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Short communication

Determination of nicotine, cotinine and caffeine in meconium using high-performance liquid chromatography

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Abstract

A high-performance liquid chromatographic method with diode-array detection for the determination of nicotine and its metabolites, cotinine and caffeine, in meconium is described. This method is suitable to assess foetus exposure to tobacco smoke. The analytes were extracted by solid-phase extraction before chromatography. From among 30 meconium samples 11 were positive for cotinine (20–86 ng/g) and 27 for caffeine (10–45 ng/g). No nicotine was present in the samples because of its rapid metabolism into cotinine. © 1998 Elsevier Science B.V.

Keywords: Nicotine; Cotinine; Caffeine

1. Introduction

Respirable particles and toxic gases released in tobacco combustion and exhaled by active smokers may have important public health implications [1].

It is now well established that the degree of exposure to tobacco smoke among smokers and non-smokers can be estimated by the measurement of nicotine and its major metabolite, cotinine, in body fluids (serum, urine and saliva) [2,3].

The monitoring of nicotine exposure in individuals can be used to control active smoking behaviour or validate abstinence from smoking. Cotinine can be taken as an indicator of chronic nicotine exposure because of its long biological half-life (37 h), while the excretion of nicotine, which is rapidly detoxified, provides information about recent exposure [4].

The negative effects of maternal active smoking on the foetus are multiple, including spontaneous abortion, preterm labour and lower birth weight. It is less clear whether similar hazards in the foetus occur with passive maternal smoking [5]. Many previous studies were designed to measure maternal exposure to tobacco smoke and included methods such as maternal interview, measurement of maternal serum thiocyanate levels, maternal urinary nicotine or cotinine concentrations, or amniotic fluid catecholamine metabolites. The common shortcoming of all these approaches is that they are indirect measures of fetal exposure to tobacco. Fetal exposure to tobacco smoke has been determined by direct measurement of neonatal serum or urinary cotinine and umbilical cord blood thiocyanate, but these measurements are invasive and are applicable only to recent exposure to tobacco smoke.

A large number of publications deal with the

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quantitative determination of nicotine and/or cotinine in biological fluids, among the methods used are radioimmunoassay [6–8], gas chromatography [9–13] or liquid chromatography [14–42].

In the present study we determined nicotine and cotinine contents in meconium, which gave us the opportunity to measure fetal exposure to tobacco smoke during a long period of intrauterine life. The aim was to develop a method of alkaloid extraction from meconium and their determination by HPLC. An important part of the study was to establish a procedure for the simultaneous determination of nicotine, cotinine and caffeine in meconium. It was also important to determine caffeine as this is an alkaloid present in some medicaments and drinks.

2. Experimental

2.1. Materials

Nicotine, cotinine and caffeine were purchased from Sigma (St. Louis, MO, USA).

All solvents were analytical grade (Merck, Darmstadt, Germany).

Extraction columns for solid-phase extraction were obtained from Backer-Bond (Gross Gerau, Germany).

The infants enrolled in the study were from a Neonatal Department in Upper Silesia in Poland where, based on mothers' reports, 30% of pregnant women are active smokers and 85% are passive smokers (exposed to tobacco smoke at home or place of employment). Of the 30 mothers and their infants enrolled in our study, six mothers were smokers, 15 were passive smokers and nine were non-smokers.

Meconium is a dark greenish mass of desquamated cells, mucus and bile that accumulates in the bowel of a foetus and is discharged shortly after birth.

2.2. High-performance liquid chromatography

Chromatography was performed on a system equipped with Merck-Hitachi L6200 intelligent pump, a Merck-Hitachi L 4500 A diode array detector. A steel column (25 cm×4.6 mm I.D.) packed with Lichrosorb C_{18} (particle size 5 μm) was obtained from Merck.

The column was equilibrated with the mobile phase for at least 30 min prior to analysis of samples. The mobile phase was water–methanol—buffer acetate (pH 4.66)–acetonitrile–acetic acid (50:29:20:2:1, v/v). It was adjusted to pH 4.3 with diethylamine and a flow-rate of 1.0 ml/min was used.

