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Measurement of nicotine in hair by reversed-phase high-performance liquid chromatography with electrochemical detection

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Abstract

We have developed an assay for nicotine in hair based on reversed-phase HPLC with electrochemical detection. The method uses a low-metal, high-purity silica reversed-phase column. We have investigated the washing, digestion and extraction procedures and discuss the important points in the HPLC method development. The assay is presented as an application in a population of exposed and non-exposed children. Analytical parameters are satisfactory with linearity, recoveries, limit of quantitation and precision all suitable for epidemiological studies involving environmental tobacco smoke exposure assessment.

Keywords: Nicotine

1. Introduction

Accurate assessment of exposure to tobacco smoke in various population groups is an aim of interested epidemiologists. Self reports of environmental tobacco smoke exposure from others have been found to be unreliable [1]. Measurements of plasma and urine cotinine have been used extensively as biochemical markers of exposure (biomarkers). However, these measurements can at best only reflect the last few days exposure to tobacco smoke and samples may be difficult to obtain, especially in young children and infants. Therefore, the development of a reliable biomarker for tobacco smoke exposure reflecting more than 1 week's exposure and using non-invasive samples, would confer significant benefit to researchers studying environmental tobacco smoke exposure. Nicotine in hair has recently been validated as such a marker [2–6], and therefore we set out to develop a relatively easy and inexpensive assay for measuring nicotine in hair.

To date gas chromatography–mass spectrometry (GC–MS) [3,5,8–10], GC [7,9] and radioimmunoassay (RIA) [11,12] have all been used to measure nicotine in hair. GC, without MS, is prone to interferences [9]. GC–MS is expensive and, as with RIA, not so readily available to a wide range of laboratories. RIA measurement of nicotine also has the problem of cross reactivity, especially with cotinine [2]. As a consequence, other investigators have turned to high-performance liquid chromatography (HPLC), mostly with UV detection [14,15] as

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a cheaper and easier means of measuring nicotine in hair. We chose reversed-phase HPLC coupled to electrochemical detection because it has previously been used to measure nicotine in plasma [17] and should give better quantitation limits and selectivity than UV detection.

In a recent study [6] non-exposed infants have been shown to have levels from less than 0.1 to 4.3 ng mg^{-1} of hair while exposed infants had levels from about 2.5 to 25 ng mg^{-1}. We show that in our assay, linearity and recoveries are satisfactory, limits of detection and quantitation are more than sufficient to assess these levels, and precision is good enough ($<10\%$) to be of use in epidemiological studies, for example, the investigation of the relationship between environmental tobacco smoke exposure and asthma.

2. Experimental

2.1. Chemicals

Dichloromethane, methanol and acetonitrile were HPLC grade purchased from Lab-Scan (Dublin, Ireland). Nicotine ditartrate was purchased from the Sigma (St. Louis, MO, USA). 2-Phenylimidazole was from Aldrich (Milwaukee, WI, USA), and all other chemicals were AnalaR grade purchased from BDH (Poole, UK). Type 1 water was obtained from a Barnstead Nanopure water purification system (Barnstead/Thermolyne, Dubuque, IA, USA).

2.2. Standard preparation, calibration and quality control samples

Stock solutions of 2 g l^{-1} nicotine ditartrate and 2-phenylimidazole (internal standard) were made up in 0.01% (v/v) HCl. A seven-point standard curve was constructed by serial dilutions of the stock nicotine standard giving concentrations from 0.05 to 2.0 mg l^{-1}. The standards were then added in 100 μl to a low nicotine hair digest, equivalent to the samples, before extracting. The ratio of the nicotine peak area to the internal standard peak area was used for calculating the standard curve and for interpolating the unknowns. The ratio in the unspiked (blank) digest was subtracted from each standard before calculating the regression line. The nicotine results were reported in terms of the free base using a conversion factor of 0.3376.

Samples for use as quality controls were acquired from volunteers when they had routine haircuts. The samples were cut into 0.5–1.0 cm lengths and then randomised by mixing. These samples were run randomly throughout batches. A standard extracted without hair was also run in each batch to specifically check the extraction and chromatographic part of the procedure.

