standards of nicotine and its major metabolites were kindly donated by Dr. Peyton Jacob of the Department of Medicine and Drug Dependence Research Centre, San Francisco General Hospital Medical Centre. These were prepared in buffer for CE experiments or mobile phase for HPLC or one-tenth of the run buffer for sample stacking experiments.

Urine was obtained from five male smokers, labelled A-E, and stored at —20 °C in tissue culture flasks. Two samples, labelled, Cj and C_2 were collected from the same subject, but on different days. Prior to extraction urine samples were thawed overnight at 4 °C thoroughly mixed and filtered with a 0.45 pm Supor Acrodisc filter.

2.2. Solid-phase extraction

Solid-phase extraction was carried out using a 1 g C 18 Isolute cartridge (Jones Chromatography Ltd.). For conditioning purposes 6 ml of methanol followed by 6 ml of deionised water (Milli-Q) were passed through the SPE cartridge sequentially. Then, 100 ml of sample was introduced into the cartridge followed by 6 ml of deionised water. The sample was then eluted with 6 ml of methanol. The eluted product was dried under nitrogen gas. The dried product was reconstituted with 0.5 ml buffer (200-fold preconcentration for CE/MS) or 1 ml mobile phase (100-fold preconcentration for recovery experiment). The reconstituted product was then subjected to ultrafiltration (via a Sorval Tc centrifuge and Vivaspin Concentrator 3000 MW) at 3950 rpm for 20 min.

2.3. Buffer preparation

A suitable quantity of ammonium formate (Sigma-Aldrich) was dissolved in the solvent mixture (acetonitrile/deionised water (Milli-Q)); buffer pHs were adjusted with formic acid or HC1 (Sigma-Aldrich), as appropriate, and were freshly prepared. Buffers were degassed prior to analysis using a Branson 1210 ultrasonic bath (Branson Ultrasonics, Danbury, CT, USA) and filtered through a 0.2 pm syringe filter (Supor Acrodisk). The buffers prepared were 25 mM ammonium formate (10% acetonitrile, 90 deionised water), which was adjusted to pH 2.5 with formic acid, for CZE; and 10 mM ammonium formate (75% acetonitrile, 25% deionised water) adjusted to pH 2.5 with HC1, for sample stacking experiments.

2.4. Recovery experiments

Non-smoker's urine (100 ml) was spiked with cotinine (at 1.25 and 0.625 pg/ml) and subsequently extracted using the SPE method described above. These samples were analysed by LC-MS using a VG Quattro I (Manchester, UK) triple quadrupole mass spectrometer operating in electrospray ionisation (ESI) and selected ion monitoring (SIM) mode.

The HPLC conditions were as follows: column Phenomenex Luna 5 pi C18 column (250 mm x 4.6 mm), mobile phase 15% acetonitrile, 5% methanol and 80% buffer in deionised water (10 mM ammonium acetate, pH adjusted to 4.8 with HC1); flow rate 1 ml/min. A 50:1 split post column was employed with a flow of 20 pl/min being introduced into the mass spectrometer. The injection volume was 10 pi.

2.5. CE separation

CE was carried out in a 100 cm, 75 |xm i.d. x 365 pm o.d., untreated fused silica capillary (Composite Metal Services Ltd., Hallow, UK). The CE system used (Crystal CE System, Prince Technologies, Lauerlabs, Emmen, The Netherlands) utilizes programmable injection with pressure and voltage. Separations were achieved using +30 kV (a field strength of 350V/cm). Injections were performed either hydrodynamically (25 mbar/0.2min) or electrokinetically (30kV/0.2min) or hydrodynamically + electrokinetically (25 mbar + 30 kV/0.2 min). Initial conditioning of the capillary took place with 1M NaOH (5 min) followed by the buffer system (7 min). The capillary was conditioned prior to each run with 1M NaOH (2000mbar/l min) followed by buffer system (2000 mbar/1.5 min). The buffer system was replenished after each run cycle.

2.6. CE/MS

A VG Quattro I triple quadrupole mass spectrometer was used throughout. To date, electrospray ionzation (ESI) is the most common technique for coupling CE to MS. In these experiments, CE/ESI/MS coupling was acheived using the co-axial sheath-flow interface developed in-house and previously described by Palmer et al. [4]. The sheath liquid comprised either; 1:1 MeCN/TUO +0.1% formic acid, or run buffer and was delivered by a Harvard Model 11 syringe pump (Harvard Apparatus, Edenbridge UK) at a flow rate of 3-5 pl/min.

