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Determination of nicotine exposure in dogs subjected to passive smoking using methanol extraction of hair followed by hydrophilic interaction chromatography in combination with Fourier transform mass spectrometry

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ABSTRACT

There has been no previous assessment of the level of nicotine exposure in companion animals as a result of passive smoking. A method was developed for the determination of nicotine in dog hair where extraction was carried out by sonication in methanol. The levels of nicotine obtained by extraction with methanol were found to be comparable to the lengthier method involving digestion of the hair in 1 M NaOH. The methanol extracts were injected directly onto a ZICHILIC column coupled to an Exactive high resolution Fourier Transform mass spectrometer. Endogenous nicotine was quantified against $^{2}H_4$-nicotine spiked into the extraction medium, linearity was found over a wide range with the calibration curve having a slope close to 1 indicating an equal response for nicotine and the deuterated internal standard, precision was determined to be ±1.9%. Nicotine was present in widely varying amounts in the hair of dogs belonging to smokers and was found to be absent from the hair of dogs belonging to non-smokers. In addition to nicotine, nicotine N-oxides, cotinine, nornicotine and nornicotine N-oxide could be detected in the hair of dogs belonging to smokers. The nicotine N-oxides were only observed in methanol extracts suggesting these compounds are not stable to the NaOH digestion process.

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1. Introduction

The exposure of non-smokers to environmental tobacco smoke (ETS) has been extensively investigated [1–19]. The monitoring of nicotine and its metabolites in body fluids [1–10] has been used as one measure of nicotine exposure in non-smokers but the half life of nicotine is only a few hours and thus monitoring of physiological fluids maybe less suitable for gauging long term exposure. Several studies have indicated that hair nicotine concentration is a more useful biomarker of long term smoke exposure [11–18] than the levels of nicotine in body fluids. Hair nicotine concentrations have been determined by using a range of techniques, including high-performance liquid chromatography (HPLC) with UV detection [11] and with electrochemical detection [12]; liquid chromatography mass spectrometry [13–15] and gas chromatography mass spectrometry [16,17]. Up to 15 metabolites of nicotine including nicotine N-oxide, cotinine, norcotinine, cotinine N-oxide and trans 3-hydroxy cotinine have been detected in urine [2]. However, in hair nicotine and its metabolite cotinine have been most frequently measured. There have been no reports assessing the level of nicotine exposure in companion animals of smokers. Hydrophilic interaction chromatograph (HILIC) methods are becoming more common in analysis of drugs and their metabolites and HILIC has been recently used to analyse nicotine and its metabolites in urine [4]. The current paper demonstrates the advantages of HILIC when used in conjunction with organic solvent extraction of hair.

2. Materials and methods

2.1. Chemicals

Nicotine, cotinine, anabasine, ammonium acetate, sodium hydroxide, dioxan and m-chloroperoxybenzoate were obtained from Sigma Aldrich Dorset UK. $^{2}H_4$-nicotine (1 mg/ml in methanol) was obtained from CK gases, UK. HPLC grade methanol and acetonitrile were obtained from Fisher Scientific, UK. HPLC grade water was prepared in the lab using a Milli Q purification system.

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2.2. Dog hair sample collection

Hair was collected from dogs by clipping the neck region with the owners consent. Owners were asked to complete a questionnaire briefly outlining the age breed and sex of the dog and estimating the amount of ETS to which each dog was exposed. The hair was stored in sealed envelopes and stored to ensure that no further ETS exposure occurred after hair collection.

2.3. Extraction of dog hair

Samples of dog hair (*ca*. 30 mg) were washed with 2 ml of methanol by sonicating for 15 min at room temperature. The methanol was removed and the washings were retained for analysis. The hair was then treated with 1 M NaOH (1 ml containing 1 μg of $^2H_4$-nicotine) at 50 °C for 24 h. The sample was then loaded onto Strata X columns (30 mg, Phenomenex, UK), which had been prewashed with 1 ml of NaOH. The columns were then washed with 2 ml of water and then eluted with 1 ml of acetonitrile/water (95:5) containing 3.25 mM ammonium acetate. The sample was then injected into an Orbitrap Exactive mass spectrometer.