2.3. Sample preparation, washing, digestion and extraction

Samples were collected by cutting hair from the base of the scalp behind the ear and were stored in paper envelopes. About 1 cm of hair was then cut from the end closest to the scalp using a scalpel swabbed with methanol [9]. Hair was handled with tweezers and fresh paper was used underneath each sample. Duplicate samples of about 2–4 mg were weighed into capped 10-ml polypropylene tubes using a four-position electronic balance. Samples were then washed without agitation for 90 min in 2 ml of dichloromethane at room temperature (21°C). The dichloromethane was aspirated off using a polypropylene transfer pipette and samples dried briefly at 50°C. The hair was then digested at 50°C overnight in 2 ml of 1.0 mol l^{-1} NaOH in capped tubes. The next day the digests were checked to see if all the hair was digested and then incubated for a further 2 h.

The digests were allowed to cool and 50 μl of 750 μg l^{-1} 2-phenylimidazole was added as an internal standard. Nicotine was then extracted into 4 ml of diethyl ether in the capped tube by vortexing for 40–60 s. After transferring the ether to a second 10-ml polypropylene tube containing 100 μl of 0.1% (v/v) HCl in methanol [13,14], the ether was evaporated to dryness under a stream of air at 40°C. Once cool, the precipitate was redissolved in 100 μl of the HPLC mobile phase, transferred to autosampler vials with polypropylene inserts and 1–8 μl injected into the HPLC system. These extracts were stable for at least 3 weeks at 4°C.

2.4. Instrumental

The HPLC system consisted of the following components; a Waters 510 pump (Waters Chromatography Division, Milford, MA, USA), a Hitachi AS-4000 autosampler (Hitachi, Tokyo, Japan) and an ESA Coulochem Model 5100A electrochemical detector (ESA, Bedford, MA, USA). The detector was configured with a post-column 5021 conditioning cell and a 5011 dual detector high-sensitivity analytical cell in series. The voltage settings for the conditioning cell and detectors 1 and 2 were +0.6, +0.6 and +0.9 V, respectively. Nicotine was detected at detector 2 with a gain of 40 times 10 and baseline current between 2 and 5 μA.

The column was a Prodigy 5 μm ODS(3) 150× 4.6 mm (Phenomenex, Torrance, CA, USA) proceeded by a 0.5 μm prefilter (catalog No. A.318, Upchurch Scientific, Oak Harbour, WA, USA) and a C_{18}(ODS) 30×4 mm Phenomenex Security Guard Cartridge. The mobile phase was 50 mmol l^{-1} KH_2PO_4 with 2.5% methanol and 4% acetonitrile added by volume. The final mixture was adjusted to pH 4.8±0.1 and was filtered to 0.2 μm. The mobile phase was run through the column at 1 ml min^{-1} at about 20 MPa pressure. A SSI (Scientific Systems, State College, PA, USA) pulse dampener was used after the pump but was not regarded as essential. Data acquisition and peak quantitation was achieved with a Macintosh LC computer (Apple Computers, Cupertino, CA, USA) and a MacLab/4e with PowerChrom software (ADI Instruments, Castle Hill, Australia).

2.5. Statistics

Precision is reported as a standard deviation (SD, $n-1$ method) and relative standard deviation (RSD, 100×SD/mean).

3. Results and discussion

3.1. HPLC and sample preparation

It was found necessary to increase the detector 2 voltage from +0.75 V [16,17] to +0.9 V in order to accommodate the higher oxidation voltage of 2-phenylimidazole (Fig. 1). We confirmed that cotinine was not electrochemically active in this system [16], and as found previously [16], analytical results were more consistent if the system was allowed to run for 10 to 12 h before making quantitative analyses.

