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Measuring tobacco smoke exposure: quantifying nicotine/cotinine concentration in biological samples by colorimetry, chromatography and immunoassay methods

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Abstract

Procedures to assess tobacco smoke exposure are reviewed and biomarkers used for determining the smoking status of an individual are compared. Methods used to extract these biomarkers from saliva, urine, and blood and the advantages and disadvantages of the assays are discussed. Finally, the procedures used to measure the levels of cortisol, a stress hormone speculated to be linked to nicotine metabolism, are discussed.

Keywords: Nicotine; Cotinine; Cortisol

1. Introduction

A large body of evidence indicates that tobacco smoking has unfavorable consequences on human health [1–7]. Chronic smokers run the risk of lung cancer [2,8–17], respiratory infections [18,19], heart disease [2,20–22], and pregnancy complications [23,24] caused by inhalation of nicotine, the principal component of tobacco. The annual worldwide mortality due to tobacco use is estimated to be 3 million [25]. Many hazardous substances in mainstream cigarette smoke are also present in environmental tobacco smoke (ETS). Therefore individuals involuntarily exposed to ETS, called passive smokers, are also adversely affected. For example, there are about 3000 lung cancer deaths per year among nonsmokers [26]. Even infants nursed by smoking mothers are affected by nicotine as it is secreted in the milk [24,27–29].

Often, a distinction has to be made between smokers and nonsmokers, and between non smokers exposed to ETS and non smokers not exposed to ETS. For example, life insurance companies are interested in knowing the smoking status of potential insurance customers, since heavy smokers run the risk of decreased life expectancy. In this respect, biochemical measurements with appropriate markers have been found useful [30,31]. A threshold value of 500 ng ml^{-1} of cotinine, a major metabolite of nicotine, is used to distinguish smokers from nonsmokers [32].

This article reviews the procedures used to assess tobacco smoke exposure. Specifically, it compares different biomarkers used to determine the smoking

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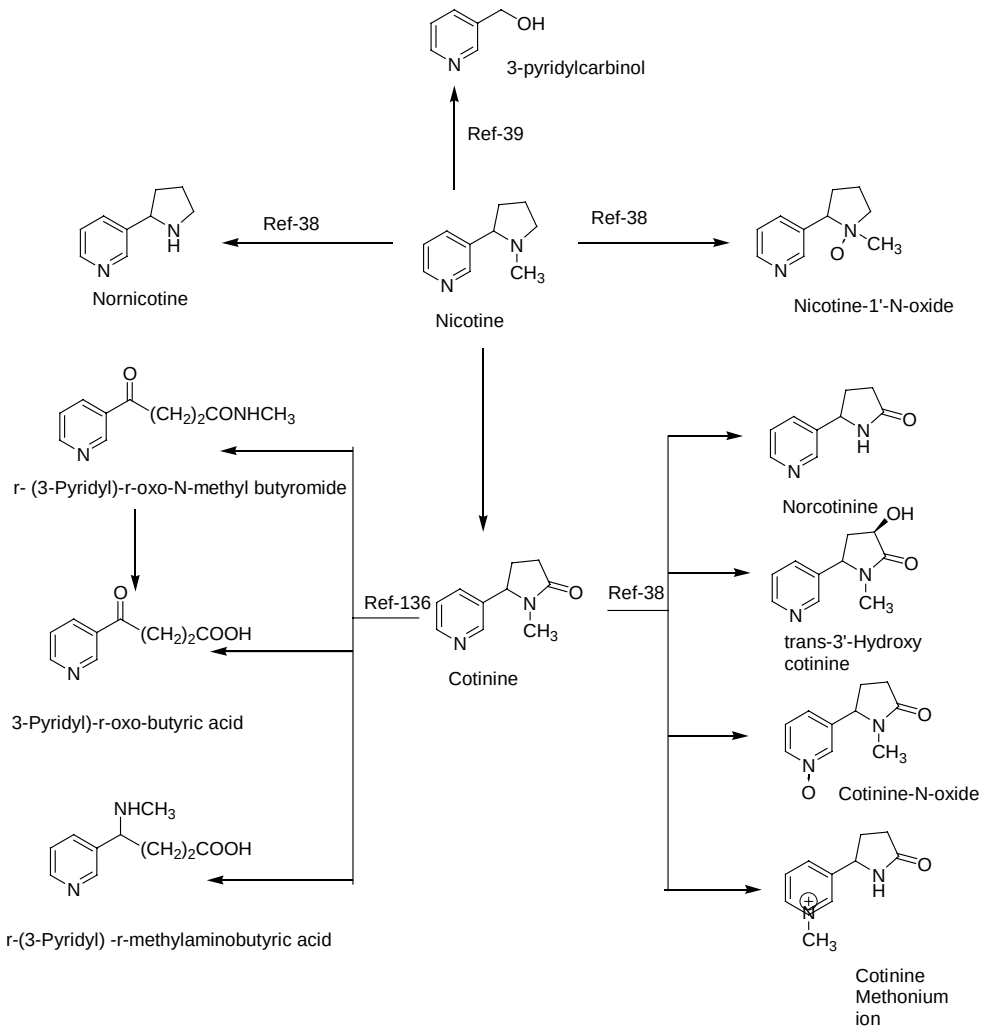
status of an individual and the different methods used to extract these biomarkers from saliva, urine, and blood. Advantages and disadvantages of all the assays currently used are discussed. The article also reviews methods for evaluating the cortisol levels in tobacco smokers; cortisol is a stress hormone that appears to be closely linked to nicotine metabolism [33]. Recent review articles regarding tobacco smoke exposure include a mini-review on the use of urinary cotinine as a tobacco-smoke exposure index [34] and a review on the use of cotinine as a biomarker of environmental tobacco smoke exposure [35].

