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Modelling nicotine intake in smokers and snuff users using biological fluid nicotine metabolites

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Data from two clinical studies involving smokers and snuff users were analysed to address the estimation of nicotine intake using urinary and salivary nicotine metabolites. Comprehensive regression modelling is performed to determine which combinations of urinary nicotine metabolites provide better estimation of nicotine intake in these subjects than the predominant practice of basing nicotine intake on urinary cotinine analysis alone. Within-subject and between-subject variability is examined with regard to reliability of measurement and replicate sampling. Salivary cotinine models are compared to urinary metabolite models. Results suggest that estimation of nicotine intake is greatly improved by measuring urinary cotinine and additional metabolites (trans-3′-hydroxycotinine, and glucuronide conjugates) rather than measuring only cotinine. Analyses also indicate that replicate sampling on subjects greatly improves the reliability of the measurement. Based on these data, a model to predict nicotine equivalents based solely on saliva cotinine was severely inferior to any of the urinary models, including that of urinary cotinine alone.

Keywords: nicotine metabolites, cotinine, *trans*-3′-hydroxycotinine, glucuronide conjugates.

Introduction

Tobacco smoke exposure has been estimated primarily from concentrations of nicotine and its metabolites in body fluids. Numerous studies have shown that, for most people, only 5–10% of nicotine is excreted unchanged in urine (Benowitz 1996). Curvall *et al*. (1991) describe phase I metabolic pathways as including C-oxidation of nicotine (NIC) to cotinine (COT), which is excreted to a small degree and then largely metabolized to *trans*-3′-hydroxycotinine (OHC). The glucuronic acid conjugates of nicotine (NICG), cotinine (COTG) and *trans*-3′-hydroxycotinine (OHCG) are formed in phase II metabolism and excreted in urine. In addition, the N-oxidation process results in nicotine-1′-*N*-oxide (NNO) and cotinine-1-*N*-oxide (CNO).

An average of 72% (with a range of 55–92%) of nicotine is converted to cotinine and then further metabolized to other metabolites (cotinine-glucuronide, *trans*-3′-hydroxycotinine, *trans*-3′-hydroxycotinine-glucuronide, and cotinine-1-*N*-oxide). However, occurrences have been noted in which subjects were identified as poor metabolizers of nicotine (Cholerton *et al*. 1994, 1996, Perkins *et al*. 1996,

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Benowitz and Jacob 1997). Researchers agree that approximately 90–95% of a nicotine dose can be accounted for in urine and can be estimated by measuring nicotine and its seven main metabolites (Byrd *et al.* 1994, Perkins *et al.* 1996).

The half-life of nicotine is approximately 2–3 h, compared with that of cotinine, which is approximately 15–17 h. Further, many studies have confirmed a relationship of increasing cotinine levels with documented increases of tobacco use or environmental tobacco smoke (ETS) exposure. This knowledge, together with the fact that only a very small percentage of the nicotine remains unchanged following phase I and phase II metabolism, explains the current preference for cotinine over nicotine as a biomarker for tobacco smoke exposure. However, *trans*-3´-hydroxycotinine constitutes by far the largest percentage of metabolized nicotine in urine samples. Hence, Benowitz (Benowitz 1988, Benowitz *et al.* 1997), amongst others, has suggested that this metabolite should be investigated as a candidate for a biomarker of tobacco exposure.

Urine, saliva and plasma are the biological media most often used for the determination of nicotine metabolites because they have all been shown to correlate well together (Andersson *et al.* 1994, 1995, 1997). The metabolism of nicotine has been more completely defined in urine than in any other body fluid. Numerous reports by Benowitz and Jacob (1997) and Andersson *et al.* (1994, 1995, 1997) have concluded that the best estimate of nicotine intake is obtained by measuring nicotine and its seven main metabolites in a 24-h urine excretion sample.

The variability of the metabolite distribution, both within and between subjects, has not been well characterized (Schwartz *et al.* 1987, Curvall *et al.* 1990, Kemmeren *et al.* 1994, Andersson *et al.* 1995, 1997, Perkins *et al.* 1996, Pritchard and Robinson 1996, Gourlay *et al* 1997, Zevin *et al.* 1997). While some authors maintain that there is a great deal of variation both within and between subjects, others have found only between-subject variation to be significant. Many demographic and biological conditions have been considered as possible confounding factors affecting the distribution of nicotine and its metabolites in biological fluids. In addition, different methods of sampling (urine pH, saliva stimulation, etc.) and data handling (treatment of data below the limits of detection and quantitation) contribute to the confusion of differing conclusions.

The analyses reported here to further investigate nicotine intake estimation using nicotine metabolites in biological fluids were performed using data generated from two studies originally designed for different objectives. Specifically, the purposes of the current investigation were as follows: to determine which combinations of urinary nicotine metabolites provide statistically improved estimation of nicotine intake in smokers and snuff users than the current predominant practice of analysing only urinary continine; to investigate the within-subject and between-subject variability; and to examine capability of saliva cotinine as an estimator of nicotine intake.

Methods

Subjects

The original objectives and details of both studies, performed by Swedish Match, Sweden, in collaboration with Lund University, have been previously described by Andersson *et al.* (1995, 1997). Male volunteers were enrolled in studies A ($n=91$) and B ($n=42$). The subjects in study A were smokers of various brands of cigarettes, while those from study B were consumers of Swedish-produced oral moist snuff (portion-packed bags). The subjects in study B were assigned to either a test group ($n=24$)

Table 1. Descriptive statistics—study A (smoke data).

