

Near-patient test for nicotine and its metabolites in saliva to assess smoking habit

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SUMMARY. Smoking is a major risk factor for periodontal disease, causing bone and tooth loss. Effective management of many oral conditions requires accurate information about smoking. We describe a disposable, 10-min, near-patient saliva test to monitor cigarette smoking. A plastic device contains the dried reagents to measure nicotine and its metabolites by a colorimetric assay. The device can be used to give a semi-quantitative assessment of tobacco consumption by observing a colour change and comparing it to a reference chart. Alternatively, the test can be quantified by measuring the light absorbance with a colorimeter and determining the concentration of nicotine metabolites by reference to a cotinine standard. This method correlates with reported daily cigarette consumption ($r=0.62$, $P<0.0001$) and nicotine yield of cigarettes smoked ($r=0.35$, $P<0.01$) and compares favourably with cotinine, as measured independently by radioimmunoassay ($r=0.57$, $P<0.005$). The method provides a simple-to-use, inexpensive way of monitoring tobacco consumption in dental surgeries.

INTRODUCTION

There is increasing awareness of the effects of smoking on the prevalence and severity of periodontal disease.¹ Smokers have greater bone loss, increased numbers of deep pockets, enhanced tooth loss and advanced calculus formation compared with non-smokers.²⁻⁶ Other oral conditions, including leukoedema, leukoplakia, stomatitis nicotina and oral cancer, are also more prevalent in smokers,^{7,8} with the severity being related to cigarette consumption. Smokers below the age of 33 years are at greater risk of periodontitis, with an increased relative risk over 14 times that of non-smokers; again the effect is correlated with cigarette consumption.^{7,9}

CLINICAL APPLICATION

Because of these wide-reaching effects, smoking prevention is becoming a priority for the dental team. It forms part of the existing commitment to disease prevention, and is coupled with more regular patient/clinician contact, especially for adolescents, than is achievable in general medical practice.¹⁰⁻¹²

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For successful treatment, accurate information about smoking habit is required. Smokers tend to under-report their cigarette consumption, or, more seriously, deny smoking altogether.¹⁰ Also, documentation of daily cigarette consumption lacks objectivity, because it does not account for the type of cigarette used, puffing behaviour, depth of inhalation and variations in metabolism.^{8,13} To overcome these problems, biochemical verification of smoking habit has become increasingly important in recent years. It is generally accepted that measurement of nicotine metabolites such as cotinine is the best approach to assess cigarette smoking.^{14,15} Cotinine can be detected in serum, urine and saliva, and measured to a high degree of sensitivity with a range of sophisticated laboratory techniques.¹⁶⁻¹⁸ Whilst cotinine has been measured in gingival crevicular fluid and saliva,¹⁹ it was not until 1990 that the pharmacodynamics of cotinine within saliva and serum were fully documented and measurement limits determined.

SAMPLE MATRIX

Cotinine is one of ten pyridine alkaloids derived from cigarette smoke, and the collective determination of all the nicotinic breakdown products

by colorimetric methods provides an alternative approach. These assays are simple and inexpensive to perform, are reliable and sensitive and do not require elaborate instrumentation or highly trained staff.²⁰⁻²³ Laboratory tests are useful research tools, but because of the considerable delay in obtaining the data the results are of little value to the patient. For the assessment of smoking habit in periodontal disease patients, urine testing seems inappropriate, whereas saliva testing, which is used extensively as a diagnostic fluid, seems more suitable.²⁴ The aim of the present investigation was to adapt the colorimetric assay to improve its sensitivity so as to measure nicotine metabolites in saliva. The concentrations of these compounds in saliva are approximately ten times less than are found in urine. We therefore needed to increase the sensitivity and investigated 2,2-dimethyl-1,3-dioxane-4,6-dione (Meldrum's acid), with its enhanced reactivity to nicotine metabolites, as an alternative condensing agent.²⁵

TECHNICAL APPROACH

Near-patient (or point-of-care) testing is becoming an increasingly important means of improving clinical management and the quality of care in a variety of clinical settings. We developed a near-patient colorimetric urine test for smoking. The test incorporated dried chemical reagents in a special disposable tube, which sealed the reagent before use and the reactants after use, enabling easy and safe disposal. The test was designed to be inexpensive and easy to use. It could be employed as a stand-alone test for semi-quantitative assessment of smoking, or with a colorimeter for a quantitative measurement. The results correlated well with self-reported cigarette consumption ($P < 0.0001$), with the nicotine yield of cigarettes smoked ($P < 0.001$) and with independent measurement of cotinine by gas chromatography ($P < 0.001$).²⁶ Our second aim was to incorporate the assay for nicotine metabolites in saliva into the near-patient testing device.