2. Nicotine metabolites

Nicotine is metabolized to more than 20 different derivatives [36]. In humans, 70% of nicotine is oxidized to cotinine, 4% is oxidized differently, 9% is excreted unchanged in the urine, and the metabolic outcome of the remaining 17% is still unknown [37–40]. (Scheme 1).

Tobacco smoking also produces metabolites other than those derived from nicotine, such as *trans*, *trans*-muconic acid [41,42] and 1-hydroxy pyrene [43]. These metabolites are produced from benzene



Scheme 1. Metabolic route to nicotine metabolites.

[41,42] and polycyclic aromatic hydrocarbons [43], respectively, which are also present in ETS.

3. Biomarkers for assessing smoking status

An ideal marker for assessing the smoking status of individuals should have a reasonable half-life, be specific, be amenable to estimation in body fluids, and be available at concentrations that can be quantified using existing analytical methods. The presence of other compounds should not interfere in the estimation of the marker and the marker should not be influenced by environmental sources other than tobacco smoke. The markers used for assessing the smoking status of individuals, and the matrices in which these markers have been estimated are: nicotine (plasma, saliva, and urine) [21,44–46]; carbon monoxide (expired air) [47,48]; carboxyhemoglobin (blood) [21,49]; thiocyanate ion (plasma, saliva, and urine) [21,49,50–55]; and cotinine (plasma, saliva, and urine) [43,45,52,56].

3.1. Nicotine

Nicotine is responsible for tobacco addiction and is the most specific component of cigarette smoke. It is present in a relatively large amount in a typical cigarette (1–2 mg per cigarette). It is absorbed and is measurable in both active and passive smokers [44,45]. However, nicotine levels in the blood fluctuate and the duration of urinary nicotine excretion is short [46]. Also, the half-life of nicotine is about two hours and hence it is poorly suited as a marker for monitoring chronic exposure [45].

3.2. Carbon monoxide

The short half-life of carbon monoxide limits its usefulness in determining the smoking status of individuals [47]. Besides, there exists a possibility of ambient environmental contamination of carbon monoxide during sample acquisition, due to automobile and domestic emissions [47,48].

3.3. Carboxyhemoglobin

Inhaled carbon monoxide is quickly absorbed into the blood to form carboxyhemoglobin (COHb). The blood COHb can be used as a biomarker in evaluating exposure to ETS, but is generally not used for this purpose [21] as it has a short half-life of 3–4 h [49].

3.4. Thiocyanate ion

Thiocyanate concentration in blood has been used as a chemical indicator for distinguishing smokers from nonsmokers [49]. In fact, thiocyanate measurement, as an index of cigarette smoke, has become increasingly popular [50–55] and has been used in large epidemiological studies. The thiocyanate estimations, however, are complicated by dietary influences. Foods rich in thiocyanate, eggplants, potatoes, and tomatoes, can produce thiocyanate levels similar to those found in habitual smokers [34].

3.5. Cotinine

Cotinine is the major metabolite of nicotine, and is the analyte of choice as it fulfils the prerequisites of specificity and retention time (18–20 h) and is found at detectable levels (in all the matrices) [45,49,57–61]. It can be used for tobacco exposure quantification in both actively and passively exposed individuals [62,63]. Cotinine, however, is biotransformed into secondary metabolites such as cotinine glucuronide, 3-hydroxy-cotinine and 3-hydroxy-continine glucuronide [62,64]. Total cotinine plasma concentration is therefore determined by the summation of all four metabolites. The general consensus is that cotinine is superior to thiocyanate as a biomarker in validating cigarette smoking.