Variable	ng ml^{-1} (urine or saliva) Mean	Min.	Max.	Std Dev.	CV (RSD)	% of total ng ml^{-1} urine Mean, %	CV (RSD), %
Total nicotine equivalents	18768	4124	51713	8231	43.9%		
Nicotine (NIC)	1643	237	5336	1038	63.2%	9.4	60.7
Cotinine (COT)	1817	304	3932	780	42.9%	9.3	27.5
Trans-3´-hydroxycotinine (OHC)	8120	577	22242	4525	55.7%	35.9	30.0
Nicotine glucuronide (NICG)	834	43	2560	551	66.0%	4.5	56.1
Cotinine glucuronide (COTG)	2762	158	9064	1546	56.0%	13.8	40.0
Trans-3´-hydroxycotinine glucuronide (OHCG)	5276	490	18143	3443	65.3%	23.2	45.2
Nicotine-1´-*N*-oxide (NNO)	597	18	3826	578	96.9%	3.0	69.5
Cotinine-1-*N*-oxide (CNO)	190	0	1492	233	122.5%	0.9	102.0
Salivary cotinine (S-COT)	261	29	538	116	44.5%		

who normally consumed a full-nicotine brand of snuff or a control group (n=18) who normally consumed a low-nicotine brand. Study A was designed to evaluate the association between apparent exposure to nicotine and actual nicotine uptake and to observe oral mucosal changes for association with exposure to cigarette smoke. Study B was designed to determine whether switching from a full-nicotine product to a low-nicotine product had any long-term or short-term effects on consumption, nicotine intake, or oral mucosal lesions.

Treatment and sampling

Each smoker in study A participated in a preliminary examination and then smoked his preferred brand for 1 week, recording the number of cigarettes smoked daily. On the seventh day, the smokers visited the clinic for a final examination which included a 2 ml saliva sample, a visual examination of oral mucosa, delivery of a 24-h urine sample taken the previous day, and consumption records.

The test subjects in study B used their regular full-nicotine snuff brand at will for the first 2 weeks of the study. For the following 10 weeks they switched to the same low-nicotine product used by the control subjects, one which contained half the amount of nicotine of the full-nicotine brand. The control group consumed their low-nicotine brand for the first 2 weeks of the study and was then dismissed. All snuff products were provided to the subjects for the course of the study. All study participants visited the clinic for a preliminary examination and returned on day 7 of weeks 1, 2, 4, 8, and 12 (control subjects visited on weeks 1 and 2 only) for analysis of the oral mucosa and collection of the following: 2 ml saliva samples; 24-h urine samples from the previous day; and weekly consumption records (grams per day and hours per day).

Data collection

As reported by Andersson *et al.* (1995, 1997), saliva samples from both studies were analysed to provide saliva continine concentration expressed in units of nanograms per millilitre (ng ml^{-1}) of saliva. Saliva pH was also measured for the subjects from study A. Urinary samples from both studies were analysed to provide measures of volume and pH, and measurements of nicotine and seven of its major metabolites (cotinine, *trans*-3´-hydroxycotinine, glucuronic acid conjugates of nicotine, cotinine and *trans*-3´-hydroxycotinine, and nicotine-1´-*N*-oxide and cotinine-*1*-N-oxide) expressed in units of ng ml^{-1} of urine. The dependent or response variable, total nicotine equivalents, was estimated as a sum of the total concentration of urinary nicotine and metabolites in the 24-h urine sample (with conversions to nicotine equivalents by molecular weight, as appropriate) and expressed as ng ml^{-1} of urine.

Note that the precise nicotine intake is unknown but is estimated as a linear combination of the observed (measured) independent urinary variables. A saturated model is the result when urinary nicotine and all seven nicotine metabolites are included in a regression model. Therefore, caution must be taken in attempting to generalize statistical results because different results may have been obtained had the response variable been a more independent determination of nicotine intake (such as precise dosing).

Additional data provided from both studies included subject age, years of tobacco or snuff use, and average daily consumption records (cigarettes smoked or grams consumed). Data collected from study A also included the machine-rated tar and nicotine levels of the brands smoked.

Table 2. Univariate analysis results—study A (smoke data).

Variable	p-value	R^2
NIC	0.0001	0.197
COT	0.0001	0.650
OHC	0.0001	0.617
NICG	0.0001	0.310
COTG	0.0001	0.378
OHCG	0.0001	0.535
NNO	0.0001	0.417
CNO	0.0014	0.111
S-COT	0.0001	0.167
Urine pH	0.5771	0.004
Urine volume	0.0001	0.186
Saliva pH	0.9750	0.000
Age	0.8453	0.000
Cigarettes per day	0.0001	0.157
Years of use	0.4576	0.006
Pack tar	0.3799	0.009
Pack nicotine	0.0533	0.042

Data analysis—study A

The urinary and salivary metabolite concentrations exhibited extreme variability, with the coefficient of variation (CV) or relative standard deviation (RSD) ranging from 42.9% to 122.5% (table 1). Even expressed as a percentage of the total nicotine equivalents, the distribution of urinary metabolites features high variability. As is traditional with laboratory data similar to these, where the distribution is asymmetric (specifically, these data are bound on the lower end), the data were transformed to a logarithmic scale for the remaining analyses. A Box–Cox transformation assessment indicated that a logarithmic transformation was adequate. A principal components analysis was performed to create a new, smaller set of variables to explain the variance–covariance structure of the data. However, the results did not accomplish this objective.