2.3. Extraction procedure

First-day meconium samples ~10 g were collected from infants after birth. Meconium was frozen at −18°C. At the time of analysis meconium was thawed and mixed. A meconium aliquot, 2.0 g, was weighed, mixed with ephedrine (used as an internal standard) and emulsified in 20 ml of 0.1 mol/l phosphate buffer (pH 8.0). Each sample was analysed three times ($n=3$). The suspension was agitated by vortex mixing and then centrifuged. The supernatant was recovered and filtered. The alkaloids being studied were extracted from supernatant using chloroform (3×2 ml). Chloroform extracts were evaporated to dryness, and the residue was dissolved in 10 ml boric buffer (pH 9.0). The prepared sample was then passed through an additional solid-phase extraction as follows: a C_8 column (3 ml) was pre-treated by passing methanol (5 ml) followed by water (5 ml) through it. The C_8 column was then placed onto a silica gel column (3 ml) which had been pre-treated with chloroform (5 ml). The sample was passed through C_8 and then the silica gel column. The C_8 column was removed and the alkaloids were eluted from the silica gel column with 3 ml of 70% methylene chloride plus 30% methanol, containing 1% ammonium hydroxide.

After solid-phase extraction, the organic phase was evaporated to dryness under nitrogen and redissolved in 100 μl of water, and 20 μl were injected into the HPLC column.

3. Results and discussion

There are many articles describing methods for nicotine and cotinine measurement, but only one of them reports that the presence of caffeine in a sample interferes with cotinine determination [37]. The HPLC method described here allows simultaneous

determination of the three alkaloids. We used a Lichrosorb (C_{18}) column and the mobile phase was: water–methanol–buffer acetate (pH 4.6)–acetonitrile–acetic acid (50:29:20:2:1, v/v).

The chromatographic separation of nicotine, cotinine and caffeine is shown in Fig. 1. In order to optimise the separation of the analytes on the reversed-phase column, we investigated the effect of pH. The choice of pH is critical in avoiding the co-elution of cotinine and caffeine. The best separation was obtained at pH 4.3 and the retention times were: 3.75 min for nicotine, 7.29 min for cotinine and 7.99 min for caffeine.

The detection limit observed with this method was 10 ng/ml. The calibration curve ($n=3$) for nicotine was linear over the range 10–500 ng/ml ($r=0.9927$), for cotinine over 10–500 ng/ml ($r=0.9438$), and for caffeine over 10–250 ng/ml ($r=0.9303$).

The analytical procedure described, which involves chloroform extraction followed by an additional solid-phase extraction, enabled us to obtain a clear chromatographic separation in which the three alkaloids were not contaminated by the presence of other compounds present in meconium. No attempt was made to identify other constituents of the meconium. Analysis of meconium sample is presented in Fig. 2. HPLC analysis should be carried out as quickly as possible after alkaloid extraction from meconium. Meconium samples can be stored at −18°C for at least 1 week without changes in alkaloids concentrations occurring.

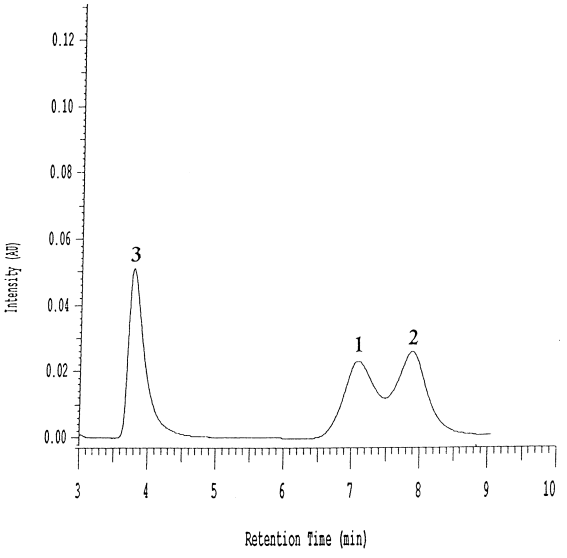

Fig. 1. Chromatogram of the mixture of cotinine (1), caffeine (2) and nicotine (3).