4. Collection of body fluids

The receptacle for collecting body fluids should be made of either glass or polypropylene, should be disposable, should be kept in a sealed package protected from the environment, and precleaned and siliconized before use [6]. A polypropylene receptacle with a screw cap closure is preferred since it can withstand breakage during transportation to a distant laboratory [65]. Different biological fluids are collected as follows.

4.1. Saliva

A simple method of collecting a saliva sample (5–8 ml) [6,56] is to instruct volunteers to accumulate saliva in their mouths for about five minutes before expelling it into a disposable glass/plastic tube. This method reduces frequent expelling froth that contains little liquid [66]. An alternate method consists of placing highly absorbent dental rolls between the lower cheek and gum, for about 15 min. The dental roll is then placed in a tube containing bacteriostatic solution (0.8 ml, prepared in organic pure water) and the saliva is recovered by squeezing the roll or by placing it in a glass syringe compressed by a glass plunger [67]. Often, a cotton swab impregnated with citric acid is used for collecting saliva samples [68,69]. Citric acid, a saliva stimulant, ensures adequate flow of saliva. The same objective is achieved by instructing volunteers to chew on a saliva inducing wax pellet [70] or a piece of teflon tape [71] after rinsing their mouths with water.

Some investigators prefer to collect the ultra filtrate of saliva with a device that has a semipermeable membrane enveloping an osmotically active substance. The device, when kept in the mouth, stimulates the flow of saliva. Sample collection is claimed [66] to be more convenient, more aesthetically pleasing, and provides saliva ultrafiltrate that is free from potential enzymatic degradation.

A detailed procedure has been described in the literature [72] for collecting saliva samples. The researchers use a paper pad (1 ml holding capacity) saturated with a solution of sodium chloride (3.5%), citric acid (0.3%), potassium sorbate, sodium benzoate and gelatin (each 0.1%), with pH adjusted at 7.2 by the addition of sodium hydroxide. The processed pad is dried and placed for about two minutes between the lower cheek and gum, and rubbed gently back and forth to ensure it is moistened. Saliva is recovered as described before and centrifuged at 10,000 × *g* for 1–2 min or 1200–2000 × *g* for 10–15 min to remove any interfering debris or non-food particulate matter [73]. The clear saliva obtained in this manner is analyzed or frozen at −20 °C for future use [74].

4.2. Blood and urine

Blood samples are withdrawn by standard venipuncture technique [72] and collected in tubes containing clot activator [75]. The precipitate obtained is separated at 4 °C by centrifugation (2000 × *g*) for 10 min [76]. The plasma is frozen at −20 °C until required for analysis. Urine samples are easy to collect and are immediately analyzed or frozen at −20 °C until the time of analysis to prevent bacterial degradation [65].

Among body fluids, saliva is the matrix of choice for assessing the concentration of nicotine and its metabolites in humans exposed to ETS. Saliva sample collection is simple, convenient, and stress-free. It is less intrusive than urine collection and is neither painful nor traumatic compared to blood collection (done by stress-inducing venipuncture). Use of saliva is especially useful for monitoring the exposure to ETS in the young, old, and infirm individuals. It is interesting to note that some employees subject to ETS exposure at their workplaces prefer to give saliva samples for analysis, since they fear that urine testing may reveal their illicit drug use [6]. A recent report however, concludes that urinary cotinine concentration is a more accurate biomarker than salivary cotinine concentration for ETS. The researchers compared the cotinine concentration in urine and saliva of 94 subjects using gas chromatography (GC), high performance liquid chromatography (HPLC) and enzyme linked immunosorbent assay (ELISA). They found that cotinine levels in urine using HPLC correlated well with levels measured using ELISA and GC (nitrogen phosphorus detection) but salivary cotinine levels measured by ELISA did not correlate well with either HPLC or GC-NPD measurements [77].

Other materials used for quantification of cotinine (in active smokers) include hair, amniotic fluid, and spinal fluid (see Section 6.3.1). Toenails have also been used to estimate nicotine concentration [78].

