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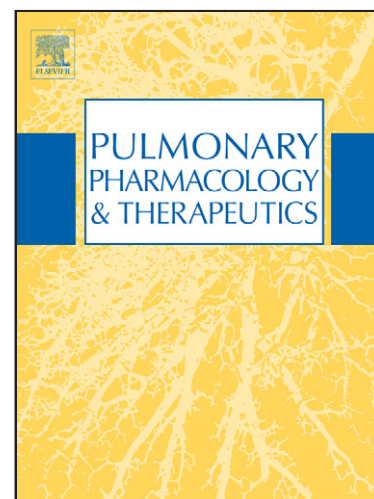
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GC-MS-determined cotinine in an epidemiological study on smoking status at delivery

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Abstract

The objective of this study was to measure plasma cotinine levels in pregnant women and their newborns using a gas chromatography-mass spectrometry (GC-MS) method in an epidemiological delivered population with a wide range of tobacco intakes. Nearly one thousand pregnant women from regional maternity wards ($n = 1007$) were selected for the study. Each patient kept a tobacco diary and underwent a blood test to assess cotinine levels at the same time that newborns' cordonal plasma was taken. These values were then cross-checked.

Cotinine was estimated using a selected-ion monitoring mode with a 1.5 ng/ml quantification limit. Cotinine levels in mothers and newborns were highly correlated, whatever the mother's smoking status with a calculated cut-off for cotinine levels in active smokers of 21.5 ng/ml. Finally, cotinine determined through this GC-MS method offered a sensitive and accurate measure of tobacco exposition of pregnant women and their babies.

Key-words: cotinine, pregnancy, newborn, smoking status,

1. Introduction

The association between maternal smoking, retarded maternal condition and birth outcome is well known. Smoking during pregnancy increases the risk of spontaneous abortions, preterm premature membrane ruptures, stillbirths, preterm deliveries, and sudden infant death syndrome. Prenatal exposure to tobacco smoke is a risk factor for respiratory infections, asthma, allergy and childhood cancer (1) Risks for most of these conditions have been found to increase with the number of cigarettes smoked (2). In order to have a reliable measure of this exposition, a survey was conducted on nearly a thousand pregnant women from regional maternity wards.

Cotinine presents a longer biological half-life than nicotine, and is the best biochemical marker for segregating smokers from non-smokers. Its rate is correlated with the daily cigarettes consumption (3, 4). Among all biomarkers used for routine diagnosis of *in utero* tobacco-smoke exposure, cotinine levels in umbilical cords are probably the most reliable and non-invasive for the newborn (5). Cotinine levels were determined in cordonal plasma and blood of new mothers and cross-checked against the mother's answers to a questionnaire on maternal smoking status.

Since the GC-MS method is recognized as the standard of reference in cotinine analysis, particularly when concentration levels are close to 1 ng/ml, we have examined serum cotinine in pregnant women with a wide range of cigarette intakes and in their newborns, to determine fully its value in assessing tobacco consumption in specific populations.

2. Methods

2.1. Epidemiological study and sample collection

The study was conducted from July 2003 to June 2004 in all the delivery units ($n = 16$) of a French administrative area in Central France (Auvergne). This multicenter study was approved by the local ethics committee in 2003. Eligible women ($n = 1007$) were self-questioned on their smoking status at delivery, and maternal and cord blood-serum samples were collected in heparinised tubes. Samples were then centrifuged at 3,000 g for 10 min to remove plasma, then frozen until assay.

2.2. Gas chromatography and mass spectrometric detection

Cotinine analyses were performed on an HP5973 MS, with an HP6890 series GC (Agilent Technologies, Atlanta, GA). Injections were performed using an HP6890 autosampler. The glass liner was equipped with a Siltek deactivated inlet glass liner (Restek, Bellefonte, PA). The GC was operated in splitless injection mode, with a constant flow of 0.5 ml/min of helium through the HP-5MS column (30 m x 0.25 mm i.d. with a 0.25- μ m film thickness (J&W, Folsom, CA). Splitless injection (1 μ l) was performed at 260°C. The transfer line temperature was 290°C. The GC oven temperature was programmed to start at 80°C for 0.5 min, then increase to 285°C at a rate of 20°C/min, and remain at 285°C for a further 5 min. MS detector parameters were: transfer line temperature 290°C; solvent delay 3 min; electron energy 70 eV. The MS was run in Selected-Ion Monitoring mode (SIM) for quantitative analysis. HP Chemstation® software controls the equipment and processes the data.