As suggested by Hosmer and Lemeshow (1989), a univariate analysis was performed on each potential variable to assess the likelihood of inclusion in the model. The evaluation of the p-value was relaxed to 0.25 (versus a more traditional 0.05) to allow for the possibility that some variables which might not meet the 0.05 criteria, when taken together in a model, may become more important predictors. In addition, while the t-test and its corresponding p-value assess the regression relationship between the independent and dependent variables, the R^2 measure assesses how well the independent variable explains the variation in the response. A very low p-value (<0.05) may indicate a significant regression, while the model may be lacking in completeness, as indicated by a very low R^2. Even with a 0.25 p-value guideline, each of the metabolites provides high significance in regression against the nicotine uptake when taken individually (table 2). Of the potential covariates, the only variables with reasonable p-values are urine volume, cigarettes per day, and machine-rated nicotine level of the brand; however, the R^2 associated with each of these variables is very small. Because all of the metabolite variables met the suggested criteria of $p<0.25$, stepwise regression was performed offering all the urinary metabolite variables and the three potential covariates.

As the R^2 statistic can never decrease when variables are added to the model, a higher R^2 for a model with more predictors may not be a true indicator of better fit. The adjusted R^2 (R^2_{adj}) slightly penalizes the statistic for the number of parameters in the model and is therefore more appropriate when comparing models with different numbers of parameters. In addition, the predicted R^2 (R^2_{pred}), removes the influence of the particular data points used to fit the model and provides an indication of the prediction capability of the model for new data (Myers 1990).

In a stepwise regression, COT was the best single predictor of this nicotine uptake data, explaining approximately 65% of the variation in the response variable ($R^2_{adj}=0.6503$) of total urinary nicotine equivalents (table 3). The next best options were OHC and its glucuronide conjugate (OHCG). The best two-variable model for nicotine uptake consisted of OHC and its glucuronide conjugate (OHCG). However, the two other possible combinations of the three variables discussed as single predictors were also reasonable models.

The best three-variable model included all three of the variables discussed above: COT, OHC, and OHCG ($R^2_{adj}=0.9233$; $R^2_{pred}=0.9157$). However, several other three-variable models provided similar results. Models with more than three predictor variables provided little improvement in either the amount of variation explained or the prediction capability. For example, the best four-variable model resulted in $R^2_{adj}=0.9545$ and $R^2_{pred}=0.9489$ (compared to $R^2_{adj}=0.9233$ and $R^2_{pred}=0.9157$ for the three-

Table 3. Regression—study A (smoke data)—best one-variable and two-variable models.

Numbers of variables in model	Variable(s) in model	R^2_{adj}	R^2_{pred}
1	COT	0.6503	0.6386
	OHC	0.6168	0.5806
	OHCG	0.5376	0.5187
2	OHC and OHCG	0.8412	0.8247
	COT and OHCG	0.8300	0.8214
	COT and OHC	0.7815	0.7616

Table 4. Regression—study A (smoke data)—best *n*-variable models.

n (number of variables)	NIC	COT	OHC	NICG	COTG	OHCG	NNO	CNO	Volume	R^2_{adj}	R^2_{pred}
1		✓								0.6503	0.6386
2			✓			✓				0.8412	0.8247
3		✓	✓			✓				0.9233	0.9157
4		✓	✓	✓		✓				0.9545	0.9489
5		✓	✓	✓		✓	✓			0.9631	0.9581
6		✓	✓	✓	✓	✓	✓			0.9624	0.9592
7	✓	✓	✓		✓	✓	✓		✓	0.9704	0.9593
8	✓	✓	✓		✓	✓	✓	✓	✓	0.9746	0.9668
9	✓	✓	✓	✓	✓	✓	✓	✓	✓	0.9765	0.9681

variable model). As the models approach saturation with independent variables, the R^2_{adj} and R^2_{pred} approach levels that indicate nearly perfect fit and prediction. This result is not surprising because the response variable, total nicotine equivalents, is a linear combination of the independent variables (table 4).

Before further investigation of these models for completeness (need for quadratic or interaction terms), they were addressed relative to analytical feasibility. The use of the one-variable model of COT alone, a current practice, was considered further. While the two-variable model of OHC and OHCG did not contain COT and was disregarded, the three-variable model containing COT, OHC, and OHCG was examined further. Because methods to analyse glucuronide conjugates also provide measures of the free (unconjugated) metabolites, chemically analysing the three-variable model would allow the inclusion of COTG as well. Therefore, this four-variable model, including COT/ONC, and their glucuronide conjugates (COTG and OHCG), was also considered for further evaluation. The next logical model would add NIC and its glucuronide conjugate (NICG) to form a six-variable model. The final eight-variable model includes the complete suite of metabolites analysed by adding NNO and CNO.

Because the inclusion of urine volume adds little to the completeness of the models and because, it is already confounded in the measurements of the metabolites in terms of ng ml^{-1} urine, it was not included in the models. However, this step should not be interpreted as implying that 24-h samples can be replaced with spot samples.

Further analysis was restricted to the following candidate models:

COT (current practice)
3-variable model—COT, OHC, and OHCG
4-variable model—COT, OHC, COTG, and OHCG
6-variable model—NIC, COT, OHC, NICG, COTG, and OHCG
8-variable model—NIC, COT, OHC, NICG, COTG, OHCG, NNO and CNO

Partial residual plots for each variable in each model failed to suggest a need for quadratic terms in any of these models (figure 1 is a sample of these plots).