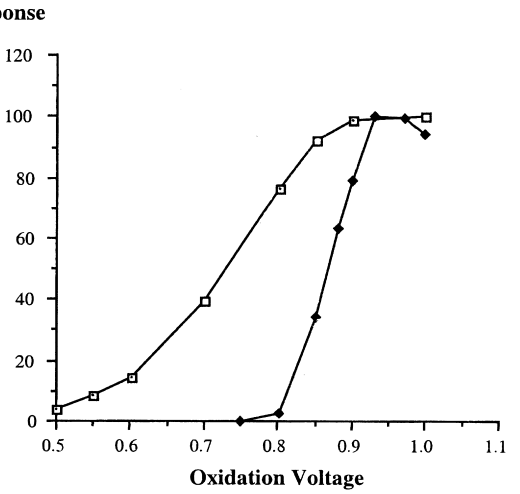

Fig. 1. Electrochemical voltagrams for nicotine and 2-phenylimidazole. These were achieved by injecting constant amounts of the pure standard into the HPLC system, without the column connected, and recording the peak areas while increasing the voltage settings in a stepwise manner. % Response was calculated by comparing each peak with the maximum peak area for each compound.

A good internal standard should be chemically similar, and have preparation characteristics that resemble the compound of interest [13]. This suggests close analogues of nicotine are optimal, however, many of these are not electrochemically active [16] or difficult to obtain. After some initial experimentation we chose 2-phenylimidazole which has been suggested to have similar characteristics to that of *N*-ethylnorcotinine when used as an internal standard with nicotine [13].

After initial experimentation we chose a low metal content, high purity silica reversed-phase column [Phenomenex, Prodigy 5ODS(3)] for the analysis which reduced peak tailing and found that the ion pairing reagent, octyl sulfate, used in earlier methods [16,17] was not required. The concentration of KH_2PO_4 was increased to 50 mmol l^{-1} to improve retention time stability and a pH of 4.8±0.1 was chosen to give good peak shape. We used acetoni-

trile, which is essential for the electrochemical reaction [16], to fine-tune the retention times. Experience with real hair samples, showed that increasing the acetonitrile concentration above 4% caused both nicotine and 2-phenylimidazole to sometimes overlap with small nearby peaks, making quantitation difficult. Nicotine and 2-phenylimidazole were thus separated from each other and nearby peaks on the Prodigy column (Fig. 2) under these conditions.

It was found possible to reuse the mobile phase between two batches, however, recycling within a batch was not used as there was some evidence of drift in the quantitation over the length of the run.

The measurement of environmental tobacco smoke exposure has similar difficulties to the analysis of other drugs in hair. It is our opinion that exposure is likely to be both systemic and by passive absorption to the exterior of the hair, with contributions from each being variable. There is still debate in the literature about the importance of each, but, among those supporting passive absorption of nicotine by hair, Nilsen et al. [21] were in fact studying the adsorption of nicotine vapour to cut samples of human hair by controlled dynamic chamber exposure, which obviously does not allow for studies of systemic absorption. More recent literature support that it is mainly incorporated through the systemic circulation [22–24]. Furthermore, several other studies have based their analyses on this conclusion [12,14,25,26]. Nonetheless we chose to wash the hair to remove externally attached nicotine contaminating the hair from handling of the hair or non-systemic exposure. This is also consistent with the approach used by most other investigators.

A range of solvents, including ethanol, methanol, and dichloromethane, detergent washes and different wash temperatures and washing times [2,8,14,19] have been used to wash nicotine from the hair. It has recently been shown that dichloromethane was an effective solvent for washing nicotine from hair compared to a range of solvents [14], and hence dichloromethane was chosen for our assay. We initially used two, 30-s washes [14] but problems with precision suggested that the time period for washing the hair was not optimised. When the time dependency of the wash was investigated (Fig. 3), the nicotine levels decreased to a plateau, making it likely that short washing times were contributing to