2.3. Sample preparation

To extract cotinine, 0.6ml of potassium hydroxide solution (1M) was added to 1 ml of plasma, 10 μ L of the stock solution of deuterated cotinine (10 μ g/ml), and 5 ml of dichloromethane in a 10 ml screw-capped glass centrifuge tube. The centrifuge tube was capped and shaken on a mechanical shaker for 15 min, then centrifuged for 10 min at 3,000 rpm. The organic layer was removed and put into a new glass tube containing 50 μ L HCl

0.25M. This tube was shaken on a mechanical shaker for 15 min then centrifuged for 10 min at 3,000 rpm. The organic layer was evaporated to dryness at ambient temperature, and the residue was reconstituted with 60 μ L of ethyl acetate and transferred for GC-MS analysis.

Calibration and QC standards were from separate stock solutions and extracted before each set of dosages by mixing various known amounts of unlabeled cotinine with a fixed amount of deuterated cotinine (100 ng) in 1 ml of human blank plasma. Standard concentrations of 0, 10, 25, 50, 100 and 250 ng/ml were used. QC sample concentrations were 5, 30 and 75 ng/ml.

2.5. Validation of the GC-MS method

The validation program included linearity, limit of quantification (LOQ), precision and accuracy studies of cotinine in human plasma. The LOQ was defined as the lowest concentration of cotinine that could be measured with acceptable precision and accuracy. Recovery after the extraction procedure was determined by comparing the areas of extracted cotinine with that of the nonextracted standard samples representing 100% recovery. Standard samples were checked for linearity over ten days. To determine intra- and inter-day precision and accuracy of the assay, a replicate set of spiked samples containing known cotinine concentrations (5, 150 and 300 ng/ml) were analyzed.

2.6. Statistical analysis

Data analysis was performed using the SAS® statistical software package. Statistical tests used a 5-percent two-sided risk α . Given the distribution of the variables, the results of the corresponding statistical analyses are generally expressed as means \pm standard deviation (*SD*). Minimum and maximum values are also given. Spearman correlation coefficients (*rs*) were used as nonparametric measures to analyze relationships between i) smoking status/mother

cotinine levels, ii) mother/newborn cotinine-level test results for nicotine dependence and other variables.

3. Results

3.1 GC-MS analysis

Cotinine and deuterated cotinine had a retention time of 8.3 min. Drug-free plasma samples were tested to confirm the absence of cotinine. In SIM mode, the molecular ion of m/z 176 was the quantification ion for cotinine and the confirmation ions were the molecular ions of m/z 98, 104 and 118. For cotinine-D3, the molecular ion of m/z 179 was used as quantification ion and the m/z 101 and 121 ions were the confirmation ions. Limit of quantification was 1.5 ng/ml. The assay was linear over the range of 10-250 ng/ml. Extraction recoveries were between 49 and 63%. Correlation coefficient and y-intercept of the calibration curves for plasma ($n = 10$) were 0.9996 ± 0.0003 and 0.0049 ± 0.0178 , respectively.

3.2 Epidemiological application

Mean age of the recruited mother population was 29.5 years ($SD = 4.3$). Cotinine levels of newborns and their mothers in smoking and non-smoking subgroups appear in Table 1. Cotinine concentration is significantly higher ($p < 0.001$) in the smokers subgroup, as expected. The same results were obtained with the corresponding babies. Mother and paired-newborn plasma cotinine concentrations were highly correlated ($r_s = 0.95$, $p < 0.0001$, $n = 961$). For non-smoking mothers, this correlation was 0.70 ($p < 0.0001$), thus confirming a high cotinine exposure of the foetus. Moreover, a dose-response relationship was found between maternal cotinine levels and number of cigarettes smoked per day (Fig 1).