When all possible two-way interactions were offered to their respective stepwise regressions, forcing the initial metabolite terms into the model, several of the interactions were brought into the models as significant terms. The curvature of the planes in interaction plots confirmed the existence of two-way interactions in the models (figure 2 is a sample of the plots). Addition of the significant interaction terms had little effect on the completeness of the model however, as evidenced by small increases in R^2_{adj}, yet it caused the models to become notably unstable. Note the extremely high variance inflation factors, some changes in parameter estimates (including some sign changes), and some higher standard errors of the coefficient estimates (table 5 is an example of the results). Therefore, further analysis of the proposed models did not include interaction terms.

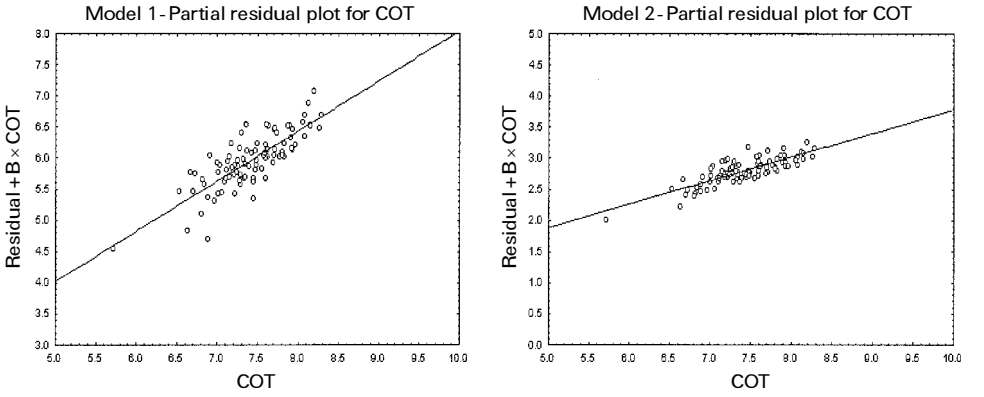

Figure 1. Regression—study A (smoke data)—quadratic terms assessment—partial residual plots.

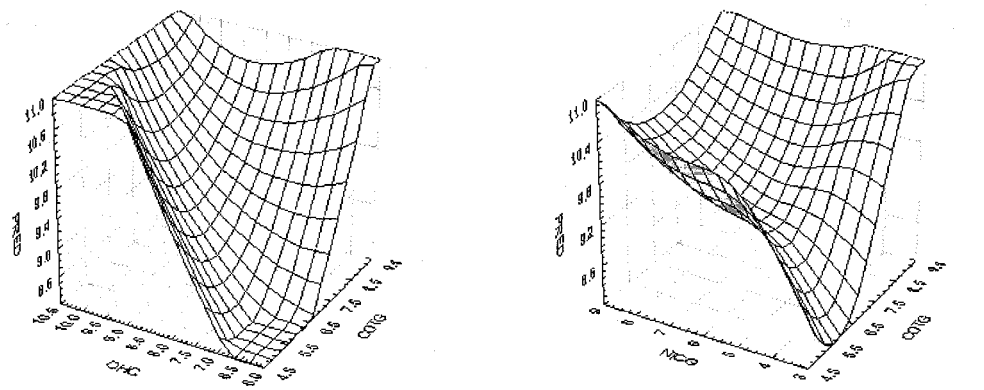

Figure 2. Regression—study A (smoke data)—two-way interaction terms assessment—interaction plots.

In building a model for nicotine equivalents using saliva cotinine, three potential covariates were identified as significant by stepwise regression: cigarettes per day, mg nicotine per cigarette, and mg tar per cigarette ($R^2_{adj}=0.3063$; $R^2_{pred}=0.2500$). Partial residual plots failed to suggest a need for quadratic terms. None of the six possible two-way interaction terms were significant in a stepwise regression. The acquisition of the covariates creates a burden that would limit the appeal of this model while providing little additional information. Therefore, any further consideration of a saliva cotinine model was without these terms.

The addition of OHC and OHCG to the model with COT provided a significant increase in both the completeness and the prediction capability of the model (table 6). The other three urinary models may not add enough value to warrant the required analytical work. The saliva cotinine model paled in comparison even to the model with urinary cotinine alone.

A modified data-splitting technique was performed by using only two-thirds of the original 91 observations to refit and confirm the adequacy of all the previously chosen models. The remaining one-third of the observations was used as 'new' data to validate how well the regression equations can predict 'future' values. The graphs of predicted versus observed values for total nicotine equivalents confirmed that the three-variable model offers significant improvement in prediction over the COT model (figure 3(A and B)). Improvement beyond the three-variable model was less impressive (figure 3(C–E)). The last graph (figure 3(F)), the result of the saliva cotinine model, has the poorest fit.

Data analysis—study B

Because the test subjects experienced a change in snuff brand after two visits, the data obtained at the baseline visits were analysed first using similar methods to those performed on the smoke data.

Table 5. Regression—study A (smoke data)—interaction—model 4.

	Without interaction	With interaction
R^2_{Adj}	0.9620	0.9771
Largest variance inflation factor	2.74	648.23
Intercept: β(SE)	2.21(0.17)	1.21(1.03)
NIC: β(SE)	0.04(0.02)	–0.19(0.13)
COT: β(SE)	0.21(0.03)	0.51(0.15)
OHC: β(SE)	0.27(0.02)	0.07(0.10)
NICG: β(SE)	0.08(0.02)	0.48(0.12)
COTG: β(SE)	0.08(0.02)	–0.32(0.10)
OHCG: β(SE)	0.26(0.01)	0.69(0.10)

Table 6. Regression—study A (smoke data)—model comparison.