MATERIALS AND METHODS

All chemical reagents were analytical grade. Sodium citrate, citric acid, chloramine-T, 2,2-dimethyl-1,3-dioxane-4,6-dione (Meldrum's acid), cotinine and potassium cyanide were obtained from Sigma-Aldrich Co Ltd (Poole, UK).

Meldrum's acid (20 mg) and potassium cyanide (7.5 mg) were combined with a buffer of citric acid (18 mg) and sodium citrate (20 mg) to maintain the pH at 4.2. Chloramine-T (11 mg) was used as the chlorine-donating agent, but for stability reasons could not be mixed with the other chemicals before use and had to be physically isolated.

The smoking test device (STD) consists of a plastic tube designed to contain the chemicals safely before use and to seal the reactants after use, so allowing easy and safe disposal (Fig. 1). It comprises a sampling device, which is a fixed-volume syringe and cap, and a reaction chamber. Because the chloramine-T requires isolation, it is contained within a seal, consisting of a washer-like annulus, with a space between two tightly fitting plastic discs (Fig. 1b).

In use, 2 mL of unstimulated saliva are aspirated by the syringe, which is then inserted into the barrel, forming a permanent seal that prevents subsequent detachment. As the syringe passes through the barrel, the tip breaks the seal and dispenses the chloramine-T into the reaction chamber below (Fig. 1c). The saliva is injected onto the reagents, which dissolve, so starting the reaction.

The STD can be used to give a semi-quantitative assessment of nicotine intake by comparison to a colour chart, or with a simple colorimeter to measure the concentration of nicotine metabolites with reference to a cotinine standard.

Semi-quantitative assessment of smoking

Saliva samples were collected from 100 healthy individuals (46 men and 54 women, with an average age of 27.5 years) of whom 55 were non-smokers and 45 were regular smokers with an average consumption of 13.2 cigarettes per day. Details of current smoking habit were obtained by self-completed questionnaire. Whole saliva was collected by expectoration into a 30-mL plastic container to a marked volume of 5 mL. The saliva was allowed to settle for 5 min to facilitate sedimentation of gross particulate matter, after which 2 mL were aspirated by the sampling device. This was inserted over the reaction chamber, the sample injected onto the reagents and shaken for 150 s or vortexed for 90 s to allow the reagents to dissolve. If the sample contained nicotine or its breakdown products, the reaction produced a yellow/orange end-point in a concentration-dependent manner. A colour change was usually observed within

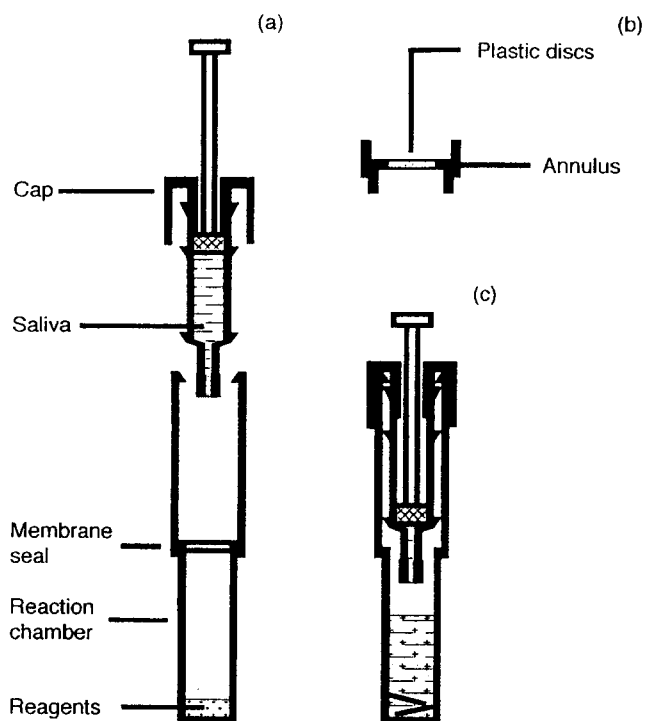


FIGURE 1. The smoking test device (a) before use, comprising a sampling syringe and cap, and a reaction chamber containing dry reagents. (b) shows an enlargement of the membrane seal, which comprises a plastic annulus and two discs and acts as an inner chamber to isolate a reactive reagent. (c) shows the assembled device after addition of the sample.