Positive ions were generated through the application of 3.5 kV to the probe tip, with a source cone voltage of 25 V. Desolvation was aided by nebulising gas (~401/h) and bath gas (~2251/h). Capillaries employed for CZE/MS were 50 |xm i.d. x 375 pm o.d. and 100cm in length.

Data was either acquired by selected ion recording of the analyte protonated molecules employing a 1Da window with a dwell time of 0.08 s or in full scan mode from 70 to 200 Da in 0.5 s.

3. Results and discussion

Table 1 summarises the data obtained from the LC/MS recovery experiments. Percentage recoveries were calculated from a linear calibration plot of eight points, recorded in duplicate. The *R2* value for the calibration data was 0.9993. The 1.25 and 0.625 pg/ml cotinine-spiked urine samples were analysed in triplicate and an overall mean percentage recovery of 98% was obtained.

Fig. 3(a) shows non-optimised CE/MS mass electropherograms obtained from the analysis of a standard mixture of the major nicotine metabolites and Fig. 3(b) the corresponding optimised separation for comparison. We have found that several factors are important in obtaining reproducable high quality eleetropherograms when using the co-axial CE/MS electrospray interface (Table 2).

In order to obtain good signal to noise in CE/MS and hence good detection limits a stable electrospray is required. We were able to improve the stability of the electrospray over our earlier work [4] by: (a) withdrawing the fused silica capillary into the stainless steel sheath capillary until it was level with the sheath capillary and (b) replacing the previously used sheath liquid, i.e. 1:1 acetonitrile/deionised water + 0.1% formic acid, with the buffer system. By withdrawing the fused silica capillary into the stainless steel capillary, the length of the fused silica that can be surrounded by the sheath capillary is maximised. Hence, the maximum electrical contact between the sheath capillary and the fused silica capillary is obtained. The use of the run buffer as sheath liquid improved beam stability presumably due to the homogeneous nature of the sheath liquid and the buffer reducing mixing effects.

A second major issue in obtaining reproducible high quality CE/MS data using the co-axial arrangement is drying out of the electrophoresis capillary. One possible approach to solving this is the application of a supplementary pressure to the head of the column [21], In our earlier work [4], a supplementary pressure of 50 mbar was in fact employed but used in conjunction with sample stacking to improve sensitivity and peak shape. If we examine Fig. 3a a CZE separation where a supplementary pressure of 50 mbar is used without sample stacking. It can be seen this has a markedly detrimental effect on separation efficiency. This is presumably since the flow profile now has the characteristic parabolic shape of a pressure driven system rather than the plug flow profile of an electrically driven system. Careful arrangement of the relative height of the buffer reservoir and the CE/MS probe allowed us to reduce the supplementary pressure to 20 mbar. Best results were obtained when the buffer reservoir was approximately 1cm above the CE/MS probe. Combining all of the slight modifications to our previous methodology with sample stacking gives stable and reproducible high quality eleetropherograms (Fig. 3b).

Table 1
Percentage recovery of cotinine from a 1g C18 SPE cartridge (n = 3)

	Unknown concentration (pg/ml)	R.S.D.	Recovery (%)
0.625 pg/ml cotinine 100 times preconcentration (Isolute Ig C18)	63.86	0.07	102.17
1.25pg/ml cotinine 100 times preconcentration (Isolute Ig C18)	118.34	0.07	94.67
Mean			98.42

SIR of 7 Channels ES+

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Table 2
A study of the reproducibility of the developed sample stacking CE/MS separation method

	Mean resolution	Resolution S.D.	Resolution R.S.D. (%)
Sample stacking			
Sample A (cot-5HC)	2.59	1.17 x 10_I	3.15
Sample B (cot-5HC)	1.41	1.44 x 10~2	4.52
Sample Ci (cot-5HC)	2.21	9.78 x 10~2	1.02
Sample D (cot-5HC)	2.16	7.70 x 10~2	3.57
Sample E (cot-5HC)	2.18	8.39 x 10~2	3.85
CZE			
Sample C2 (cot-5HC)	2.36	6.10 x 10~2	2.57
Sample stacking (cot-3HC)			
Hydrodynamic injection	0.75	1.89 x 10~2	2.51
Electrokinetic injection	1.26	9.98 x 10"3	0.80
Hydrodynamic + electrokinetic injection	0.77	2.42 x IQ-2	3.15

The resolution, *R,* between two adjacent peaks (cotinine and S'-hydroxycotinine for A-E and cotinine and trans-3'-hydroxycotimne for the three injection methods) was calculated as follows: $R = 2(t2 - t\backslash)/(w\backslash + wj)$; where *ty* and *ti,* and uq and W_2 are the retention times and peak widths of adjacent peaks, respectively. For smokers urine samples A-E, $n = 3$; $n = 5$ for CZE; $n = 5$ for injection methods; where n is the number of repeat analyses.