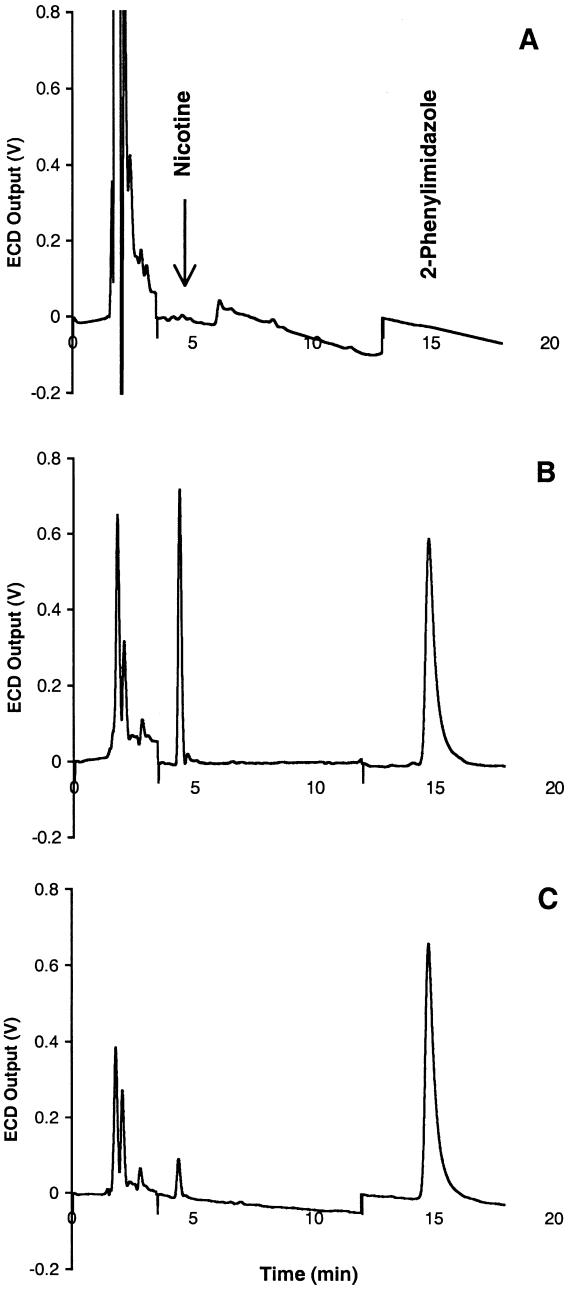

Fig. 2. Representative chromatograms. (A) Blank; extracted from NaOH without hair. (B) Standard; low nicotine hair digest spiked with nicotine (equivalent to 16 ng mg^{-1} of hair). (C) A child's sample (1 ng mg^{-1} of hair). Running conditions as in text. Baselines were reset to zero at 3.8 and 12 min to allow for any drift in the baseline and to keep nicotine and 2-phenylimidazole peaks on scale.