2.4. Calibration

Calibration curves were prepared by spiking 1 μg of $^2H_4$-nicotine into 1 ml amounts of 1 M NaOH and 0.05, 0.1, 0.2, 0.4, 0.8 and 1.6 μg amounts of nicotine. The samples were then treated in the same way as the hair samples. The precision of the analyses was assessed for the NaOH digestion and methanol extraction procedures by repeating analysis of the hair from dog 7 three times.

2.5. Mass spectrometric analysis

The extracts were analysed by using an Exactive Mass Spectrometer (ThermoElectron, UK) operated in positive ion ESI mode with a needle voltage of 4.5 kV, a heated capillary temperature of 275 °C, sheath gas flow of 50 arbitrary units and auxiliary gas flow of 17 arbitrary units. The instrument was operated at 50,000 resolutions and scanned from 75–1000 amu. Chromatography was carried out using a Dionex 3000 binary HPLC pump fitted with a ZICHILIC column (150 mm × 4.6 mm; 5 μm particle size, Hichrom, UK) a gradient was used with a flow rate of 0.5 ml/min. Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. The gradient was as follows: 60% B 0 min to 20% B at 20 min followed by re-equilibration for 10 min.

2.6. Synthesis of nicotine N-oxides

Nicotine (20 mg) in 1 ml of chloroform was cooled in ice and 20 mg of m-chloroperoxybenzoic acid in 2 ml of chloroform was then added a drop at a time to the nicotine solution shaking after each addition [19]. The mixture was left at room temperature for 2 h. The sample was used directly by diluting it to 10 ml with methanol. The stock solution was diluted 1–100 with mobile phase before analysis.

3. Results

3.1. Consideration of the extraction method

In order to distinguish between nicotine adhering to the surface of the hair and nicotine within the hair, hair samples are generally washed with organic solvent prior to being digested. The procedures used for the analysis of nicotine in hair are generally similar to those developed by Pichini et al. [11]. Digestion is most often carried out by gentle heating in 1 M NaOH following an initial wash

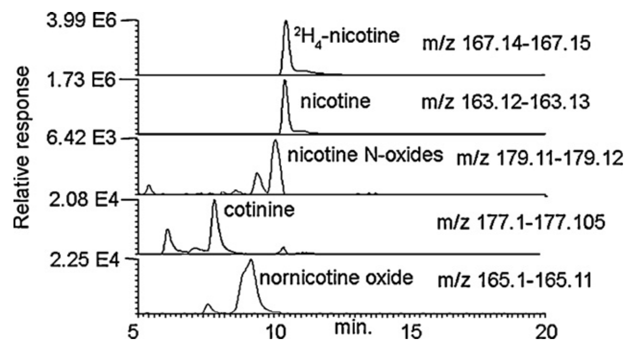

Fig. 1. Extracted ion traces showing $^2H_4$-nicotine, nicotine, cotinine and nicotine N-oxides extracted from the hair of dog 7 following 2 h digestion in 1 M NaOH followed by SPE.

with dichloromethane. However, there is really no definitive way to distinguish nicotine within the hair and that on the surface and although dichloromethane has been generally adopted on the basis of a relatively small study as the best solvent pre-treatment [11], it is not possible to be sure that it is optimal either in terms of removing all surface nicotine, particularly in the case of dogs where the hair character is very diverse. Dichloromethane would seem to be a less than ideal washing solvent since it is relatively non-polar, does not swell the hair [20] and is less likely to break up ion pair interactions between nicotine and acidic groups in the proteins in hair and hair melanin than methanol. It has been demonstrated in a previous study that non-swelling solvents are ineffective at completely removing cocaine applied to the surface of the hair since they do not remove drug, which has penetrated beyond the hair cuticles [21]. Such a degree of penetration is even more likely in the case of nicotine from smoking, which is applied to the hair in the vapour phase. Since animals can also absorb nicotine via licking the surface of their fur the total nicotine levels in the hair are also relevant.

