Journal of Chromatography, 620 (1993) 55–60
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 7063

Determination of 4-(methylnitrosamino)-4-(3-pyridyl)-butyric acid in tobacco, tobacco smoke and the urine of rats and smokers

A. Pachinger*, H. Begutter, I. Ultsch and H. Klus

Ökolab, Gesellschaft für Umweltanalytik, Hasnerstrasse 124a, 1160 Vienna (Austria)

(First received November 6th, 1992; revised manuscript received July 16th, 1993)

ABSTRACT

The potential endogenous nitrosation of nicotine and/or nicotine metabolites has led to speculation on the possible formation of 4-(methylnitrosamino)-4-(3-pyridyl)butyric acid (iso-NNAC) in smokers. A gas chromatographic method with thermal energy analytical detection is described for the determination of iso-NNAC in tobacco, tobacco smoke and urine. Sample pre-concentration is performed using C_{18} extraction cartridges prior to esterification of iso-NNAC using ethereal diazomethane solution. Sample clean-up includes chromatography on aluminum and silica, and fractionation using high-performance liquid chromatography. The detection limits for iso-NNAC in tobacco, tobacco smoke and urine are 2 ng/g tobacco, 0.1 ng/cigarette and 20 ng/l urine, respectively.

INTRODUCTION

4-(Methylnitrosamino)-4-(3-pyridyl)butyric acid (iso-NNAC; Fig. 1), a tobacco-specific N-nitrosamine, has been identified in tobacco [1] and in tobacco smoke [2] using gas chromatography and thermal energy analytical detection (GC–TEA). The structure of iso-NNAC in tobacco has been confirmed by GC–MS after enrichment of the methylated fraction. Sample preparation included liquid–liquid extraction at pH 4, methylation, and chromatography on an alumina column. The transfer rate of iso-NNAC from spiked cigarettes into mainstream smoke was determined to be *ca.* 1.0% (*ca.* 1 μg/cigarette) [2]. It has been speculated that endogenous nitrosation of nicotine and/or nicotine metabolites could result in the formation of iso-NNAC *in vivo* [1]. Iso-NNAC is not carcinogenic in animal bioassays [3].

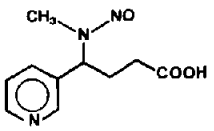

Fig. 1. Structure of 4-(methylnitrosamino)-4-(3-pyridyl)butyric acid (iso-NNAC).

Therefore, a sensitive and selective procedure for the determination of iso-NNAC in human urine using GC–TEA is presented (detection limit 20 ng/l). This procedure is also applicable for the determination of iso-NNAC in tobacco and tobacco smoke. A simplified method for the determination of iso-NNAC in urine (detection limit 30 μg/l) of rats administered iso-NNAC is also described.

* Corresponding author.

0378-4347/93/$06.00

EXPERIMENTAL

Chemicals and reagents

Bond-Elut C_{18} cartridges (1 g packing material) and Mega Bond-Elut C_{18} cartridges (10 g packing material) were purchased from Analytichem International (Harbor City, CA, USA). P.A. grade anhydrous sodium sulfate, cyclohexane, extra pure silica 60, l-ascorbic acid, disodiumhydrogen phosphate, phosphoric acid (85%) and citric acid were from Merck (Darmstadt, Germany). HPLC grade ethyl acetate was purchased from Rathburn (Walkerburn, Scotland). P.A. methanol was obtained from J.T. Baker (Deventer, the Netherlands) and basic aluminum hydroxide (activity II–III) from M. Woelm (Eschwege, Germany). The Cambridge filters were purchased from Borgwaldt (Hamburg, Germany). Ethereal diazomethane solution was prepared by the action of potassium hydroxide on N-nitrosomethylurea in diethylether [4].

The buffer solution (buffer I) used in the analysis of mainstream smoke was composed of 0.2 *M* Na_2HPO_4 adjusted to pH 4.0 with 0.1 *M* citric acid. Iso-NNAC was a gift of Dr. A. R. Tricker (Analytisch-Biologisches Forschungslabor, Prof. Dr. med. F. Adlkofer, Munich, Germany). A standard stock solution of iso-NNAC (1 mg/ml) was prepared in methanol. Working standard solutions used for the spiking of samples were prepared by dilution of the standard stock solution with methanol. Working standard solutions of iso-NNAC methyl ester were prepared in ethyl acetate after esterification [5].