5. Methods for the extraction of biomarkers

Several organic solvents have been employed to extract organic constituents in plasma, saliva, and urine. From the organic solvent the basic constituents, namely, nicotine and cotinine are recovered by salt formation with a mineral acid ($HCl/H_2SO_4/H_3PO_4$) [73]. Cotinine and/or nicotine can be re-extracted from the corresponding salts by basification by NaOH (or Na_2CO_3–$NaHCO_3$ [79–82]/K_2CO_3–NH_4OH [74]). These are then assayed either by GLC [79,83,84],

Table 1
Summary of extractive procedures

Compound	Matrix	Solvents extraction	Use	Internal standard	Method of analysis	Reference
Nicotine	Plasma urine	Diethyl ether	*n*-Heptane (nitrogen detector) methylene chloride (flame ionization)	Aqueous solution quinoline	Rapid GLC	[76]
Cotinine	Plasma saliva cotinine	Methylene chloride	Acetone (plasma or saliva) methylene chloride (urine)	Aqueous solution of lignocaine hydrochloride N (2 methoxy ethyl) nor cotinine succinate	Packed column GLC	[74]
Cotinine nicotine	Plasma saliva urine	Methylene chloride	Methylene chloride	5-Methyl cotinine (aqueous solution)	Rapid gas–liquid chromatography	[79]
Cotinine nicotine	Plasma saliva[a]	Methylene chloride	HPLC solvent	2-Phenyl imidazole	High performance liquid chromatography	[73]
Nicotine and its metabolites	Urine	Chloroform–MeOH (9.5:0.5; v/v)	Diethyl ether	Norephedrine in methanol	High performance liquid chromatography	[80]
Cotinine	Serum urine saliva	Toluene–butanol (9:1; v/v)	Methylene chloride toluene–butanol (9:1; v/v)	Deutero-cotinine (methyl D3) in HCl	Capillary GC–MS	[69]
Cotinine	Serum[a]	Methylene chloride	Toluene	{G-H} cotinine	LC–APER–MS–MS	[81]
Nicotine cotinine and oxamide[b]	Serum urine	Methylene chloride	Methylene chloride	^{3}H-Nicotine ^{125}I-derivative of cotinine and oxamide	Radio-immuno assay	[70]

[a] Deproteination was made by the addition of trichloroacetic acid + the metabolites include, cotinine, *trans*-3′-hydroxy cotinine, nicotine-1′-*N*-oxide and 3-pyridylcarbinol.
[b] Oxamide: γ-(3-pyridyl)-γ-oxo-*N*-methylbutylbutyramide.

HPLC [39,65], GC–MS [74,85], LC–APCI/MS–MS [38] or radioimmunoassay [86]. The various extractive procedures adopted in these investigations are summarized in Table 1.

6. Methods of assay

6.1. Colorimetry

For monitoring the smoking status of humans, colorimetry is a desirable method of analysis. It is simple, inexpensive, and gives (after taking appropriate precautions) [87,88] an estimate of total metabolites produced from nicotine inhaled during smoking [77,89,90]. It, however, lacks specificity and the estimated urinary concentration is reported to be higher than that obtained by gas or liquid chromatography. Moreover, drugs containing the pyridine nucleus (e.g., isoniazid, nicotinamide, nicotinic acid) [73,88–91] may interfere in the colorimetric estimation. Accordingly, colorimetry is not a foolproof method for monitoring exposure to ETS.

6.2. Chromatography

Chromatographic techniques have proven to be useful aids for quantifying the biomarkers in different biological fluids. They are preferred over other types of analysis, as they are highly sensitive, specific, and can analyze both nicotine and cotinine in a single assay [68,92]. Also, they are less susceptible to interference by non-specific factors (compared to immunoassay methods) as they require extraction and

concentration prior to analysis [75]. The reagent costs are generally low [34], and the quantification limits are about 0.1 ng ml^{-1}.

Based on the above-mentioned attributes, gas chromatography [34,65,68,74,76,79,93–110] is the method of choice for monitoring ETS exposure by quantifying cotinine concentration in plasma/saliva, particularly when concentration levels are less than 1 ng ml^{-1}. A related technique, GC–MS involves coupling a gas chromatograph with a mass spectrometer, and has been recognized as an ultimate standard of reference in analysis [111].

The chromatographic methods used for the estimation of various biomarkers are: packed column gas chromatography using a nitrogen–phosphorus flame ionization detector [79,99]; capillary column gas chromatography using silicone derivatives in liquid phase [100,102,112]; high performance liquid chromatography (HPLC) using a C_{18} reversed phase column, with paired-ion chromatography and UV detection at 237 nm [113–115,117–128]; gas–liquid chromatograph-coupled by a mass-spectrometer employing electron impact or chemical ionization [112,129–131]; selected ion monitoring gas chromatographic mass spectrometry (SIM GC–MS) [72,129,131]; and liquid chromatography atmospheric pressure chemical ionization tandem mass spectrometry (LC–APCL–MS–MS) [38,40]. The detection/quantification limits for the above mentioned chromatographic methods range from 0.1–5 ng ml^{-1}.