3.2. NaOH digestion procedure for extracting nicotine from hair

Fig. 1 shows an extracted ion chromatogram of nicotine from NaOH digested dog hair along with extracted ion chromatograms for its metabolites cotinine and nicotine N-oxide, the mass accuracy produced by the Exactive was always <2 ppm and generally <1 ppm. This meant that there was little potential for interference from other components and Fig. 2 shows an extract from dog hair, which has not been exposed to nicotine. It can be seen that even with addition of the relatively large amount of (1 μg) of deuterated internal standard there is no trace of nicotine in the blank sample. Low levels of nicotine N-oxide and cotinine could also be observed in hair from dogs exposed to smoking (Fig. 1). In addition nornicotine and an oxide of nornicotine, possibly N-hydroxyl or ring hydroxylated, could be putatively identified in some samples particularly in the

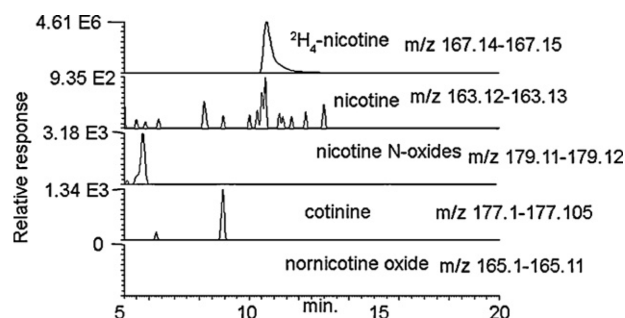

Fig. 2. Extracted ion traces of an extract from the hair of a dog not exposed to cigarette smoke spiked with 1 μg of $^2H_4$-nicotine.

Table 1
Concentrations of nicotine determined in dog hair in a 15 min methanol wash and in the NaOH digest following the methanol wash.

Dog no.	Category	Hydrolysate nicotine (ng/mg of hair)	Methanol wash (ng/mg of hair)
1	Smoker	0.0865	0.115
2	Smoker	0.109	0.278
3	Smoker	0.189	0.905
4	Smoker	0.150	0.812
5	Smoker	0.0136	0.0208
6	Non-smoker	0.000	0.0046
7	Smoker	9.03	9.80
8	Non-smoker	0.0000	0.00470
9	Non-smoker	3.08	0.101
10	Non-smoker	0.0281	0.0156
11	Non-smoker	0.000	0.000
12	Non-smoker	0.000	0.000
13	Non-smoker	0.000	0.000
14	Non-smoker	0.000	0.0
15	Smoker	0.489	2.77

dogs which had been exposed to high levels of ETS. Table 2 summarises the accurate mass data for the various nicotine metabolites in the dogs of smokers. Since there was no evidence for nicotine in the blank samples and the range of concentrations in the samples was wide the calibration curve was forced through 0 and the equation of the line was $y = 1.0238x$ with $R^2 = 0.996$. Table 1 shows the levels of nicotine extracted from dog hair following washing with methanol for 30 min and then digestion with NaOH. Varying amounts of nicotine are removed by the initial methanol wash perhaps reflecting the widely differing types of hair. The precision obtained for repeat analysis of dog 7 was ±3.2% ($n = 3$).

3.3. *Extraction of nicotine and its metabolites using a methanol wash*

Since there was some uncertainty regarding the amount of the nicotine removed during the solvent wash, the extraction of nicotine from dog hair with methanol with length of sonication was also studied for dog 7. An advantage of using HILIC was that the methanol extract could be injected into the chromatography system without any further preparation. Fig. 3 shows a plot of the release of nicotine from a sample of the dog hair over a 2 h sonication period in methanol. The nicotine released reached a plateau at around 1 h and at 30 min about half the nicotine has been released thus reflecting that which was observed for some of the samples shown in Table 1 although in some cases the rate of release appeared to be greater. The profile of the release of nicotine from the hair with sonication in methanol is very similar to that reported for the basic drug heroin [20]. The nicotine metabolites cotinine and nicotine N-oxide were also gradually released up to 1 h. Thus it is difficult to decide which is surface nicotine and which is nicotine from inside the hair particularly because the structure of hair is complex and to remove surface nicotine is important to ensure good contact between the extraction solvent and the hair. This is