Gas chromatography

System (A). Extracts of rat urine samples were analysed on a Varian (Walnut Creek, CA, USA) Model 3700 gas chromatograph equipped with a modified thermal energy analyzer (TEA) Model 502 from Thermo Electron Corp. (Waltham, MA, USA), a Hewlett-Packard (Avondale, PA, USA) Model 7673A autosampler and a HP 3396A integrator. The gas chromatograph was fitted with a 15 m × 0.54 mm I.D. fused silica capillary column coated with a 3 μm film of DB-1 (J&W Scientific, Folsom, CA, USA). The modifications of the TEA have previously been described elsewere [6]. Helium was used as carrier gas at a flow-rate of 5 ml/min (at 240°C). The injection port was maintained at 210°C. Samples (1 μl) were injected on-column with a column temperature of 150°C. One min after injection the oven temperature was programmed at 10°C/min to 240°C. Detector parameters were as follows: pyrolyzer furnace temperature 475°C and oxygen pressure 0.11 bar.

System (B). Extracts of human urine, tobacco and tobacco smoke were analyzed on a Varian Model 3700 gas chromatograph equipped with a modified TEA Model 543 (Thermo Electron Corp.) and a HP Model 7673A autosampler using a 30 m × 0.54 mm I.D. fused silica capillary column coated with a 1-μm film of DB-1301 (J&W). Modifications to the TEA Model 543 were the same as described for the Model 502. Helium was used as carrier gas at a column head pressure of 0.2 bar. The injection port was maintained at 240°C. Samples (1 μl) were injected with the column at 170°C. The injection port was equipped with a glass insert vaporizing chamber (0.04 ml) which promotes an efficient and quick sample transfer onto the column. This injection technique resembles direct injection as described by Grob [7] and Tekel' *et al.* [8]. One min after injection, the oven temperature was programmed at 10°C/min to 250°C. Detector parameters were as follows: pyrolyzer furnace temperature 470°C and oxygen pressure 0.3 bar.

Data integration was carried out using a HP Vectra QS/20 computer and HP 3365 ChemStation (version A.02.00 software).

The performance of both systems was checked daily by the injection of a 0.1 ng/μl standard solution of iso-NNAC methyl ester. Quantification was made by comparing the peak areas of chromatograms obtained from sample extracts with those of standard solutions containing iso-NNAC methyl ester at a concentration similar to the concentration of iso-NNAC methyl ester in the sample extracts. This procedure is based on the supposition that the calibration curve is linear at least within a small concentration range.

High-performance liquid chromatography

The liquid chromatograph used was a HP1090 (Hewlett-Packard) Series M fitted with a 7 μm Nucleosil 7-OH guard column (20 mm × 4 mm I.D.) and a 7-μm Nucleosil 7-OH analytical column (250 mm × 4 mm I.D.). Fractionation was performed under isocratic conditions using ethyl acetate–cyclohexane (3:2, v/v) as mobile phase at a flow-rate of 1 ml/min and a column temperature of 40°C.

Determination of the fractions containing iso-NNAC methyl ester was accomplished by the collection of 0.5 ml fractions at the end of the column after the injection (250 μl) of a standard solution (1 μg/ml). The concentration of iso-NNAC methyl ester in each fraction was determined using GC–TEA.

Samples

Method development for the determination of iso-NNAC in both rat and human urine was performed using spiked fresh urine samples from non-smokers. Tobacco and cigarettes used during the course of method development were purchased on the open market. Urine samples were stored at −20°C in the dark prior to analysis.

Analysis of rat urine

Bond-Elut C_{18} cartridges (1 g packing material) were used to concentrate rat urine samples for GLC–TEA analysis. The cartridges were conditioned with 5 ml of methanol followed by 5 ml of phosphate buffer (pH 4.0, 0.2 *M*). The urine sample (4 ml) was adjusted to pH 4.0 with phosphoric acid and passed through the cartridge. The eluate was discarded. Ethyl acetate (5 ml) was used to extract iso-NNAC and the eluent collected directly onto a column containing 1 g of anhydrous sodium sulfate. The sodium sulfate was washed with 2 ml of ethyl acetate and the combined eluates were concentrated *in vacuo* to *ca.* 2 ml using a rotary evaporator. The extract was derivatized with ethereal diazomethane. After evaporation of diethylether and excess diazomethane the remaining residue (*ca.* 2 ml) was transferred to a column containing 6 g of basic aluminum hydroxide. The extract was chromatographed using ethyl acetate as the mobile phase. The first 20 ml of the eluent containing iso-NNAC methyl ester was concentrated to *ca.* 1 ml *in vacuo* and made up to 2 ml with ethyl acetate. Sample aliquots (1 μl) were injected into the GC–system (A).