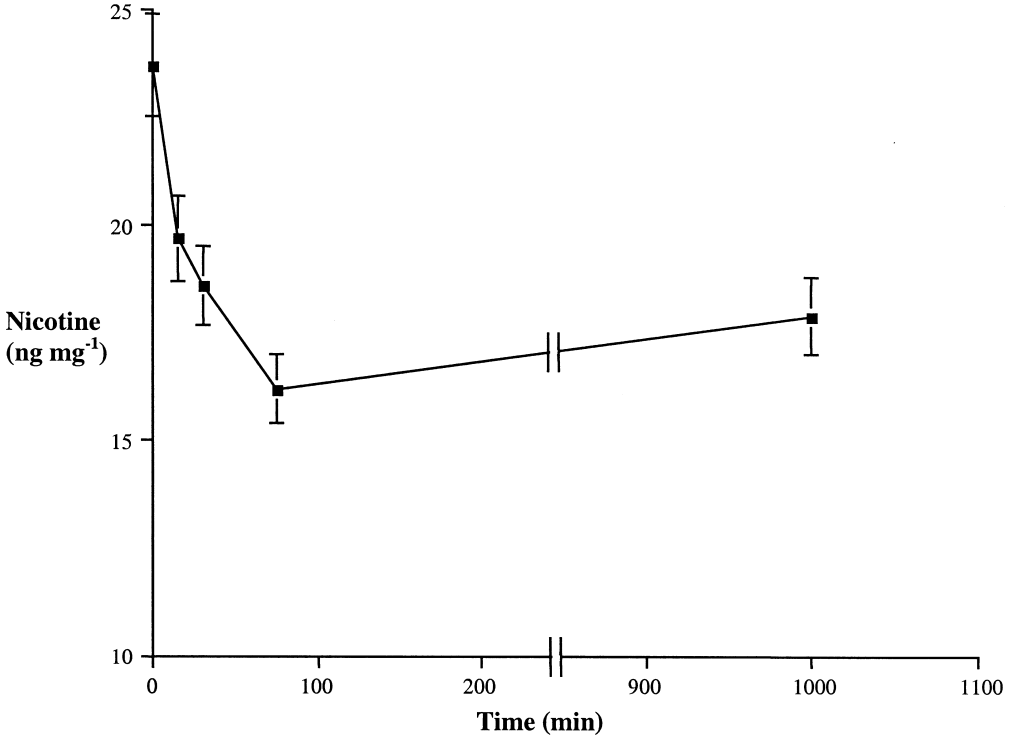

Fig. 3. Kinetics of dichloromethane hair washing step. Nicotine levels were measured in triplicate at each incubation time using the same randomised hair sample. Error bars indicate one standard deviation.

the poor precision. A similar time dependency has been found when washing other drugs from hair [18]. We chose a single 90-min wash because it was on the plateau phase and reduced the labour involved when compared to using a pair of 30-s washes. However, there are drawbacks to this choice in that this is quite a severe wash and we could be losing more nicotine than is desirable. Nonetheless, comparisons with the two, 30-s washes gave similar results and precision (Table 1).

We investigated digestion temperatures and the possibility that nicotine may not be well extracted from the melanocytes, to which nicotine is thought to associate [7] (Table 2). Microscopic examination of the digests supported the spectrometric data and we chose 50°C overnight in 1 mol l^{-1} NaOH as the optimum digestion for our method.

Nicotine has been extracted from the digest by extraction with diethyl ether [8] or by solid-phase extraction (SPE) using Extreleut (Merck, Darmstadt, Germany) [14]. Extreleut is expensive and while we managed to develop a precise and accurate extraction method based on a C_2 SPE column [13], it was much more labour intensive and there were many more peaks in the hair chromatograms, so SPE approaches were discarded.

There is evidence in the literature that nicotine will bind to glass [13,20], and that PTFE tubes seem

Table 1
The effect of different wash protocols on nicotine levels in hair[a]

Wash period	W5		W1	
	Mean±SD (ng mg^{-1} hair)	RSD (%)	Mean±SD (ng mg^{-1} hair)	RSD (%)
Unwashed	3.5±0.91	26	0.90±0.05	5.4
2×30 s	3.1±0.35	11.1	0.77±0.06	7.4
2 h	2.7±0.33	12.3	0.76±0.05	6.3

[a] Two hair samples, W5 & W1 were measured in triplicate using a single 2-h, 2 ml dichloromethane wash and a pair of 30-s, 2 ml dichloromethane washes. W5 was discarded as a quality control because of poor precision, presumably caused by non-homogenous exposure to nicotine.

Table 2
The effect of different hair digestion conditions on melanocyte breakup and nicotine recovery[a]

NaOH concentration (mol l^{-1})	Temperature (°C)	Incubation time	Precipitate size and colour	Absorbance (AU±SD)	Nicotine, mean±SD (ng mg^{-1} hair)
1	50	Overnight	Trace, white	0.366±0.020	4.87±0.05
	60	2 h	Small, brown	0.179±0.004	3.7±0.5
	100	15 min	Medium, brown	0.301±0.068	4.68±0.32
6	50	Overnight	Trace, white	0.354±0.028	
	100	15 min	Large, brown	0.244±0.032	

[a] Duplicate samples of a hair were digested at different NaOH concentrations, digestion temperatures and digestion times. The breakup of the melanocytes was assessed by (a) visual inspection of precipitate size and colour and (b) supernatant absorbance at 400 nm, after centrifugation at 6000 *g*. A wavelength of 400 nm was chosen arbitrarily after scanning the supernatant with a Unicam UV4-100 UV–Vis spectrophotometer (Unicam, Cambridge, UK), which revealed no clear absorption peaks. Absorbance measured in absorbance units (AU). Nicotine levels and precision were determined in a separate experiment. The overnight nicotine level was digested at 40°C. The 60°C digestion appears incomplete with only 2 h incubation.

to give better recoveries [13]. PTFE was not available but we found that polypropylene gave better precision than glass. As a consequence, the washing, digestion and extraction process was completed entirely in polypropylene tubes, pipettes and autosampler inserts.

