Journal of Chromatography, 414 (1987) 449–453
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3452

Note

Simplified method for the determination of plasma cotinine using gas chromatography–mass spectrometry

C. GAIL NORBURY

Area Laboratory, West Park Hospital, Horton Lane, Epsom, Surrey KT19 8PB (U.K.)

(First received August 4th, 1986; revised manuscript received October 6th, 1986)

Cotinine is the principal metabolite of nicotine [1]. It serves as a more useful index of tobacco smoking than nicotine due to the fact that whilst its concentration is linearly and directly related to that of nicotine [2], it has a much longer metabolic half-life [1,3] and also habitual smokers appear more efficient than non-smokers at metabolising nicotine [1,4].

A number of methods for the determination of cotinine have been described in the literature. Most of these involve solvent extraction, gas chromatography (GC) and detection by nitrogen–phosphorus detectors or mass spectrometry (MS) [5–9]. All except one of these methods [8] require 1 ml plasma sample. The internal standards used in the MS methods have been structural [6,8] or deuterated [7,9] analogues of cotinine, which have been prepared by time-consuming, multi-step, in-house methods. The solvent extraction procedures tend to be tedious [5–7], involve transfer of extracts and are vulnerable to low recovery and contamination. Detection by MS has helped overcome problems of selectivity. A method is described here which requires only 200 μl sample, a commercially available internal standard, one-tube extraction procedure and GC–MS. This is a simple yet specific and sensitive method.

EXPERIMENTAL

Chemicals

Cotinine was obtained from Sigma (St. Louis, MO, U.S.A.) and was stored as a 17.6 μM ethanolic solution at 4°C in the dark. The internal standard, methyprylone (Noludar®), was from Roche (Welwyn Gdn City, U.K.). The working solution was prepared by reconstituting a 10 mM ethanolic stock to give a 6 μM

0378-4347/87/$03.50

aqueous solution stored at 4°C. Dichloromethane and dichloroethane were of HPLC grade from Rathburn Chemicals (Walkerburn, U.K.). Ethanol was from James Burrough (London, U.K.). Diethyl ether and sodium hydroxide were from BDH (Poole, U.K.). Water was purified by an Elgastat Spectrum System (High Wycombe, U.K.).

Standards and controls

Working standards were prepared by reconstituting 17.6 μM cotinine (ethanol solution) dried under nitrogen in cotinine-free plasma to give a range of 0–2000 nmol/l (0, 100, 200, 400, 600, 800, 1000 and 2000 nmol/l). Controls were prepared from patient samples at approx. 200, 500, 600 and 1000 nmol/l. Standards and controls were stored at −20°C.

Extraction

Sample/standard (200 μl) together with ml working internal standard (100 μl) was treated with 1.5 ml diethyl ether in silanised, glass-stoppered tubes and mixed by vortexing for 2 min. After allowing the phases to separate on standing for 5 min, the organic phase was decanted after freezing the aqueous phase in an ethanol cold hold at −20°C. A 20-μl volume of 1 M aqueous sodium hydroxide and 2 ml of dichloromethane were then added to the thawed aqueous phase and the cotinine was extracted into the organic phase by vortexing for 2 min. The phases were separated by centrifugation at 1200 g for 5 min, the aqueous phase was then removed by aspiration and the extract dried by vortex evaporation under vacuum at room temperature or under nitrogen at 45°C. The extract was reconstituted in 50 μl dichloroethane and stored in ice and in the dark. Glass pipettes or syringes were used for the addition of organic solvent to avoid contamination with plasticisers.

Instrumentation

The gas chromatograph–mass spectrometer was a Hewlett-Packard Model 5790-5970A system. Peak areas were determined by automatic integration using a Hewlett-Packard 2672G on-line computing unit. Analyses were performed on a 25 m×0.2 mm SE30 cross-linked, bonded, fused-silica column with a silanised borosilicate glass splitless injection port liner. The carrier gas was helium at a linear velocity of 35 cm/s.