Analysis of human urine

Human urine (200 ml, pH 4.0 with phosphoric acid) was applied to two preconditioned (50 ml of methanol followed by 50 ml of phosphate buffer 0.2 *M*, pH 4.0) Mega Bond-Elut C_{18} disposable cartridges (100 ml each). Each column was washed with phosphate buffer solution (20 ml, 0.2 *M*, pH 4.0) and iso-NNAC eluted from each column with ethyl acetate (60 ml). The extracts were combined and dried over anhydrous sodium sulfate. After filtration the extract was derivatized with an ethereal diazomethane solution and concentrated to *ca.* 5 ml *in vacuo*. The derivatized extract was chromatographed on aluminum hydroxide (10 g packing) using ethyl acetate as the mobile phase. The first 20 ml of the eluent containing iso-NNAC methyl ester was concentrated to *ca.* 3 ml *in vacuo*. The extract was transferred to a column packed with 3 g of silica. The silica column was washed with cyclohexane–ethyl acetate (75:25 v/v; 50 ml) and iso-NNAC methyl ester was eluted with ethyl acetate (100 ml). The eluate was concentrated to *ca.* 0.5 ml *in vacuo* and made up to 1.0 ml with cyclohexane. The extract was fractionated by HPLC (chromatographic conditions as above). The fractions containing iso-NNAC methyl ester (4.5–6.5 min) from three injections (250 μl each) were combined and concentrated to 50 μl under a gentle stream of nitrogen. The exact final volume was measured with a microlitre syringe. Sample aliquots (1 μl) were injected into the GC system (B).

Analysis of tobacco

Tobacco (2 g) was extracted with a phosphate buffer solution (50 ml, pH 7.0, 0.01 *M*) by vigorous shaking for 1 h. After addition of Celite (3 g) the sample was filtered through a glass frit and the residue washed with a further 50 ml of phosphate buffer solution. The combined extracts were adjusted to pH 4.0 with phosphoric acid

TABLE I

RECOVERY AND VARIABILITY DATA FOR ISO-NNAC

Samples were spiked with iso-NNAC prior to sample isolation and then prepared as described in text.

Sample	Spiked concentration	Measured concentration (mean)	Recovery (mean ± S.D.) (%)	*n*
Inter-assay				
Human urine	500 ng/l	360.5 ng/l	72.1 ± 11.1	4
Human urine	50 ng/l	34.2 ng/l	68.4 ± 14.5	5
Rat urine	100 ng/ml	89.9 ng/ml	89.9 ± 8.5	4
Tobacco	25 ng/g	21.9 ng/g	87.6 ± 8.6	5
Tobacco smoke	100 ng/cig.	84.3 ng/cig.	84.3 ± 9.4	5
Intra-assay				
Human urine	500 ng/l	340.5 ng/l	68.1 ± 13.4	5
Human urine	50 ng/l	37.1 ng/l	74.2 ± 8.9	4
Rat urine	100 ng/ml	84.5 ng/ml	84.5 ± 7.9	3
Tobacco	25 ng/g	21.0 ng/g	84.0 ± 8.9	6
Tobacco smoke	100 ng/cig.	88.6 ng/cig.	88.6 ± 5.9	5

and applied to a single Mega Bond-Elut C_{18} cartridge. The extraction, derivatization, concentration and fractionation procedure for the determination of iso-NNAC in tobacco was the same as described above for the analysis of human urine.

Analysis of tobacco smoke

Fourty cigarettes were smoked on a 20-port rotary head Model RM20 smoking machine (Borgwaldt). The mainstream smoke was collected in two wash bottles arranged in tandem followed by a Cambridge filter. Each wash bottle was filled with 90 ml of buffer I and ascorbic acid (0.3 g) to prevent N-nitrosamine formation [9]. The Cambridge filter was impregnated with a solution of ascorbic acid (3 g/l) and allowed to dry at room temperature before use. The Cambridge filter was extracted under sonication with 20 ml of buffer I for 30 min and the filter extract added to the combined contents of the two wash bottles. The extract was applied to two Mega Bond-Elut C_{18} extraction cartridges (100 ml each). The following procedure was the same as described above for the analysis of human urine.