4 min, but 10 min were required for adequate and reproducible colour development. A colour chart consisting of seven panels of graduated colour density, ranging from colourless to yellow/orange, equivalent to cotinine solutions varying from 0 to 3 mg/L, was generated using a computer program (Microsoft Paint for Windows 95). The colour developed by the saliva smoking test at 10 min was compared to the colour chart and a result ranging from 0 to 6 was assigned to each test. On occasions when the colour was perceived to be between two shades, it was allocated a midway score. To assess inter-operator variability three different operators assessed 'blind' colour changes in the same 20 saliva samples taken from non-smokers and a range of smokers.

Quantitative assessment of smoking

Saliva samples were collected from a further 106 healthy individuals (62 men and 44 women, average age 28.3 years) of whom 28 were non-smokers and 78 were regular smokers with an average consumption of 13.7 cigarettes per day.

Details of current smoking habit were again obtained by self-completed questionnaire.

For the quantitative assessment of the STD, whole saliva was collected as before, but the sample was cleared either by allowing the saliva sample to stand at room temperature for 30 min or, preferably, by centrifugation at 500 g for 5 min. Saliva was aspirated as before, added to the device and mixed, then placed in a portable battery-operated colorimeter (6030 colorimeter, Jenway, Felsted Dunmow, UK). The test was allowed to settle for a further 30 s; then, 3 min after addition of the sample, the first absorbance reading at 495 nm was taken, and a further reading every minute thereafter until 10 min had elapsed. The result was taken as the absorbance change during this period.

For active smoking, a three-point cotinine standard curve was used, with cotinine concentrations of 0, 1.25 and 2.5 mg/L (14.2 $\mu\text{mol/L}$) plotted against absorbance change. The change in absorbance obtained from an unknown saliva sample was read from the curve, and the appropriate 'cotinine equivalent' concentration

was determined. Comparison of the smoking test result with various factors of reported smoking habit was investigated in all subjects for whom details were available.

Comparison between STD device and cotinine

The smoking test results were evaluated by comparison with a 'gold standard' for cigarette smoking, namely cotinine measured by radioimmunoassay (RIA). Twenty-four saliva samples previously measured with the STD were assayed 'blind' by an independent laboratory using an ^{125}I -based RIA for cotinine.¹⁸ Aliquots (10 μL) of sample or cotinine calibrators were incubated overnight with cotinine antibodies and radiolabelled cotinine. Bound and free fractions were separated using a polyethylene glycol-assisted second antibody technique. The radioactivity of the bound fraction was counted and cotinine concentrations were estimated using a four-parameter logistic curve-fitting program. Within- and between-batch imprecision were 5.1% and 7%, respectively.²³

Statistical analysis was undertaken using Minitab[®] statistical software (release 9.2). Relationships to smoking status were analysed by two-sample *t*-test, and variable parameters by Pearson's correlation coefficients, with $P < 0.05$ taken as statistically significant. A comparison of medians was made using the Kruskal-Wallis non-parametric test. For measuring the reproducibility of results from several operators, the average of the correlation coefficients between pairs of operators is used here.

RESULTS

Semi-quantitative assessment of smoking

Table 1 shows the distribution of results for the visual comparison of the saliva test with a colour chart consisting of seven panels, numbered 0–6, of graduated colour density, ranging from

colourless to yellow/orange. There was a steady increase in visual scores against reported daily cigarette consumption ($r = 0.76$, $P < 0.001$). There was also a significant difference in median visual scores across the different categories of reported daily cigarette consumption ($\chi^2 = 69.09$; d.f. = 5, $P < 0.001$). The majority of non-smokers gave a negative result, with a wide spread of values in the different categories of cigarette consumption. Analysis of questionnaire data also showed a good correlation with cigarette consumption on the day of the test ($r = 0.56$, $P < 0.001$) and with cigarette consumption on the day prior to the test ($r = 0.63$, $P < 0.001$). Also significant were the correlation with time since the last cigarette ($r = -0.40$, $P < 0.001$) and nicotine yield of the cigarettes smoked ($r = 0.33$, $P < 0.02$). The sensitivity (the proportion of true positives correctly identified by the test) was 91% and the specificity (proportion of true negatives correctly identified by the test) was 89%, providing a diagnostic accuracy of 90% and positive/negative predictive values of 87%/93%, respectively (data derived from 2×2 contingency table).