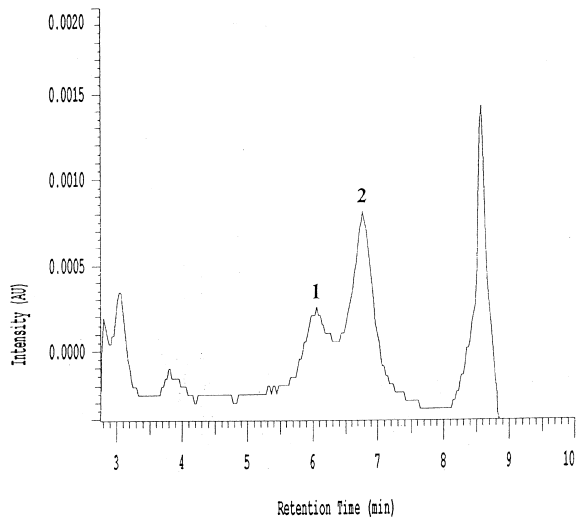

Fig. 2. Chromatogram of the meconium: (1) cotinine, (2) caffeine.

When the meconium samples were subjected to HPLC, 90% (27/30) were positive for the presence of caffeine (range 10–45 ng/g) and 11 of them were positive for the cotinine (range 20–86 ng/g). (Table 1). Cotinine was found in meconium samples obtained from children whose mothers belonged to both the active and passive smoker groups. No cotinine was found in meconium samples obtained from children whose mothers did not smoke.

Nicotine presence was not found in any of the samples examined. However, nicotine can be determined by the method described if it is introduced into meconium. The overall uncorrected mean recovery of cotinine-supplemented meconium was 85%, of nicotine 90% and caffeine 92%.

In conclusion we can state that meconium can be recommended as a suitable material for assessment of fetal exposure to tobacco smoke. The presented method is non-invasive, the material easily available, and present at an age when the foetus is very sensitive to the negative influence of environmental factors, located on the fetal side of the placental barrier. The procedure has the additional advantage that no parental consent is necessary to take samples of meconium.

Table 1
Mean concentration of alkaloids in meconium samples obtained from children whose mothers were active, passive and non-smokers ($n=3$)

Subject	Exposure	Nicotine (ng/g)	Cotinine (ng/g)	Caffeine (ng/g)
1	Passive	0.0	20.0	10.0
2	Passive	0.0	20.0	15.0
3	Active	0.0	28.0	10.0
4	Passive	0.0	21.0	20.0
5	Active	0.0	19.0	25.0
6	Active	0.0	40.0	28.0
7	Active	0.0	78.0	10.0
8	Passive	0.0	20.0	45.0
9	Active	0.0.	20.0	39.0
10	Passive	0.0	25.0	36.0
11	Active	0.0	86.0	20.0
12	Passive	0.0	0.0	10.0
13	Passive	0.0	0.0	10.
14	Passive	0.0	0.0	15.0
15	Passive	0.0	0.0	40.0
16	Passive	0.0	0.0	18.0
17	Passive	0.0	0.0	25.0
18	Passive	0.0	0.0	28.0
19	Passive	0.0	0.0	30.0
20	Passive	0.0	0.0	10.0
21	Non-smokers[a]	0.0	0.0	14.0
22	Non-smokers	0.0	0.0	38.0
23	Non-smokers	0.0	0.0	26.0
24	Non-smokers	0.0	0.0	10.0
25	Non-smokers	0.0	0.0	10.0
26	Non-smokers	0.0	0.0	26.0
27	Non-smokers	0.0	0.0	20.0
28	Non-smokers	0.0	0.0	15.0
29	Non-smokers	0.0	0.0	20.0
30	Non-smokers	0.0	0.0	35.0
Mean±S.D.		0.0	34.3±20.0	21.9±10.0

[a]Non-smokers, not exposed passively to tobacco smoke.

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