Women were then classified as non-smokers or active smokers, depending on cotinine levels (mean cotininemia = 101.3 ng/ml; $SD = 78.4$) (non-smokers: cotinine levels below 15

ng/ml; n = 766 with n = 600 samples < LOQ, active smokers: cotinine levels above 15 ng/ml; n = 195). A receiver operator characteristic (ROC) curve (Fig. 2) and a sensitivity plot (true positive rate) against 1-specificity (false positive rate) was used to allow the selection of the best possible cut-off point between passive/active smokers, *i.e.* the one which maximizes the sensitivity and specificity of the test. The calculated cut-off for cotinine levels in an active smoker was 21.5 ng/ml. This value showed a specificity of 95.3% and a sensitivity of 83.2%.

4. Discussion

Plasma was a specimen of choice for cotinine measurement, since it is not subject to bacterial degradation or sample concentration. Admittedly, assay in urine presents some advantages, such as the reliability of the results and weak variations during a 24-hour cycle, but this matrix was not used, as it was difficult to collect during delivery.

Numerous methods have been reported for cotinine analysis in biological samples. To summarise, these methods include radioimmunoassay (6), high-performance liquid chromatography (7, 8), as well as gas chromatography-mass spectrometry (GC-MS) (9-14). Since the GC-MS method is recognized as the standard of reference in drug analyses, it was used in this study. Limit of quantification was 1.5 ng/ml, which was lower than values obtained with the HPLC/UV method, *e.g.* 10 ng/ml with Ghosheh *et al.* 2000 (7). It bears stressing that previously published values in GC-MS methods with a liquid-liquid extraction were higher than ours: 2 and 5 ng/ml (9, 10). However, using the same detection, Man *et al.*, 2006, (12) Shin *et al.*, 2003, (13) and Cognard and Staub, in 2003, (9) presented lower or similar LOQ values than us, 0.5, 1 and 1 ng/ml, respectively, although in the last article, extraction was performed using the Tox tubes® system, making it more expensive and not realistically employable in our large-scale clinical study. Using HPLC with MS or MS/MS detection, Gunter in 1997 (15) or

Stolker *et al.* in 2003 (8) reported either lower or similar LOQ values, *i.e.* 0.05 and 1 ng/ml, respectively. The results of this technique provide a satisfactory reflection of the reality of maternal smoking status due to the low LOQ.

The dose-response relationship found between maternal cotinine levels and the number of cigarettes smoked by the mother each day is consistent with results presented by McDonald *et al.* in 2005 (16). Moreover, our results confirm the high accuracy of self-reported smoking status during pregnancy.

Our cut-off value for passive/active smokers, 21.5 ng/ml, has been validated by a good specificity, with only moderate loss of sensitivity, and is consistent with published results. There is still no real consensus on a precise target value, with ranges from 10 ng/ml (17) to 15 ng/ml (18, 19), or between 10-15 (20), and up to the 10-20 range (21). To conclude, we believe the GC-MS is a reliable and sensitive method, useful in epidemiological studies to assess maternal and newborn tobacco-smoke exposure.

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Cotinine concentration ng/ml , mean (S.D.)	Smoking mothers (n = 226)	Newborns of smoking mothers (n = 226)	Non-smoking mothers (n = 781)	Newborns of non-smoking mothers (n = 781)
	92.0 (80.5)	76.0 (81.0)	7.4 (18.1)	7.5 (18.7)

Table 1: Mean and standard deviation (S.D.) plasma cotinine values of pregnant women and newborns according to smoking status as declared by mothers.

p<0.001: Smoking mothers / Non smoking mothers

p<0.001: Newborns of smoking mothers / Newborns of non smoking mothers)