6.2.1. Gas–liquid chromatography (GLC)

The concentration of nicotine (up to 0.1 ng ml^{-1}) in the plasma and urine samples of nonsmokers exposed to ETS can be estimated by rapid GLC [84]. A procedure has been described [79] for the determination of cotinine, down to 100 pg ml^{-1} in biological fluids (plasma, urine, and saliva) of non-smokers by packed column GLC. An improved method for rapid and simultaneous estimation of cotinine and nicotine in biological fluids has also been reported [34,56,68,85] (Table 2). This method involves a single rapid extraction step (1 min) without any further concentration, purification or evaporation, thus allowing 150 samples to be analyzed per day. The lower limit of detection claimed for cotinine and nicotine is 0.1 ng ml^{-1} [85]. The salivary cotinine concentration in nonsmoking school children has been found to vary depending upon the smoking habits of their parents [85]. Thus, when neither parent smokes, only father smokes, only mother smokes, or both parents smoke, the cotinine concentrations are 0.79, 1.98, 2.72, and 4.46 ng ml^{-1}, respectively.

Table 2
Cotinine and nicotine concentration in biological fluids

Constituent	Plasma	Concentration (ng ml^{-1})	
		Urine	Saliva
Cotinine	27	100	130
Nicotine	23	23	115

Another study [74] relates to the determination of cotinine in biological fluids of nonsmokers, passive smokers, and active smokers (Table 3).

An investigation [65] to quantify salivary cotinine in adolescent girl smokers (11–14 years old) was done over three consecutive years using GLC. At the beginning, the mean cotinine concentration for the adolescent girls was found to be 87.8 ng ml^{-1}, whereas for adult smokers, the mean concentration was 350 ng ml^{-1}. Two years later, the mean cotinine concentration in the adolescent girls went up to ~233 ng ml^{-1} (2/3 of that reported for adults).

A workshop on determining cotinine (<1 ng ml^{-1}) in human body fluids as a measure of passive exposure to tobacco smoke recommends the use of GLC [68]. It reports that saliva cotinine determination by GLC discriminates smokers from non-smokers, with great accuracy. Its sensitivity and specificity are claimed to be greater than 95% [74]. The reagent costs are generally low and the limits of detection are also low. These attributes make GLC the method of choice for studies related to passive smoking.

Table 3
Cotinine concentration level by smoking status

Smoking status	Mean cotinine concentration (ng ml^{-1})		
	Serum	Saliva	Urine
Non-smokers			6.0
Passive smokers			9.2
Active smokers 10 cgts per day	78.0	66.9	646.8
Active smokers >10 cgts per day	301.2	283.7	1100.7

Note. The specimen of biological fluids had been taken from the active smokers in the morning before smoking their first cigarette. Cgts: cigarettes.

Table 4
Cotinine concentration in biological samples before and after exposure to nicotine

Exposure	Plasma	Cotinine concentration ($ng\,ml^{-1}$)	
		Saliva	Urine
Before	0.82	0.73	1.55
After	2.04	2.48	7.71

Various GLC studies have been reported [68,84,103,110] with respect to mean cotinine concentration in the biological fluids of nonsmokers, before and after exposure to ETS. The result of one such study is reported here [103] (Table 4).

It is apparent that the cotinine concentration in plasma and saliva are almost comparable, but the cotinine concentration in urine is often twice as much [71].

6.2.2. High performance liquid chromatography (HPLC)

Since the late 1970s, there have been many reports in the literature about the determination of nicotine and its metabolites in the biological fluids of smokers by HPLC. According to one investigation, pre column derivatization was done before HPLC [115]. A fast and low cost method to assess the smoking status of an individual utilizes 2-thio barbitaric acid (DBTB) as a derivatizing reagent for nicotine and its metabolites [116]. Doubts however, have been expressed regarding the use of cotinine as a marker for identifying the intermittent smoker, because the time elapsed between smoking and sample collection, is of prime importance before considering use of this marker [111].

6.2.3. High performance thin-layer chromatography (HPTLC)

HPTLC has been used for determining urinary cotinine with 1-methyl-2-pyrrolidinone as an internal standard. Prior to the HPTLC analysis, cotinine is extracted from urine by solid phase extraction (SPE). The lower detection limit of cotinine by this method is $6\,\mu g\,l^{-1}$. In a pilot study, cotinine measurement by SPE-HPTLC method has been used for assessing hazard from home ETS on the health of elementary school boys [128].