Model	R^2_{Adj}	R^2_{Pred}
1—COT	0.6503	0.6386
2—COT, OHC, OHCG	0.9233	0.9157
3—COT, OHC, COTG, OHCG	0.9476	0.9372
4—NIC, COT, OHC, NICG, COTG, OHCG	0.9620	0.9506
5—NIC, COT, OHC, NICG, COTG, OHCG, NNO, CNO	0.9729	0.9634
6—Salivary COT	0.1673	0.1403

Variation comparable to that observed in study A was observed. Because no systematic difference was observed between the first and second visits, the two baseline observations for each subject were averaged to provide more precise estimates of the measures (tables 7 and 8). As with the previous data, all variables were transformed to their natural logarithms, and all subsequent analyses were performed with the natural log of all the data. Again, principal components analysis performed on these baseline averages did not yield a reduced number of components that highlighted specific variables.

The results of the univariate analyses for the baseline averages were similar to those for study A, with each of the metabolites and all potential covariates except age exhibiting strong regression relationships (table 9). For most variables, the R^2 values were higher than in study A, probably due to the averaging of two observations for each subject to obtain better estimates.

Stepwise regressions were performed, offering all the metabolite variables and the four potential covariates, to build appropriate models. Results obtained with these data were slightly different from those obtained with the previous data, as OHC was the best single predictor of nicotine uptake. However, COT and COTG were both very close in terms of R^2_{adj} and R^2_{pred} (table 10). The best two-variable model for nicotine uptake consisted of COT and OHC, with an R^2_{adj} of approximately 0.92. However, three other two-variable models also resulted in R^2_{adj} between 0.91 and 0.92. The best three-variable model included OHC, COT, and COTG with an R^2_{adj} of approximately 0.96. However, seven additional three-variable models provided an R^2_{adj} of at least 0.94. As with the previous dataset, models with more than three predictor variables provided little improvement in either the amount of variation explained or the prediction capability (table 11).

For the remaining analyses, the five models proposed in the previous section were utilized. Partial residual plots confirmed the linearity of the terms in the models and therefore failed to suggest the need for quadratic terms in any of the models. Stepwise regressions offering two-way interactions into models with the initial metabolites resulted in some significant interactions for the six-variable and eight-variable models. Although the curvature in the interaction plots again confirmed the presence of two-way interactions, the inclusion of these terms added little to the model completeness and caused instability. Therefore, the five proposed models were further assessed without the inclusion of higher orders terms.

Table 12 compares the adjusted and predicted R^2 values of the models applied to the baseline average data from study B. While cotinine alone again provided only modest fit and prediction, better results were obtained with this data than with the smoke data, probably due to averaging of observations. Again, the addition of OHC and OHCG provided a significant increase in both measures. Adding COTG to form the third model increased the R^2_{adj} to 0.9811 and the R^2_{pred} to 0.9754. Adding NIC and NICG and then the N-oxides (NNO and CNO) to form the fourth and fifth models provided minor increases in the R^2 values.

As with the previous data, saliva cotinine provided a very poor model of nicotine equivalents for the baseline averages, with $R^2_{adj} = 0.4506$ and $R^2_{pred} = 0.3936$.