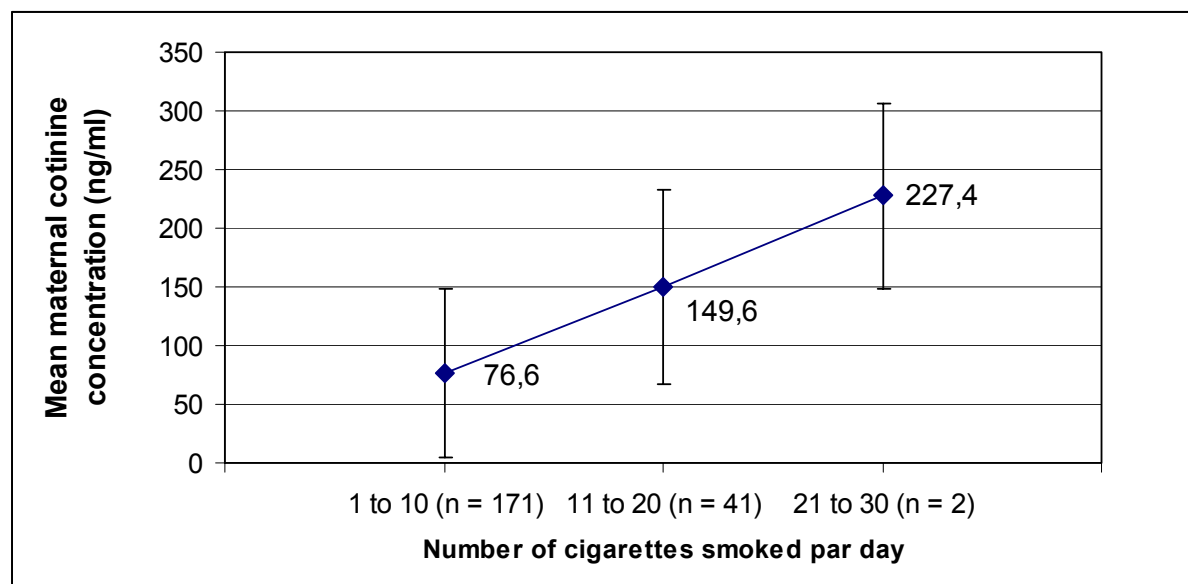


Figure 1: Relationship between cotinine levels and number of cigarettes smoked per day (Student's t test, $p < 0.0001$)

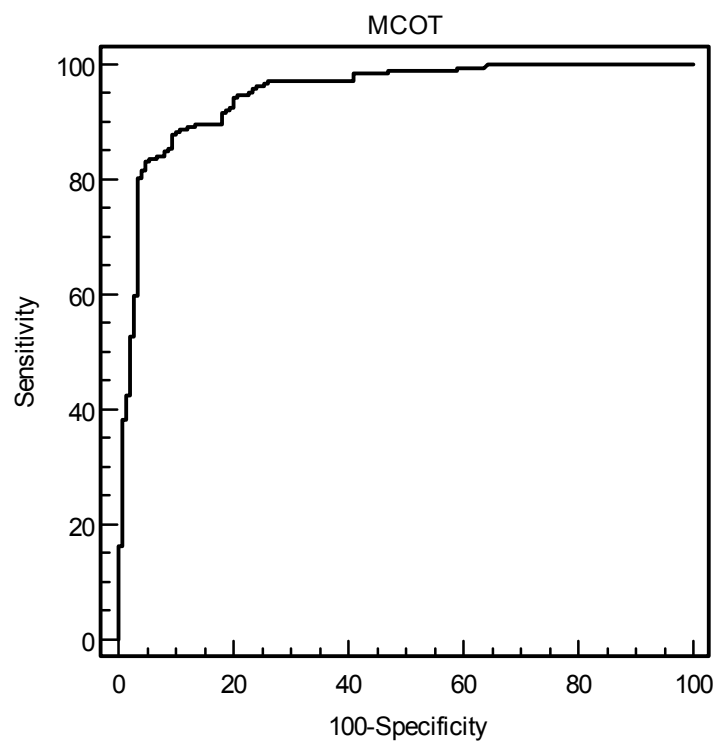


Figure 2: Receiver operating characteristic (ROC) curves for maternal cotinine concentration and smoking status