6.2.4. Selected ion monitoring gas chromatographic mass-spectrometry (SIM GC–MS)

SIM GC–MS methods have been described in the literature for the determination of cotinine in serum and urine samples [72]. The quantification was done using the most abundant fragment ions at *m*/*z* 98 and 101 corresponding to cotinine and deutero cotinine (Methyl-d_3, internal standard), followed by their integration [72]. The limits of detection ($ng\,ml^{-1}$) of cotinine in the three biological media are: saliva (5), serum (10), and urea (50) [72,129–131].

6.2.5. Liquid chromatography–atmospheric pressure chemical ionization tandem–mass spectrometry (LC–APCI–MS–MS)

LC–APCI–MS–MS has been described as a sensitive and specific method, and has been developed for evaluating the serum cotinine concentrations in nonsmokers potentially exposed to ETS. It consists of a HPLC coupled with an atmospheric pressure chemical ionization tandem mass spectrometer. The limit of detection is claimed to be $\sim 50\,ng\,l^{-1}$ and about 100 samples per day can be analyzed by this method [38].

6.3. Radioimmunoassay (RIA) and enzyme linked immunosorbent assay (ELISA)

RIA and ELISA are used for estimating cotinine in serum and saliva. The detection limit ($0.1\,\mu g\,l^{-1}$) [71] is similar for both methods. The principal advantages of the immunological methods are a low sample volume requirement and little sample manipulation [68,132]. These methods have been found to be 100% effective in discriminating smokers from nonsmokers. The merits and demerits of RIA and ELISA are given next.

6.3.1. Radioimmunoassay

RIA involves the use of two radioisotopes—iodine (^{125}I) [133–142] and tritium (^{3}H). The technique is specific, sensitive, and easy to perform; it can be applied with appropriate dilution directly to the sample without taking recourse to extraction, evaporation, and concentration steps. It is suitable for the determination of cotinine in biological [86,133–136,141,143–146] fluids such as amniotic fluid, blood serum, saliva, spinal fluid, and urine. The lower limit of detection depends upon the nature of the fluid analyzed and is, for

example, 0.5 ng ml^{-1} for cotinine in serum or plasma, and 10–15 ng ml^{-1} in saliva and urine [147,148]. It should, however, be noted that the RIA method failed to reveal any trace of nicotine and cotinine in amniotic fluid collected from nonsmoking women [68]. Male smokers, on the other hand, are reported to exhibit a significant level of cotinine (∼10–250 ng ml^{-1}) in the spinal fluid, while non-smokers exhibit none.

Although RIA is easy to perform, the procurement of antisera presents a bottleneck in obtaining a metabolite profile of nicotine. RIA requires specific antibody not only for nicotine, but also for each of its metabolites [73]. Also, the method is time consuming (>48 h). However, with the introduction of automation, the number of analyses carried out per day can be increased. The sensitivity of the method can further be increased using tracers of higher specific activity [147,148]. Another major concern is the disposal of a large volume of radioactive ^{3}H scintillation fluid [68]. It may be noted that cotinine levels attained by conventional RIA are reported to be higher by 30–50% compared to chromatography. The higher values have been explained partly due to cross reactivity of anticotinine with *trans*-3′-hydroxycotinine present in the smokers urine. Its concentration is reported to be three times higher than cotinine [101]. There is a significant variation in the concentration of cotinine in the various body fluids [112]. In adult nonsmokers the cotinine concentration (expressed in terms of μg l^{-1}) in serum, saliva, and urine are 1.5, 1.7 and 5, respectively [105]. In smokers the value increases 180-fold in plasma and saliva and 280-fold in urine. An interesting observation has been reported about the significant difference in cotinine concentration between white and black American children. The cotinine ratio between white and black children is 1:9.3. Since the black American kids come mostly from a poor socio-economic background where smoking is more prevalent, they show higher levels of cotinine [146].

RIA has been employed for measuring cotinine in serum and urine of habituated smokers. The RIA protocol has been suitably modified so that it is applicable to non-smokers (exposed to ETS) as well. The method is claimed to have a lower sensitivity and the range of detection is 0.2–0.4 μg l^{-1}.

RIA of hair [141] has also been used for distinguishing nonsmokers from smokers. The environmentally derived nicotine (from tobacco smoke) is absorbed on the exterior of the hair shaft and can be removed by hexane washing. The nicotine and cotinine present in the inner shaft of the hair can be extracted with acetone. The solvent is evaporated and the residue analyzed by RIA. The average value of total nicotine and cotinine, of the scalp hair samples are reported [141] (Table 5).