We found no difference between the use of air and nitrogen for evaporating the diethyl ether, so air was used thereafter. This presumably reflects the increased stability of the chloride salt of nicotine compared to the free base [13].

3.2. Assay parameters

3.2.1. Linearity

The detector cell has good linearity but the working standard range was limited by the detector electronics when requiring a low limit of detection. With a 2 mg hair sample and 1 μl injection, linearity could be demonstrated between 4 and 640 ng mg^{-1} [nicotine=51.0×(nicotine to 2-phenylimidazole peak area ratio)−0.01, RSD (r^2)=0.991, n=7]. With routine batches we used 8 μl injections, less internal standard and more instrument gain, which gave a measuring range between 0.1 and 20 ng mg^{-1} hair. If required, we reinjected with 1 μl to extend the range to 160 ng mg^{-1}. A routine standard curve is represented by the linear regression line; nicotine (ng 2 mg^{-1})=71.91×(nicotine to 2-phenylimidazole peak area ratio)+0.21, [S.E.; slope, intercept, y estimate=1.78, 0.831, 1.46, respectively, and RSD (r^2)=0.997, n=7].

We only found one hair sample below the 0.1 ng mg^{-1} quantitation limit and one above 200 ng mg^{-1} (an adult smoker) in several series totalling 600 samples, justifying the selection of our measurement range for environmental tobacco smoke exposure assessment.

3.2.2. Limit of detection and interferences

The limit of detection was lower than 0.05 ng mg^{-1} hair for 2 mg of sample and 8 μl injections (signal-to-noise ratio 3:1), however the more relevant parameter, the limit of quantitation, based on within-batch RSD <20% was about 0.1 ng mg^{-1}. The ability of the assay to specifically measure nicotine was good to the extent that there was no obvious interference found in the 600 hair samples assayed to date.

3.2.3. Recoveries

Recoveries across a typical standard curve were high (Table 3). An extracted nicotine standard had a recovery of 105±6.5% (at 4.2 ng mg^{-1}, n=18) when treated as an unknown. Standard curves prepared in hair digest were superimposable upon those extracted from pure solutions once the blanks were subtracted. We continued to use standards prepared in the hair digest as using the same matrix is good analytical practice. There was a small blank for nicotine (Fig. 2A) when extracting 1 mol l^{-1} NaOH without hair, however, this was well below the quantitation limit. We did not think this was critical to accuracy.

Table 3
Recoveries of nicotine and the internal standard, 2-phenylimidazole[a]

Nicotine standard (ng added prior to extraction)	Nicotine recovered (%)	2-Phenylimidazole recovered (%) (37 ng added)
64.9	88.3	83.5
48.6	88.1	81.3
32.4	93.1	89.4
16.2	93.7	87.2
8.1	89.1	88.0
4.1	95.3	88.8
2.0	112.7	88.0
Mean±SD	94.3±8.6	87.9±5.5

[a] Recoveries of nicotine and 2-phenylimidazole were assessed as the mean of five typical batches for each point. Recovery at each point was calculated from a non-extracted standard curve run in the same batch.

3.2.4. Precision

Within- and between-batch precisions are documented in Table 4. Analytical variability is less than 10%. Sample homogeneity can be a problem with hair samples, especially when the hair has had variable exposure along its length. We had to discard several quality control hairs because of this. We chose not to attempt to create artificially spiked hair for quality control material [14], rather using randomised hair samples as better reflecting real samples. To reduce the effect of random error, e.g., potentially created by the electrostatic difficulties found when weighing hair, we measured each hair in duplicate and repeated the measurement if the RSD between duplicates was greater than 15%.