Chromatography

The capillary GC–MS analysis of cotinine was performed using splitless injection. The injection port was maintained at 250°C, the detector at 200°C and the column was programmed from 80 to 200°C at 30°C/min. Injection volume was 3 μl and the purge time was 0.9 min. The electron multiplier was set at 2200 V and the detector turned on 4.0 min after injection. To avoid carry-over, the injection port was purged with 5 μl dichloroethane at the end of each sample run before the solenoid valve opened and again with the valve open to purge the head of the column of any residual sample. The selected ions monitored were at 140 m/z for Noludar and 176 m/z for cotinine with dwell times of 100 and 300 ms, respectively.

The peak-area ratio of cotinine/Noludar was used as the basis for quantitation. A standard curve was established for each batch by calculating the least-mean-squares line of regression for standards in the range 0–1000 nmol/l.

Recovery

This was estimated from the ratio of the gradients of the standards curves obtained from the extracted plasma standards and from aliquots of stock dried under nitrogen and reconstituted in 50 μl of 720 nmol/l Noludar (dichloroethane). The extracts were reconstituted in the same way. By introducing Noludar only at the reconstitution step, it corrected only for variation in injection volume and not for differences in recovery during extraction (i.e., it was an external standard).

Reproducibility

Intra- and inter-batch variations were determined for each of three levels of plasma cotinine.

RESULTS

Extraction

Diethyl ether extraction was necessary to remove lipid from the plasma which otherwise could cause problems in the chromatographic system. Nicotine extracted mainly into diethyl ether (60%), whilst cotinine was virtually insoluble due to its greater polarity. Alkali was added to dichloromethane to improve the extraction recovery of cotinine. The final evaporation step had to be carried out with great care to avoid loss of cotinine and to a lesser extent internal standard. The reconstitution volume was 50 μl to ensure complete recovery of extract from the side of the tube. This dilute sample necessitated the use of splitless injection onto the GC column together with selected-ion monitoring as a means of detection. Noludar was selected as internal standard on the basis of its chromatography, ion mass spectrum, non-radioactivity and extraction characteristics.

Chromatography

Noludar eluted first with a retention time of 5.5 min. The peak was narrow and symmetrical. The retention time of cotinine was 6.4 min. Whilst the peak shape was reasonable there was some tendency to broaden and tail particularly at low levels if chromatographic conditions (liner and column activity) were not optimal. The Kovats retention index of Noludar under the described chromatographic conditions was 1532 [coefficient of variation (C.V.) 0.4%, $n=10$] and that of cotinine was 1682 (C.V. 0.7%, $n=10$). Overall run time allowing for the temperature gradient was 17 min. Chromatograms of samples from a non-smoker and smoker are shown in Fig. 1.

Ion monitoring

The base peak of cotinine is 98 m/z. However, at this relatively low ion mass interference from other species including Noludar is likely. By monitoring the

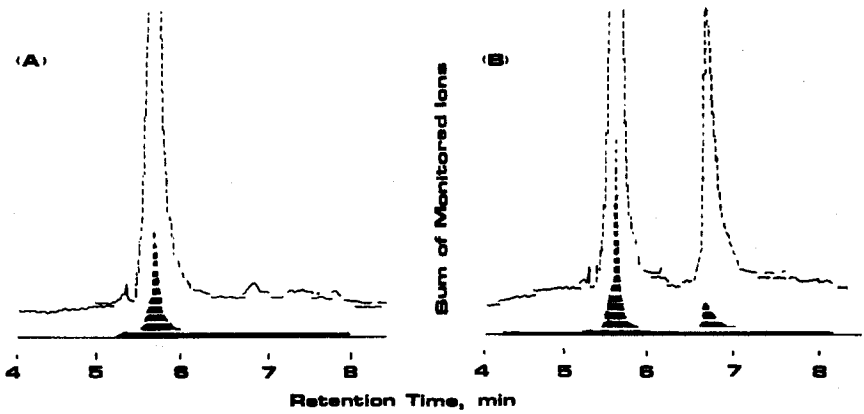

Fig. 1. Chromatograms of GC–MS determination of plasma cotinine. (A) Non-smoker, (B) smoker with a cotinine level of 805 nmol/l.