RESULTS AND DISCUSSION

Extraction

Due to the polar and amphoteric nature of iso-NNAC, quantitative partition of iso-NNAC into organic solvents by repeated liquid–liquid extraction is a time-consuming und cumbersome procedure. Djordjevic *et al.* [1,2] have used ethyl acetate (4 × 1 l) for the extraction of iso-NNAC from 1.25 l of aqueous solution at pH 4, the approximate isoelectric point of iso-NNAC. Before extracting iso-NNAC from an aqueous tobacco extract these authors removed organic acids and bases by liquid–liquid partitition at pH 1.5 and pH 9, respectively. The partition coefficient of iso-NNAC (phosphate buffer pH 4/ethyl acetate) determined in our laboratory was 0.67 ± 0.09. Liquid–liquid extraction of iso-NNAC on Extrelut 3 (phosphate buffer pH 4.0–ethyl acetate) resulted in a recovery of less than 1%. A less time consuming method with a higher degree of extraction efficiency for iso-NNAC was achieved using a solid phase procedure with Bond-Elut C_{18} disposable cartridges. Recovery of iso-

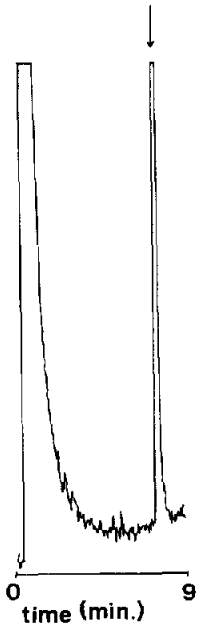

Fig. 2. GC of rat urine containing 1.1 μg/ml iso-NNAC. Arrow indicates retention time of iso-NNAC.

NNAC from Bond-Elut C_{18} cartridges (1 g packing material) using standard solutions (0.1 μg/ml iso-NNAC in 10 ml phosphate buffer pH 4.0) showed a recovery of 97 ± 3.4%. Breakthrough of iso-NNAC on Bond-Elut C_{18} cartridges occured only when the ratio of sample volume (in ml) to sorbent weight (in g) exceeded a value of 15.

Detection in rat urine

To investigate the excretion of iso-NNAC by rats which had been administrated 100 μg of iso-NNAC, a simple procedure using solid phase extraction followed by esterification, chromatography on basic aluminum, and GC–TEA was applied. The aluminum column was necessary only to prevent early deterioration of the GC-column. Matrix interference was not observed even without basic aluminum. The determined detection limit (signal-to-noise ratio of 3:1) for iso-NNAC in rat urine was 30 μg/l. The results obtained from a recovery study are presented in Table I. A chromatogram of rat urine containing 1.1 μg/ml iso-NNAC is shown in Fig. 2.

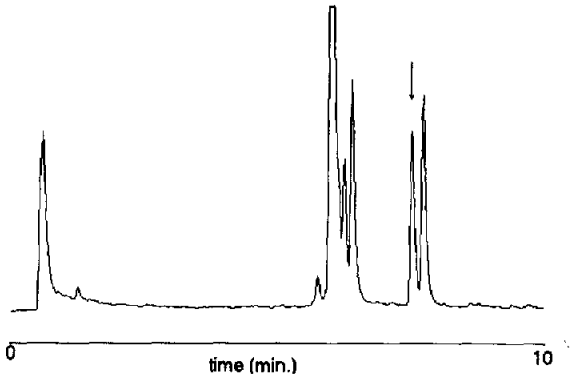

Fig. 3. GC of tobacco containing 43 ng/g iso-NNAC. Arrow indicates retention time of iso-NNAC.