Table 5
Nicotine and cotinine concentration by smoking status

Status	Concentration (μg g^{-1})	
	Nicotine	Cotinine
Smokers	12.8	0.5
Non-smokers	2.24	0.12

RIA (with ^{3}H and ^{125}I radionucleides) in fluid as well as solid phase systems, involving the use of monoclonal antibodies specific for cotinine, have been developed [137,138]. In the latter case, the cotinine polyglycine conjugate is passively adsorbed to the surface of 96-well plastic microtiter plates. Monoclonal antibodies in fluid phase have likewise been prepared for ELISA and florescent immunoassay (FIA) [137,138].

Nicotine like cotinine has been assayed in the physiological fluids by RIA methods.

6.3.2. *Enzyme-linked immunosorbent assay (ELISA)*

Unlike RIA, ELISA does not involve costly equipment, such as scintillation or gamma counters [73], and avoids the use of radiolabeled compounds, expensive reagents [149], and long working time [71]. Since the time required for analysis is short (<5 h) [68], ELISA is well suited for epidemiological investigations that seek to assess human tobacco risk.

The estimation of cotinine by ELISA involving the use of polyclonal rabbit antinicotine antisera is beset with difficulty, namely *trans*-3′-hydroxy cotinine cross-reacts with the antisera by about 30% [150]. This, however, does not seem to be the case with monoclonal antibody immunoassay in the detection of cotinine in saliva and urine of passively exposed children [134–136,143,144,151].

While using isotopic or non-isotopic immunoassay, caution should be exercised in interpreting the data obtained from urine analysis. Interference in the assay might arise from several factors, such as the possible

non-specific inhibition of antigen–antibody reaction resulting from the effect of pH or high concentration of salts or urea. A worthwhile recommendation has been made to employ cotinine perchlorate (for fumarate) as a primary standard in immunoassay in place of presently used cotinine base [68]. It is non-hygroscopic and therefore presents no problem in accurate weighing.

7. Cortisol

Cortisol is one of the most frequently used steroid hormones for assessing adrenal disorders. Serum and urinary cortisol concentrations have been used to monitor adrenocortical function as well as in the diagnosis of chronic fatigue and depression [152,153]. Cortisol is also used as a biomarker of stress [154–157]. A study performed on rats concluded that stress lowers circulating nicotine levels [33]. In humans, the relationship between stress and smoking is well documented (Fig. 1).

It is speculated that stress might increase smoking by increasing the removal of unmetabolized nicotine by the kidneys, thereby decreasing nicotine availability in the body. This would trigger the craving for smoking so that a steady nicotine level in the body can be maintained. Cortisol thus appears to play a role in the metabolism of nicotine. Salivary cortisol has been suggested as a stress biomarker since saliva collection is easy and stress-free and does not require medical supervision. It is an excellent indicator of unbound cortisol concentration in serum [158] and has close correspondence in circadian fluctuations with plasma cortisol [159]. The only drawback is that salivary cortisol concentrations are lower than serum and urine cortisol concentrations. The reference salivary cortisol concentrations are 1–8 $\mu g\,l^{-1}$ in the morning and 0.1–1 $\mu g\,l^{-1}$ in the evening. These values are about two orders of magnitude lower than those found in serum [160]. Thus salivary cortisol can be used to monitor stress in large populations, provided the detection method is very sensitive. Various procedures used to assess the concentration of cortisol in body fluids are discussed next.

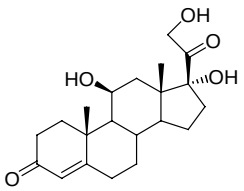


Fig. 1. Hydrocortisone/11,17,21-trihydroxypregore-4-ene-3,20-dione.

7.1. Methods of measurement

7.1.1. Chromatography

A number of analytical methods have been developed for determination of cortisol. A nonspecific color reaction for serum cortisol determination, called the Porter-Silber, reaction is interfered with certain drugs, steroids, and non-steroid metabolites [161]. The same is the case with fluorometric analysis [162–166]. Fluorometric analysis is a liquid chromotagraphic assay for serum cortisol and exploits sulfuric acid induced fluorescence. Each analysis is completed in seven minutes and only 50 μl of the serum is required. The lower detection limit of cortisol by this method is 10 $\mu g\,l^{-1}$ [166].

A method based on analytical liquid chromatography has been described for the estimation of serum plasma/cortisol. This method offers specificity and sensitivity and requires 15 min analysis time per sample. The lower limit of detection of cortisol by this method is 5 $ng\,l^{-1}$. One milliliter of serum/plasma is analyzed isocratically on a reverse phase column and the mobile phase consists of acetonitrile–phosphate buffer (30:70; v/v) with a flow rate of 2 $ml\,min^{-1}$. The cortisol absorption is monitored at 254 nm and the concentration is estimated by measuring the peak area in the chromatogram [161].