Inter-operator variability was assessed by three different operators. The results showed a significant average correlation between operators ($r = 0.79$, $P < 0.05$), indicating good reproducibility between observers.

Quantitative assessment of saliva test and smoking

Figure 2 shows a typical standard curve of changes in light absorbance at 495 nm against changes in aqueous cotinine concentrations of 1.25 and 2.5 mg/L (71 and 142 $\mu\text{mol/L}$, respectively). This range of cotinine concentrations was used for analysis of active smokers, but the lower limit of sensitivity of the assay was 0.125 mg/L (7.1 $\mu\text{mol/L}$), below which no absorbance change could be

TABLE 1. Semi-quantitative assessment of smoking habit by visual classification of 100 saliva samples from smokers ($n = 45$) and non-smokers ($n = 55$)

Daily cigarette consumption	<i>n</i>	Category of visual classification (arbitrary units)					Median	Quartile range
		0	0.5–1.0	1.5–2.0	2.5–3.0	3.5–6.0		
0	55	49	4	1	1	0	0	0–0
1–5	10	3	4	3	0	0	1.0	0–2.0
6–10	12	0	5	4	3	0	1.75	1.0–2.75
11–15	9	1	2	6	0	0	2.0	0.75–2.0
16–20	11	0	1	4	4	2	2.5	2.0–3.0
20+	3	0	1	0	1	1	2.5	1.0–5.0

The number in each category is shown together with the median and upper and lower quartiles for each group.

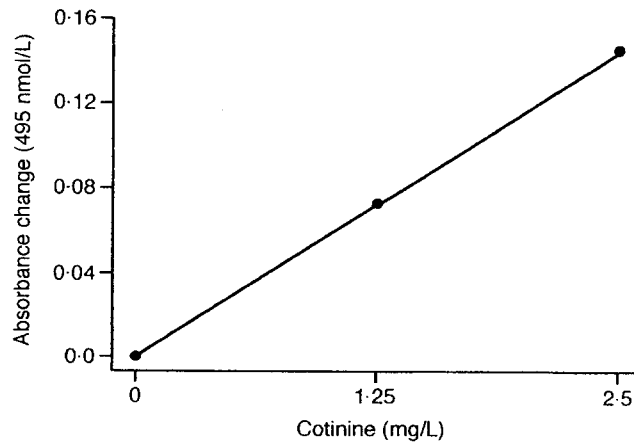


FIGURE 2. Typical cotinine standard curve obtained by plotting cotinine concentrations of 0, 1.25 and 2.5 mg/L against changes in light absorbance at 495 nm, measured over a 10-min period.

reproducibly detected. A standard curve was produced for each batch of samples, usually ten in each batch, to take account of operator dependency and variations in reagent activity, operating temperature and atmospheric moisture. Inter-assay variation was 9.5% ($n=20$) and intra-assay variation 15.4% ($n=25$).

The results from the 106 saliva samples from smokers and non-smokers produced a range of 'cotinine equivalent' concentrations of 0–4.9 mg/L (0–27.8 $\mu\text{mol/L}$), with a median of 0.62 mg/L (3.5 $\mu\text{mol/L}$). The majority of

samples from reported non-smokers produced no colour change, and so gave a negative result. A plot of the results against categories of daily cigarette consumption is shown in Fig. 3. The data included results from 28 non-smokers and 78 current smokers. There was a broad spread of results in each category of cigarette consumption, but there was a significant correlation between the result and reported consumption (Pearson $r=0.62$, $P<0.0001$; Spearman $r=0.76$, $P<0.0001$). There was a progressive increase in median values with increasing reported smoking.