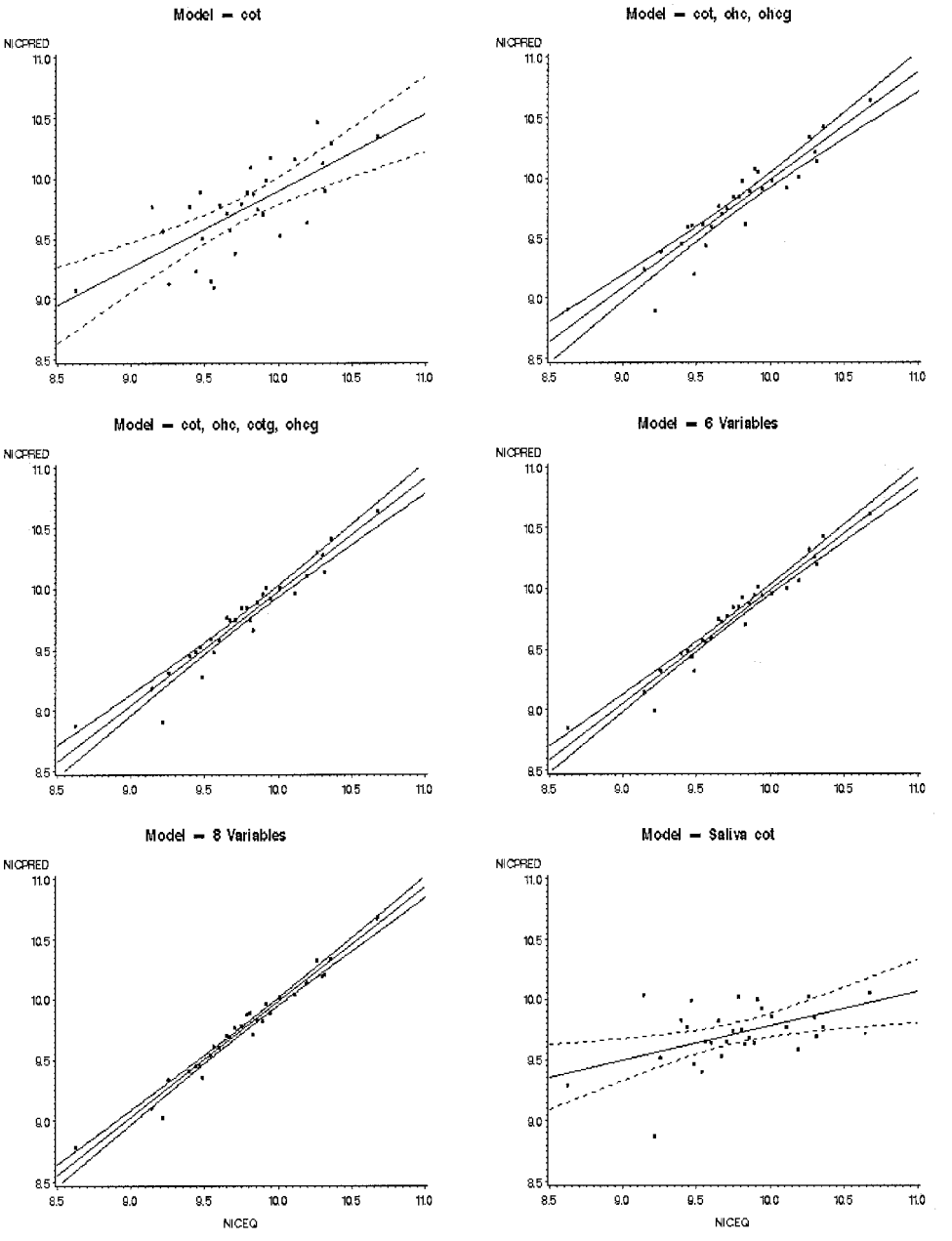

Figure 3. Regression model comparison—study A (smoke data)—data splitting. A, cotinine alone; B, three metabolites; C, four metabolites; D, six metabolites; E, eight metabolites; F, salivary cotinine.

Mixed models theory removes the standard linear model's restriction that the errors be independently distributed and of constant variance and allows modelling of not only the mean(s) but also the variances and covariances of the data. Mixed models regression analysis of the repeated baseline observations (not averaged) confirmed the adequacy of the proposed models and indicated that simple common slopes models (versus different slopes for control subjects and test subjects) were appropriate. Various diagnostics, including REML Log Likelihood, Akaike's Information Criterion, Schwarz's Bayesian Criterion, and –2 REML Log Likelihood, indicated that each model with more parameters than the previous model was preferable. Formal Likelihood-ratio tests confirmed that each larger model

Table 7. Descriptive statistics—study B (snuff data), baseline averages—test subjects.

	ng ml^{-1} (urine or saliva)					% of total ng ml^{-1} urine	
Variable	Mean	Min.	Max.	Std Dev.	CV (RSD)	Mean, %	CV (RSD), %
Total nicotine equivalents	19032	6463	46434	9584	50.4%		
Nicotine (NIC)	1702	255	6313	1526	89.7%	3.9	47.0
Cotinine (COT)	2163	427	5015	1162	53.7%	3.8	35.1
Trans-3´-hydroxycotinine (OHC)	7529	2610	14910	3947	52.4%	10.7	30.7
Nicotine glucuronide (NICG)	732	73	2146	546	74.7%	1.8	47.1
Cotinine glucuronide (COTG)	2829	420	8056	2236	79.0%	5.5	43.0
Trans-3-´-hydroxycotinine glucuronide (OHCG)	4429	639	13045	3141	70.9%	8.9	44.8
Nicotine-1´-*N*-oxide (NNO)	1318	169	3477	974	73.9%	3.8	54.9
Cotinine-1-*N*-oxide (CNO)	647	65	2358	602	92.9%	2.5	79.0
Salivary cotinine (S-COT)	336	78	723	157	46.7%		

Table 8. Descriptive statistics—study B (snuff data), baseline averages—control subjects.

	ng ml^{-1} (urine or saliva)					% of total ng ml^{-1} urine	
Variable	Mean	Min.	Max.	Std Dev.	CV (RSD)	Mean, %	CV (RSD), %
Total nicotine equivalents	10726	1823	21005	5199	48.5%		
Nicotine (NIC)	767	87	2766	626	81.6%	4.4	59.2
Cotinine (COT)	1073	228	2264	503	46.9%	2.4	24.5
Trans-3´-hydroxycotinine (OHC)	5662	873	14619	3343	59.0%	7.3	16.9
Nicotine glucuronide (NICG)	345	44	736	196	56.7%	1.6	47.8
Cotinine glucuronide (COTG)	1318	192	2932	824	62.5%	4.5	40.7
Trans-3´-hydroxycotinine glucuronide (OHCG)	2229	566	6020	1410	63.3%	5.9	33.4
Nicotine-1´-*N*-oxide (NNO)	505	62	1097	259	51.3%	2.8	55.5
Cotinine-1-*N*-oxide (CNO)	271	31	819	201	74.0%	0.9	38.8
Salivary cotinine (S-COT)	158	56	432	87	55.0%		

Table 9. Univariate analysis results—study B (snuff data), baseline averages.

Variable	*p*-value	R^2
NIC	0.0001	0.622
COT	0.0001	0.757
OHC	0.0001	0.772
NICG	0.0001	0.666
COTG	0.0001	0.746
OHCG	0.0001	0.513
NNO	0.0001	0.625
CNO	0.0001	0.579
S-COT	0.0001	0.464
Urine pH	0.0124	0.146
Urine volume	0.0016	0.224
Age	0.7776	0.002
Grams per day	0.0677	0.081
Years of use	0.0037	0.192

Table 10. Regression—study B (snuff data), baseline observations—best one-variable and two-variable models.