High performance liquid chromatography (HPLC) offers the advantage of specificity. By employing this powerful separation technique, it is possible to estimate serum cortisol in the presence of elevated levels of steroids [167]. For facilitating sample preparation and preventing HPLC column contamination, the biological sample is subjected to solid phase extraction in place of the traditional liquid–liquid extraction. The extraction column is packed with silica-bonded to octadecylsilane and the mobile phase consists of aqueous methanol (40:60; v/v). A study reports that the saliva sample need not be refrigerated before analysis, and can be preserved at room temperature

for an extended period (∼6 months) with the addition of citric acid [168].

Sensitive and reliable GC–MS and LC–MS methods for simultaneous determination of cortisol and cortisone in plasma using deuterium labeled internal standard have been reported [169–171]. Isotope dilution mass spectrometry is a highly reliable analytical tool for measurement of endogenous and synthetic steroids in biological fluids [172].

A method for determining cortisol production rate in the serum involves solid phase extraction and derivatization of cortisol followed by gas chromatographic separation and detection by selective negative ion monitoring mass spectrometry [173]. A recent study describes a capillary GC–MS method for the simultaneous determination of endogenous cortisol and cortisone and their ^{13}C labeled analogues. The separation of the tetra hydrocorticoids is achieved by the bis-methylene dioxy-pentafluor propionyl (BMD-PFP) derivatization. Selected ion monitoring of the molecular ion of BMD-PFP derivative of cortisol is used for quantification. Sensitivity limit for cortisol by this method is found to be 150 pg per injection [174].

Liquid chromatography–tandem mass spectrometry (LC–MS–MS) is now widely used in the analysis of the organic substances. Due to high selectivity and sensitivity of this method, it is possible to rapidly analyze complex biological materials at low concentrations. LC–MS–MS methods for urinary cortisol analysis [175–177] as well as salivary cortisol have been reported [178]. For determining the salivary cortisol content by this method, saliva is collected, centrifuged, and deuterium labeled; cortisol is added as an internal standard. Proteins are precipitated out using acetonitrile and the supernatant is evaporated. Residue is dissolved in MeOH and acidified with acetic acid. Analysis is then done by LC–MS–MS and the limit of quatification is 0.5 $\mu g\,l^{-1}$ [178].

7.1.2. Immunoassays

In order to evaluate the dynamic endocrine function, salivary cortisol is quantified by immunoassay method [167,179–183]. The method is claimed to be simple, quick, sensitive, and specific. Also, the assay involves collection of saliva, which provides a convenient alternative to the stressful and invasive procedure of repeated collection of venous blood from human subjects. Accordingly, measurement of steroids in saliva rather than plasma is gaining popularity. Most of the earlier methods reported for salivary cortisol measurement were based on RIA [184–189].

A non-isotopic, heterogeneous, competitive immunoassay has been reported for serum cortisol determination and is called carbonyl metallo-immuno assay. Two stereo isomers of a cobalt carbonyl complex have been employed as the organo-metallic tracers. Using the carbonyl metallo-immunoassay, complete characterization of the two different polyclonal anticortisol antibodies was possible. Therefore, reliable and reproducible assay with as little as 50 μl of serum is possible.

A convenient, accurate, and reliable method of salivary cortisol measurement has been reported [173]. This is a non-isotopic immunoassay and is called, dissociation enhanced lanthanide fluoroimmunoassay (DELFIA). It is based on the competitive reaction between sample cortisol and europium labeled cortisol for limited amount of binding sites on biotinylated monoclonal antibodies specific for cortisol. The salivary sample is centrifuged to remove any particulate material and the clear sample is extracted with cold methylene chloride followed by reconstitution in 0.1 ml of cortisol free serum. Cortisol extract aliquots (30× 25 μl) are then analyzed by DELFIA. Extraction procedure was not necessary and in fact the intra assay precision of unextracted sample was better than the extracted sample. The detection limit of the assay is 2 ng/ml. DELFIA assay provides a reliable and convenient alternative for salivary cortisol assay.

8. Conclusion

Of the different body fluids, saliva is the matrix of choice for assessing the presence of nicotine and its metabolites in humans exposed to ETS. Of the different biomarkers, cotinine appears to be the analyte of choice, as it fulfills the prerequisites of specificity and retention time and is found in detectable concentrations in all three matrices. GC–MS is preferred for analyzing nicotine and its metabolites in smokers whereas GLC is preferred for studies related to passive smokers.

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