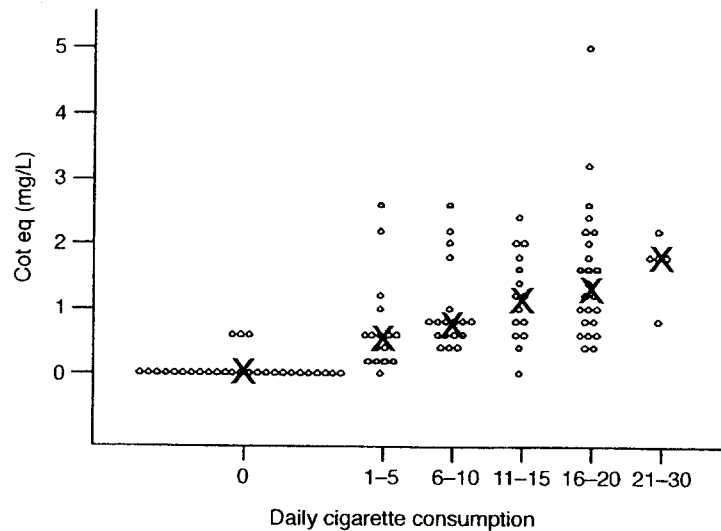


FIGURE 3. Shown are 106 smoking test device results ['cotinine equivalents' (cot eq), mg/L] plotted against different categories of daily cigarette consumption: 0 ($n=28$); 1–5 (16); 6–10 (18); 11–15 (15); 16–20 (24); 20+ (5). The individual results are represented, together with a cross to denote the median point for each category. The results of one-way analysis of variance are $F=13.74$, $P<0.0001$.

A cut-off ratio value of 0.25 mg/L was used, above which the result was classified as positive. The data summarized in Table 2 include results from 28 non-smokers, of whom 25 (89.4%) were 0 mg/L and three (10.7%) were 0.5 mg/L. The table also shows results for 78 smokers, of whom 73 (93.6%) were classified as positive and five (6.4%) as negative. These latter patients reported smoking fewer than 5 cigarettes a day and had not smoked on the day of the test. Calculation of sensitivity produced a result of 89.3% and specificity of 93.6%, with positive and negative predictive values of 96% and 83.3%.

Comparison between STD results and cotinine

Figure 4 shows a graph of cotinine equivalent results as measured with the STD against cotinine concentrations as measured by radioimmunoassay on 24 parallel samples. A significant correlation was observed between the

TABLE 2. Quantitative assessment of smoking habit using the colorimetric method (results from 78 smokers and 28 non-smokers)

Self-reported smoking	Positive smoking test		
	Yes	No	Total
Yes	73	5	78
No	3	25	28
Total	76	30	106

Using a cut-off value of 0.25 mg/L, above which the result was classified as positive, the test produced a sensitivity of 93.6% and a specificity of 89.3%.

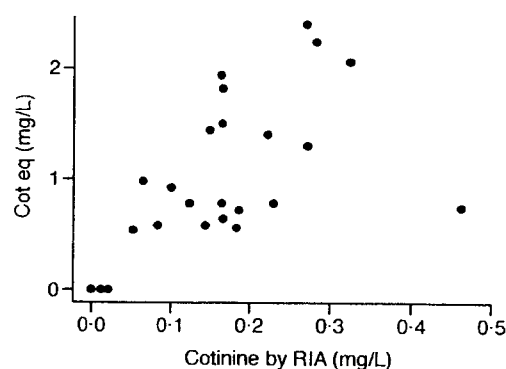


FIGURE 4. 'Cotinine equivalent' concentration (cot eq, mg/L), as measured with the near-patient saliva test, plotted against cotinine concentration (mg/L) measured by radioimmunoassay (RIA) on parallel saliva samples ($n=24$). A significant correlation ($r=0.57$, $P<0.005$) was obtained.

STD results and cotinine concentration ($r=0.57$, $P<0.005$). The cotinine concentrations ranged from 0 to 0.46 mg/L with a median of 0.17 mg/L, while the 'cotinine equivalent' results ranged from 0 to 2.4 mg/L with a median of 0.78 mg/L.