3.3. Application to epidemiological studies

Once an assays analytical parameters are satisfactorily determined it is then necessary to demonstrate that the assay can be applied to a particular use [27]. To do this we gathered hair samples from 297 children, aged 3–27 months, admitted for a wide range of conditions to the three public hospitals in the Wellington region of New Zealand. Environmental tobacco smoke exposure was assessed by questionnaires filled out by their caregivers, including numbers of cigarettes smoked in the home by themselves or visitors. Analysis of the questionnaires allowed us to divide the children into two groups, one of whose households had no tobacco smoke exposure (non-smokers, $n=101$) and those who were exposed (smokers, $n=196$). Analysis of the nicotine levels in the hair samples in the two groups is presented (Fig. 4), and demonstrates that our assay gives good discrimination between the two groups. Additionally, the hair nicotine levels as measured by our assay in the smoking group were strongly correlated to the number of cigarettes smoked by household members and visitors at the children's

Table 4
Within- and between-batch precision for nicotine in hair[a]

	Within-batch			Between-batch		
	Nicotine±SD (ng mg^{-1})	RSD (%)	*n*	Nicotine±SD (ng mg^{-1})	RSD (%)	*n*
W3	0.25±0.026	10.2	10	0.32±0.06	19.8	44
W2	1.37±0.98	7.1	10	1.32±0.12	9.3	22
W4	25.7±1.22	4.7	10	22.5±1.96	8.7	44
Nicotine standard				4.2±0.27	6.5	18

[a] Mean and variation (±standard deviation, SD and relative standard deviation, RSD) measured at three different levels using randomised hair samples. Two of the quality control hair samples, W3 and W4, were measured twice in each batch. An undigested nicotine standard was also run in each batch.

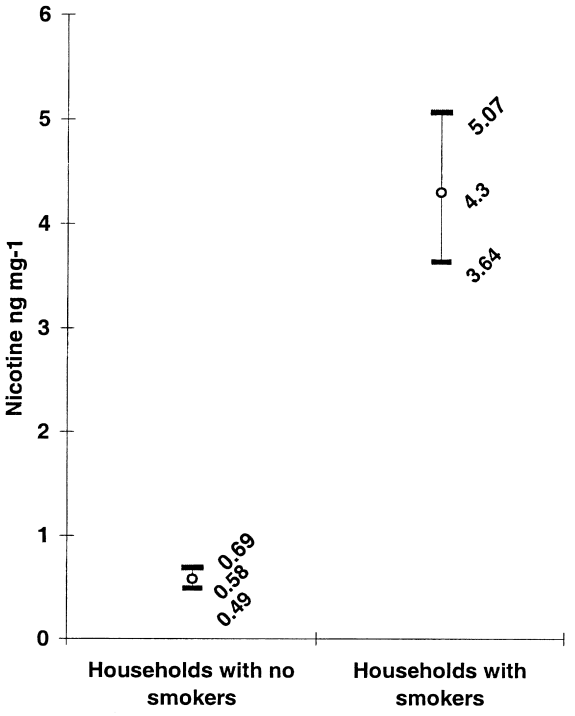

Fig. 4. The geometric means and 95% confidence limits of the geometric mean (error bars) of nicotine measured in hair in two groups of children according to their reported exposure to environmental tobacco smoke at home (see text for details of groups).

homes ($r=0.7$ $P<0.0001$). This evidence supports our contention that our assay is suitable for its intended use in assessing environmental tobacco smoke exposure.

The application of this assay in epidemiology is the subject of several papers currently under consideration for publication in public health journals. The hair nicotine biomarker may have applications in the assessment of exposure–disease associations involving environmental tobacco smoke exposure. Coronary heart disease, respiratory illnesses, and sudden infant death syndrome are just a few of the illnesses that can be studied in relation to environmental tobacco smoke exposure using the hair nicotine biomarker. This biomarker may be also applied for assessment of exposure to environmental tobacco smoke exposure in relation to certain smoking behaviours, for example a paper is being considered for publication which specifically looked at exposure levels of bar and restaurant workers in relation to the levels of environmental tobacco smoke exposure at their work environment. In addition, intervention studies such as smoke cessation will find that the use of this objective biomarker offers advantages that are not possible using questionnaires.

4. Conclusion

The nicotine in hair assay we have developed has good analytical parameters with excellent between batch precision, recoveries, and similar or better quantitation limits to other HPLC hair nicotine assays. Linearity and specificity are good. We have presented evidence that data generated by this assay gives good discrimination between groups of children living in exposed and non-exposed households and therefore is appropriate for its intended use in epidemiological assessment of environmental tobacco smoke exposure.

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