molecular ion of cotinine, which has a higher ion mass of 176 *m/z* and a relative abundance of 32%, interference by other species including Noludar is avoided. Noludar was monitored at its base peak of 140 *m/z* at which there is no interference from cotinine. It is proposed that this ion fragment of Noludar is formed as a result of loss of a keto and methyl group from the parent structure and subsequent rearrangement. The sites of cleavage are indicated in Fig. 2. The sensitivity lost by not measuring cotinine at its base peak, in order to increase selectivity, was compensated for by increasing the dwell time for cotinine.

Recovery

The recovery of cotinine as described was 111%. This reflects the relative errors in the standard curves of extracted and non-extracted standards over the concentration range 0–1000 nmol/l and the relative loss of cotinine during solvent extraction and evaporation steps.

Standard calibration curve

A plot of cotinine/Noludar peak-area ratio ($\times 10^{-1}$) against cotinine concentration ($\times 10^{2}$ nmol/l), gives a typical regression line described by the equation $y = 6.76 \cdot 10^{-4} x - 0.014$ (standard error of estimate = 0.0143). The curve is linear upto 1000 but not 2000 nmol/l.

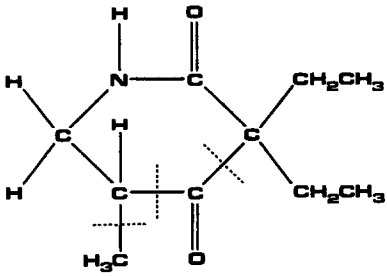

Fig. 2. Proposed sites of cleavage involved in the formation of the ion fragment used to monitor Noludar.

TABLE I

INTRA- AND INTER-BATCH REPRODUCIBILITY FOR THE GC-MS DETERMINATION OF PLASMA COTININE

Concentration (nmol/l)	*n*	Coefficient of variation (%)
Intra-batch		
218	6	16
489	6	7
617	6	9
Inter-batch		
231	11	16
512	11	14
1051	11	6

Detection limit

This was considered to be in practice 100 nmol/l on the basis that it could be confidently distinguished from a blank sample and was, in this procedure, the level of the lowest standard.

Reproducibility

The intra- and inter-batch precision data for plasma cotinine are summarized in Table I.

ACKNOWLEDGEMENT

The author wishes to thank Martyn Egerton for his excellent guidance.

REFERENCES

1 A.H. Beckett, J.W. Garrod and P. Jenner, J. Pharmacol. (Suppl.), 23 (1971) 625.
2 R.L. Galeazzi, P. Daenens and M. Grugger. Eur. J. Clin. Pharmacol., 28 (1985) 301.
3 N.L. Benowitz, F. Kynt, P. Jacob III, R.T. Jones and A.L. Osman, Clin. Pharmacol. Ther., 34 (1983) 604.
4 H. Ashton and R. Stepney, The Importance of Nicotine, Tavistock Publications, London, 1982.
5 N. Henegen and M. Henegen, Clin. Chem., 24 (1978) 50.
6 P. Jacob III, M. Wilson and N.L. Benowitz, J. Chromatogr., 222 (1981) 61.
7 J.A. Thompson, Mind-Shan Ho and D.R. Petersen, J. Chromatogr., 231 (1982) 53.
8 M. Curvall, E. Kazemi-Vala and C.R. Enzell, J. Chromatogr., 232 (1982) 283.
9 P. Daenens, L. Laruelle and K. Callewaert, J. Chromatogr., 342 (1985) 79.