Number of variables in model	Variable(s) in model	R^2_{adj}	R^2_{pred}
1	OHC	0.7658	0.7457
	COT	0.7504	0.7268
	COTG	0.7393	0.7091
2	COT and OHC	0.9217	0.9117
	OHC and COTG	0.9208	0.9087
	OHC and NICG	0.9165	0.9064
	OHC and NNO	0.9132	0.9041

Table 11. Regression—study B (snuff data), baseline observations—best *n*-variable models.

n (Number of variables)	NIC	COT	OHC	NICG	COTG	OHCG	NNO	CNO	pH	R^2_{adj}	R^2_{pred}
1			✓							0.7658	0.7457
2		✓	✓							0.9217	0.9117
3		✓	✓		✓					0.9626	0.9548
4			✓		✓	✓	✓			0.9824	0.9795
5	✓		✓		✓	✓	✓			0.9882	0.9851
6	✓	✓	✓		✓	✓	✓			0.9912	0.9894
7	✓	✓	✓		✓	✓	✓		✓	0.9923	0.9889
8	✓	✓	✓		✓	✓	✓	✓	✓	0.9927	0.9895

Table 12. Regression—study B (snuff data), baseline observations—model comparison.

Model	R^2_{Adj}	R^2_{Pred}
1—COT	0.7504	0.7268
2—COT, OHC, OHCG	0.9486	0.9418
3—COT, OHC, COTG, OHCG	0.9811	0.9754
4—NIC, COT, OHC, NICG, COTG, OHCG	0.9845	0.9764
5—NIC, COT, OHC, NICG, COTG, OHCG, NNO, CNO	0.9918	0.9870
6—Salivary COT	0.4506	0.3936

was a statistically better fit than its predecessor. Although the improvement from the four-variable model to the six-variable model may be considered small (and likewise from the six-variable model to the eight-variable model), this method found the difference to be statistically significant (table 13).

To obtain estimates of within- and between-subject variation, the entire set of five observations from each of the 24 test subjects was used. The five proposed models were used to choose the appropriate covariance structure and analyse the variance of the components. Of three common types of covariance structures assessed (variance components, compound symmetry, and first-order autoregressive), compound symmetry was the most appropriate for each of the five proposed models, as determined by traditional comparison diagnostics (REML Log Likelihood, Akaike's Information Criterion, Schwarz's Bayesian Criterion, and –2 REML Log Likelihood).

The results of the above procedures were used to estimate the variability within each subject (or between measures within a subject) and between subjects by specifying the $\boldsymbol{R}$ matrix to have compound symmetric structure. The off-diagonal component of the $\boldsymbol{R}$ matrix is the variability between any two measurements within a subject (within-subject variation). The diagonal component defines the total variability of any one measurement, which is the sum of the off-diagonal component and the repeated measures residual component (between-subject variation). The total variability of any one measurement decreased as the model became more complete (table 14). In addition, until the final model was reached, the within-subject component decreased at a faster rate than the between-subject component. This difference resulted in the within-subject variation becoming a smaller proportion of the total, indicating that each subject's results are estimated better as the model becomes more complete. The failure of the trend to continue with the final model may be an indication that the last two terms added (NNO and CNO) are not reliable measures. Fleiss (1986) describes how the within-subject and between-subject

Table 13. Mixed models regression—study B (snuff data), baseline observations—Likelihood -Ratio tests.

Metabolites		–2 REML log likelihood				
First model	Second model	First model	Second model	Test χ^2 (difference)	df	Critical χ^2
COT only	3	54.2105	–37.5398	91.7503	2	5.99
3	4	–37.5398	–82.8830	45.3432	1	3.84
4	6	–82.8830	–92.4335	9.5505	2	5.99
6	8	–92.4335	–99.3968	6.9633	2	5.99

Table 14. Repeated measures—study B (snuff data), test subjects—variability estimates and reliability results.

	Variability				Number of replicates required, *m*, for:		
Model	Total	Between-subject: s^2_T, % of total	Within-subject: s^2_s, % of total	Reliability ($\hat{R}$)	R_m = 0.95	R_m = 0.90	R_m = 0.85
COT	0.1487	0.0782,53	0.0705,47	0.53	18	9	6
3-Variable	0.0358	0.0194,54	0.0164,46	0.54	17	8	5
4-Variable	0.0226	0.0145,64	0.0081,36	0.64	11	6	4
6-Variable	0.0153	0.0106,69	0.0048,31	0.69	9	5	3
8-Variable	0.0120	0.0080,67	0.0040,33	0.67	10	5	3

components of variability can be used to estimate the '*intraclass correlation coefficient of reliability* (the *reliability*, for short) by:[2]

$$\hat{R}=\frac{s_T^2}{s_T^2+s_e^2}$$

where s_T^2 is the estimate of between-subject variability and s_e^2 is the estimate of within-subject variability. Essentially, this reliability value defines the proportion of total variability of a measurement that is due to subject-to-subject differences as opposed to within-subject variability. If the value is high, most of the variability is due to actual differences between subjects, as opposed to unreliable measures on each subject. Given the estimated reliability of a single measurement, $\hat{R}$, the required number of replicate measurements, *m*, to obtain a desired reliability of the mean can be determined by inverting:

$$R_m=\frac{m\hat{R}}{1+(m-1)\hat{R}} \text{ to } m=\frac{R_m(1-\hat{R})}{\hat{R}(1-R_m)}$$

The greatest improvement in reliability, and hence the most dramatic reduction in required replicates, is seen from the three-variable model to the four-variable model.