Sample injection in CE is generally performed by hydrodynamic or electrokinetic injection. During hydrodynamic injection (HE) the sample vial is pressurised, forcing the sample into the capillary. Thus, the volume of sample injected is dependent on the magnitude and duration of the pressure applied, the capillary dimensions, and the sample viscosity. Electrokinetic injection is performed by placing the capillary and the anode into the source inlet vial and applying a voltage over a period of time. Thus, ionic solutes are injected as a result of their electrophoretic mobilities, with neutrals being pushed through the capillary as a result of the electro-osmotic flow.

Electrokinetic injection provides for sensitivity enhancements in comparison to hydrodynamic injection, since the volume of sample solution that can be introduced does not limit the injected amount of the sample. However, sampling bias is a major drawback of electrokinetic injection, since larger quantities of higher mobility solutes are injected than those of lower mobility. An example of sampling bias was observed in this work. When electrokinetic injection was employed, 7-3-pyridyl-y-oxo-butyric acid and cotinine-iV-oxide were not detected presumably since they were not injected in an adequate amount to yield a sufficient response from the mass spectrometer for detection. In order to eliminate this effect the combination of electrokinetic and hydrodynamic injection was employed. The injected quantities of nicotine and cotinine for the HE method were 0.83 and 0.74 ng, respectively. Of this 0.29 ng of both nicotine and cotinine was contributed by the hydrodynamic injection element (Table 3) and hence electrokinetic injection contributes 0.54 and 0.45 ng of both nicotine and cotinine, respectively. That is 65% of the nicotine HE load and 61% of the cotinine HE load.

Sample stacking was employed in conjunction with the combined injection procedure in order to achieve the best possible sensitivity. The limits of detection, by CE-sample stacking/MS, of nicotine and cotinine, respectively, for the three injection methods were found to be; (1) 0.11 and 2.25 |ig/ml for the HE injection method; (2) 2.86 and 6.25 p-g/ml for hydrodynamic injection; and (3) 0.18 and

Table 3
Sample injection data calculated for CE-sample stacking/MS

	Injected quantity (ng)	Injected volume (nl)	Sample plug length (mm)	Mean migration time (min)	*tm* R.S.D. (%)
Hydrodynamic + electrokinetic					
Nicotine	0.83	132.04	29.89	10.27	1.52
Cotinine	0.74	118.15	26.74	12.24	2.08
Thiourea	0.51	18.6	82.17	24.58	4.04
Uracil	0.51	18.48	81.65	24.41	3.70
Hydrodynamic					
Nicotine	0.29	45.98	10.40	9.59	3.77
Cotinine	0.29	45.98	10.40	11.2	3.83
Thiourea	0.29	45.98	10.40	22.2	3.05
Uracil	0.29	45.98	10.40	22.1	2.71

The viscosity of the buffer system was calculated as 0.51 cP. $\{2,rj = (Vj/1 \times 106) \times c \times 1000$; $Lp = (A\,P \times f \times d2)/(53.3 \times i) \times L) + (\ll x/x$ $(Vjrj/V^p)$; $Vi = (r \times 1 \times 10^{-}v)2 \times (Lp \times 1 \times 103) \times n \times 1 \times 1012$; where Qnj is the injected quantity; Ki,j the injected volume; c the concentration in pg/ml; Lp the sample plug length; AP the pressure across the capillary; t the injection time; d the capillary inner diameter; tj the viscosity of the sample solution; L the total length of the capillary; v the velocity of the analyte in mm/min; Krj the injection voltage; and the separation voltage.

Fig. 4. SIR mass eleetropherograms of a smoker's urine (sample E); all conditions as Fig. 3b.