Detection in tobacco, tobacco smoke and human urine

To obtain a reasonable detection limit suitable for trace determination of iso-NNAC in man the sample preparation had to be extended by two additional chromatographical steps (silica column and HPLC). Problems arose not from matrix interference on the chromatogram but from the fact that precipitation occured in the final sample extract (50 μl) within a few hours. This was resolved by performing GC–TEA immediately after HPLC fractionation. The same procedure used for determining iso-NNAC in human urine was applied to tobacco and the mainstream smoke of cigarettes. The limits of detection (signal-to-noise ratio of 3:1) for iso-NNAC in tobac-

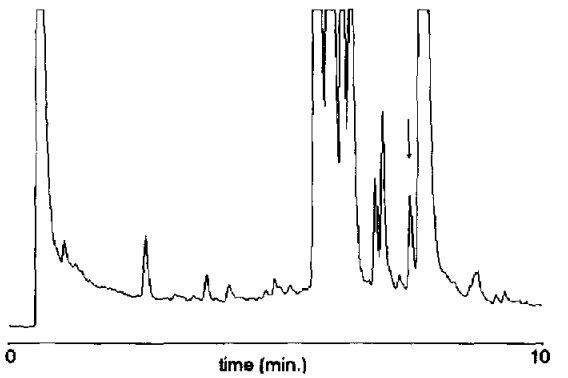

Fig. 4. GC of tobacco smoke containing 0.8 ng/cigarette iso-NNAC. Arrow indicates retention time of iso-NNAC.

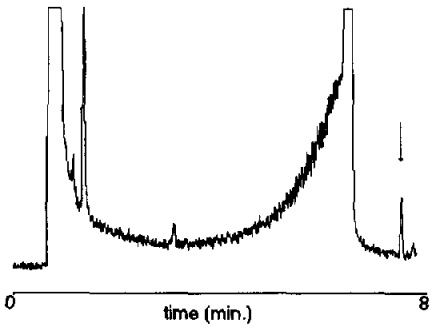

Fig. 5. GC of human urine containing 44 ng/l iso-NNAC. Arrow indicates retention time of iso-NNAC.

co, mainstream smoke of cigarettes and human urine were 2 ng/g tobacco, 0.1 ng/cigarette and 20 ng/l urine, respectively.

Tobacco extracts, combined extracts of tobacco smoke and human urine were spiked with iso-NNAC and the recovery determined using the respective isolation procedures. The results are summarized in Table I. Representative chromatograms obtained from spiked tobacco, tobacco smoke and human urine are shown in Figs. 3–5.

CONCLUSION

Sensitive and selective procedures for the determination of iso-NNAC in various samples are presented. One of these procedures has proved to be applicable to determining the excretion of iso-NNAC by rats. Though time consuming, the combination of solid phase extraction and different chromatographic sample preparation steps with GC–TEA provides a sensitive method with an adequate detection limit for the determination of iso-NNAC in tobacco, tobacco smoke and the urine of smokers. These procedures have been used to show that nicotine and/or its metabolites are not endogenously nitrosated in humans to form iso-NNAC [10].

REFERRENCES

1 M. V. Djordjevic, K. D. Brunnemann and D. Hoffmann, *Carcinogenesis*, 10 (1989) 1725.
2 M. V. Djordjevic, C. W. Sigountos, K. D. Brunnemann and D. Hoffmann, *J. Agric. Food Chem.*, 39 (1991) 209.
3 A. Rivenson, M. V. Djordjevic, S. Amin and R. Hoffmann, *Cancer Lett.*, 47 (1989) 111.
4 *"Organikum"*, VEB Deutscher Verlag der Wissenschaften Berlin, Berlin, Germany, 1981, p. 655.
5 K. Blau and G. S. King (Editors), *Handbook of Derivatives for Chromatography*, Heyden, London, 1978, p. 69.
6 H. Begutter, H. Klus and I. Ultsch, *J. Chromatogr.*, 321 (1985) 475.
7 K. Grob, *Classical Split and Splitless Injection in Capillary Gas Chromatography*, Hüthig, Heidelberg, 1986, pp. 209-212.
8 J. Tekel', K. Schultzová, P. Farkaš, J. Kovačičová and E. Brandšteterová, *HRC & CC*, 14 (1991) 423.
9 H. Egan, R. Preussmann, G. Eisenbrand, B. Spiegelhalder, I. K. O'Neill and H. Bartsch (Editors), *Environmental Carcinogens — Selected Methods of Analysis*, IARC Scientific Publications No. 45, International Agency for Research on Cancer, Lyon, 1983, p. 26.
10 A. R. Tricker, G. Scherer, C. Conze, F. Adlkofer, A. Pachinger and H. Klus, *Carcinogenesis*, 14 (1993) 1409.