Results

Recall that the response variable for both datasets, total nicotine equivalents, was a linear combination of all the measured urinary metabolites. Therefore, building models to estimate total nicotine equivalents with these data provides results which may have been different had the response variable been measured other than from the independent variables.

Although stepwise regression revealed several viable models, six models were proposed for further analysis based on analytical feasibility considerations:

COT (current practice)

Table 15. Summary of regression model fit and prediction measures.

Model	Study A (smoke data)		Study B (snuff data)	
	R^2_{adj}	R^2_{pred}	R^2_{adj}	R^2_{pred}
COT only	0.6503	0.6386	0.7504	0.7268
3 Variables (COT, OHC, OHCG)	0.9233	0.9157	0.9486	0.9418
4 Variables (COT, OHC, COTG, OHCG)	0.9476	0.9372	0.9811	0.9754
6 Variables (NIC, COT, OHC, NICG, COTG, OHCG)	0.9620	0.9506	0.9845	0.9764
8 Variables (NIC, COT, OHC, NICG, COTG, OHCG, NNO, CNO)	0.9729	0.9634	0.9918	0.9870
Salivary Cotinine	0.1673	0.1403	0.4506	0.3936

3-variable model—COT, OHC, and OHCG
4-variable model—COT, OHC, COTG, and OHCG
6-variable model—NIC, COT, OHC, NICG, COTG and OHCG
8-variable model—NIC, COT, OHC, NICG, COTG, OHCG, NNO and CNO
Salivary COT

Although the analyses performed indicated that the models were a better fit for the data obtained from study B than for those from study A, this result can be partially attributed to the averaging of the baseline observations from study B (table 15).

Comparison of the models indicated that while measuring cotinine alone resulted in a mediocre model, the three-variable model provided substantial improvement in both fit and prediction. Although adding the glucuronide conjugate of cotinine provided only a moderate improvement over the three-variable model, an analytical method to obtain the three variables would provide this fourth variable with no additional analytical work. The fourth model added the fifth and sixth variables of nicotine and its glucuronide conjugate, while the final model added nicotine-1′-*N*-oxide and cotinine-1-*N*-oxide. While these models do show incremental improvement and are substantial improvements over the current practice of measuring cotinine alone, they may not add enough value over the four-variable model to warrant the additional analytical work required. However, a cost–benefit analysis model should be used to make this determination. The salivary cotinine model demonstrated extremely poor results, both in fit and prediction, a finding that agrees with Jenkins and Counts' (1999) results of R and R^2 values in the range of 0.10 to 0.20.

A mixed models approach confirmed that the five proposed models were adequate as common slope models. Additional diagnostics indicated that each more inclusive model was statistically better than its predecessor.

Use of all five observations from the test subjects in study B resulted in the compound symmetric covariance structure as a better fit than variance components or first order autoregressive. Review of components of variance estimates indicated that while the within-subject variance was only marginally smaller than the between-subject variance, the within-subject variance became a smaller proportion of the total as more variables were included in the model.

The reliability measures on these data, which express the proportion of the variability due to actual subject-to-subject differences, indicated that the reliability

of a single measurement when analysing only cotinine is approximately 0.53. In order to obtain, for example, a 0.85 reliability of the mean of several measurements, six replicate samples would be needed. When using the four-variable model, however, the reliability of a single measurement is approximately 0.64. To obtain a 0.85 reliability of the mean, four samples would be required. With the exception of the complete model (which actually seems to decline in reliability), the more variables in the model, the less significant the within-subject variability, the higher the reliability of a single result, and hence, the fewer replicate samples required to obtain increased reliability of estimation.

Discussion

The data analysed here provided a preliminary investigation into several important issues regarding estimation of nicotine intake. A major outcome of the regression modelling is the implication that nicotine intake estimation can be greatly improved by adopting the three-variable model. However, the four-variable model appeared to offer substantial improvement in reliability because it resulted in a smaller proportion of within-subject variation than the previous model.

Based on the data analysed here, a model to predict nicotine equivalents based on saliva cotinine demonstrated severely poorer quality of both fit and prediction than any of the urinary models, including that of urinary cotinine alone. Results reported by Bentley *et al*. (1999) have found not only cotinine but *trans*-3′-hydroxycotinine in saliva samples. Since the presence of an additional nicotine metabolite in saliva may improve the outlook for saliva samples as a media for tobacco exposure estimation, any future study to investigate the adequacy of saliva samples should include the analysis of this metabolite.

Because it was not an objective of the studies analysed here to investigate the affects of urine pH on nicotine metabolism, the subjects were not given agents in an attempt to adjust or control urine acidity. Therefore, the issue of the effect of urine pH on metabolite levels could not be investigated. Additionally, the studies from which these data were obtained utilized 24-h urine samples. Therefore, it cannot be concluded that the same results would have been obtained if spot samples had been collected.

The data analysed here have come from studies that involved white male tobacco users. Therefore, the conclusions drawn may not be extended to the population of tobacco non-users or even to a more diverse population of tobacco users, as these subjects may metabolize nicotine differently (Jenkins and Counts 1999).

A study that involved dosing of subjects rather than relying on reported use and estimated exposure would more accurately measure the subjects' exposure to nicotine. Such a study would provide a means to categorize subjects by amount of exposure and a response variable that is less dependent on the measured independent variables (metabolites). Different results may be obtained under these conditions.

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