3.27 (Jig/ml for electrokinetic injection. Thus, it is clear to see that the HE injection method yields the lowest limits of detection, followed by electrokinetic, and finally hydrodynamic injection. The lowest detection limits yielded by the HE injection method can be explained by its ability to introduce the greatest quantity of sample to the capillary (Tabic 3). It should also be noted that the higher electrophoretic mobility of nicotine results in greater quantities of this solute being introduced to the capillary than cotinine. This, accompanied with the higher ionising potential of nicotine ensured its lower limit of detection. Comparison of the limit of detection obtained for nicotine by HE injection of 0.11 jxg/ml, i.e. 14.5 pg on-column (Table 3) with the reported literature values of, for nicotine in tobacco 286 fg [19] (using electrochemical detection) and for nicotine in plant cells 0.013 pmol (i.e. 2.1 pg) [20] (using ultraviolet detection), shows that the sensitivity of our CE/MS method is comparable with that of CE with ultraviolet detection.

Optimisation of the electrospray together with the application of sample stacking (via HE injection) has greatly improved peak efficiency, baseline stability, and detector response to the analytes (Fig. 3b); and the added selectivity that SIM provides ensures the clear identification of nicotine and its metabolites. The order of elution is comparable to that shown by Palmer et al. [4J.

When applying this method to urine samples, sample preconcentration via solid-phase extraction was required (with the 200-fold preconcentration of sample achieved). Data from a smoker's urine (Fig. 4) shows the clear identification of nicotine, cotinine and the other major metabolites of nicotine. Since nicotine has only a half-life of approximately 2 h, the relatively high abundance on the mass electropherogram could be attributed to the recent exposure of the smoker tobacco smoke.

Samples Ci and D (not shown) exhibited a more extensive metabolism of nicotine and hence had a relatively low abundance of this analyte on both mass eleetropherograms. The high abundance of 5'-hydroxycotinine with respect to /rans-3'-hydroxycotinine in all the mass eleetropherograms, with the exception of sample C_2, suggests that the major pathway for cotinine metabolism is via 5'-hydroxycotinine. This is further confirmed by the presence of 3-pyridyl acetic acid. However, it is possible that tran.s,-3,-hydroxycotinine could mainly exist in the glucuronide form. More work in this area (with respect to patient studies) is required.

The inter-urine sample migration times of the metabolites appeared to differ slightly. The probable cause being, the presence of urobilins and urobilinogens in urine. The presence of this compound in urine may be brought about by the derivation of bilirubin from senescent red blood cells and from other haem-containing proteins, such as cytochromes. Bilirubin is poorly water-soluble and so undergoes glucuronidation to form bilirubin diglucuronide, which is excreted into the bile. The glucronide residues are released in the terminal ileum by intestinal bacterial hydrolases. Free bilirubin is then reduced to colourless urobilinogens, which are oxidised to coloured products known as urobilins. Urobilins are mainly excreted in the faeces, but small proportions of urobilins and urobilinogens are excreted in the urine. Urobilins give urine its yellow pigment. Since ultrafiltration of the urine sample only filters proteins of 3000 MW and over, urobilins and urobilinogens (584.65 MW) are still present in the filtered urine. When preconcentrating urine by 200-fold the concentration of urobilins increase and hence the viscosity increases. Thus, an increase in viscosity will increase migration times, but not the order of elution providing the fused silica capillary is long enough for effective separation.

4. Conclusions

The 100-fold preconcentrations of 1.25 |xg/ml and 625 ng/ml of cotinine, in urine, showed a mean percentage recovery of 98% from a lg C18 SPE cartridge. Hence, the 200-fold preconcentration of sample could be achieved through drying w'ith nitrogen gas and reconstituting with buffer.

A combination of hydrodynamic and electrokinetic injection was used for sample stacking/MS due to the lower detection limit, and the reasonable number of theoretical plates achieved. Thus, sample stacking mode of CE, when compared to CZE was shown to improve peak efficiency by a mean of 131.6-fold.

LODs of nicotine and cotinine, by CE-sample stacking/MS (via HE injection), were found to be 0.11 and 2.25 p.g/ml, respectively. Thus, the LODs of nicotine and cotinine after 200-fold preconcentration would be 0.55 and 11.25 ng/ml, respectively.

The added selectivity that SIM provided ensured the clear identification of nicotine and its metabolites.

The same pattern of elution was observed for nicotine and its metabolites from smoker's urine. However, the inter-urine sample migration times of the metabolites appeared to differ slightly and were attributed to the presence of urobilinogens and urobilins. The extraction of both tetrapyrroles by acetaldehyde and petroleum ether, from urine, is currently under investigation.

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