DISCUSSION

We have developed a 10-min near-patient saliva test for nicotine and its metabolites. The test is the first colorimetric assay for smoking to incorporate Meldrum's acid (2,2-dimethyl-1,3-dioxane-4,6-dione) as the condensing agent. The mechanism of the reaction involves combination of cyanide and chloride to form cyanogen chloride, which interacts with the pyridine ring structure common to nicotine and all its metabolites. This forms a glutacodialdehyde intermediate which reacts with Meldrum's acid to form a coloured derivative.²⁵ This approach has the advantage over measurement of a single metabolite such as cotinine because there is wide inter-individual variation in the metabolism of nicotine, with ten possible derivatives being formed. Cotinine itself is the major breakdown product, but urinary excretion is small, accounting for 10–15% of nicotine metabolite excretion.²⁷

The test was developed to be easy to use and disposable, yet inexpensive, with a high degree of sensitivity and specificity for tobacco smoking. It can be used visually by comparison with a colour chart, which produces a semi-quantitative assessment of nicotine intake. Colour assessment, especially when the quality is different, can be subjective and open to misinterpretation. This was alleviated to some extent by allocating midway scores if the test colour was between two shades on the chart. The inter-operator variability was low with good correlation between examiners, indicating the potential for reliable reproducibility. These scores correlated well with average self-reported cigarette consumption, consumption on the day of the test, on the day prior to the test and, surprisingly, with the nicotine yield of the cigarettes smoked. The broad overlap of test results and different categories of cigarette consumption will be influenced to some extent by false reporting and timing of the last cigarette, but, we believe, also reflects the variation in smoking habit. Age and gender also influence the type of cigarettes smoked, puff topography and depth of inhalation. Many biochemical studies show little or no relationship between nicotine intake and yield,

suggesting compensation to maintain the same level of nicotine after brand switching.⁹ However, other studies have found that, after a period of acclimatization, nicotine intake does tend to reflect nicotine yield.^{28,29}

With minor sample preparation the smoking test can give a fully quantitative result by comparison with a cotinine standard curve. This is better suited to providing biofeedback to patients during smoking cessation programmes. Evidence from our own studies using the urine test in pregnancy (unpublished data) has shown that patients who have tried to reduce their smoking, but maintain their usual cigarette consumption, have been found to have a significantly lower nicotine intake. This reduction is possibly due to an increased awareness of smoking, reflected in a reduced smoking efficiency.

Saliva is well recognized as a suitable fluid to test for tobacco by-products. It has the advantage of being non-invasive, and yet salivary nicotine metabolites are in equilibrium with plasma levels.³⁰ The results from the smoking test suggest that salivary measurements of nicotine metabolites reflect long-term smoking habit, while the close relationship with time since the last cigarette suggests that there may be local absorption of nicotine through the buccal mucosa and parotid ducts.

Education of both patients and dental practitioners about the risks of smoking for periodontal and other oral diseases is important if the damaging effects are to be reduced. A recent feasibility study of smoking cessation advice provided by primary care dentists found that motivated dentists with staff support and access to information were able to contribute to tobacco control measures in the community.³¹ Biofeedback of tobacco biomarkers has been shown to be a useful tool to improve smoking cessation in general medicine. Nicotine replacement therapy is also a useful adjunct to smoking cessation by alleviating withdrawal symptoms. However, incorrect prescribing has contributed to poor results. Replacement therapy has been shown to be improved by biochemical measurements of baseline smoking followed by tailored prescribing.^{32,33} The near-patient saliva test described here can be used to assess these parameters quickly and easily.

The quantitative measurements using the saliva test gave results comparable with previously published data using the laboratory colorimetric urine test. Barlow *et al.* cited sensitivity and specificity of 93% and 96.6%,

respectively,³⁰ which is comparable with our results of 89.3% and 93.6%, respectively. We were able to establish a range of 'cotinine equivalent' concentrations of 0–3 mg/L, which encompassed 95% of all results, with a cut-off of 0.25 mg/L, to distinguish between active smokers and non-smokers. Hence, we conclude that the assay for nicotine and its metabolites in saliva has successfully transferred to the STD. The highly significant correlation between our results and independent RIA measurements of cotinine shows that the STD is comparable with sophisticated high technology assays. The test results were on average six times higher than with the cotinine RIA method, which is similar to results previously obtained from a similar colorimetric assay/RIA comparison.³⁴ The reason for this discrepancy is that the colorimetric assay reacts with all the nicotine metabolites, whereas the cotinine assay is specific for the one breakdown product.

We have demonstrated the feasibility of an extra-laboratory test for cigarette smoking, which may be semi- or fully quantitative. As well as verifying self-reported smoking and aiding smoking cessation programmes, it can also be used to monitor compliance with nicotine replacement therapy.

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