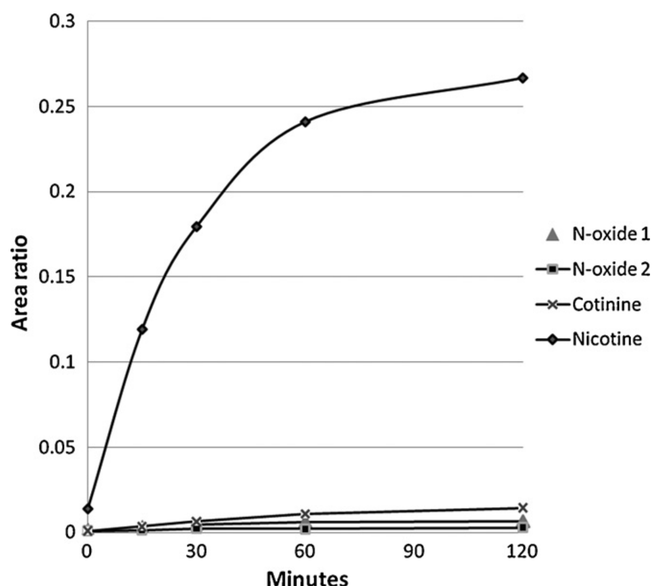

Fig. 3. Dissolution of nicotine and its metabolites from dog hair (dog 7) over 2 h of sonication in methanol.

why sonication is important since it produces efficient wetting of the hair. Fig. 4 shows the chromatogram for the methanol extract of the same dog hair as shown in Fig. 1 and it can be seen that in addition to nicotine the peaks for nicotine N-oxide and cotinine are larger than was observed for the NaOH extraction. The double peak for the N-oxide is probably due to the formation of diastereoisomers of the N-oxide since nicotine contains a chiral centre and the addition of oxygen to the nitrogen creates a second chiral centre on the nitrogen. This pattern was repeated for most of the extracts containing nicotine. Thus it may be that cotinine and nicotine N-oxide are unstable in the NaOH digestion solution to some extent. It is perhaps not surprising in the case of cotinine since it contains an amide type bond, which is unstable to strongly acidic or basic conditions. Cotinine has often been measured in hair [11,13,14,17,18] but its level of recovery has not always been rigorously assessed. Spiking of blank dog hair with 50 ng of cotinine and nicotine N-oxides resulted in good recovery when a simple methanol extraction was used. When the NaOH extraction process was used no nicotine oxide could be observed and the recovery of the cotinine was around 70% of that obtained by direct methanol extraction with a much noisier background. In some of the blank samples that were extracted with methanol there was apparently a peak for cotinine but on closer inspection this peak was due to the ^{13}C-isotope peak for the amino acid citrulline which eluted close to cotinine and had a very close mass match to it. In the case of nicotine oxide it is quite possible that it is not a metabolite of nicotine

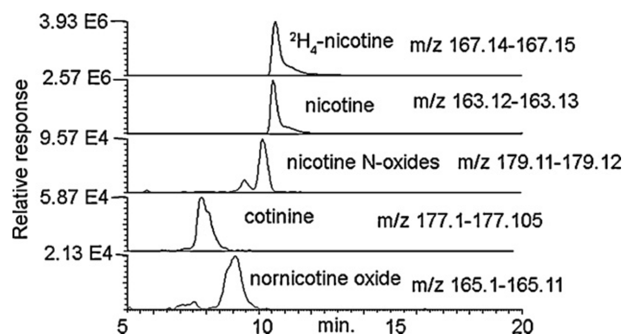

Fig. 4. Extracted ion traces showing $^2H_4$-nicotine, nicotine, cotinine and nicotine N-oxides extracted from the hair of dog 7 following 2 h sonication in methanol.

Table 2
Metabolites of nicotine observed in smoke exposed dog hair samples with mass deviations from their exact elemental composition.

Dog no.	Cotinine	Nicotine N-oxide	Nornicotine	Nornicotine N-oxide
1	−0.96 ppm	0.058 ppm	ND	ND
2	0.02 ppm	0.002 ppm	ND	−0.97 ppm
3	−0.11 ppm	−0.61 ppm	ND	−1.03 ppm
4	1.02 ppm	0.28 ppm	ND	−0.42 ppm
5	ND	ND	ND	ND
7	−0.11 ppm	−0.28 ppm	0.17 ppm	0.12 ppm
15	ND	−0.39 ppm	0.17 ppm	−0.85 ppm

Table 3
Concentrations of nicotine in dog hair determined in NaOH digests with no pre-washing of the hair and in methanol extracts prepared by 2 h of sonication.

Dog no.	Category	NaOH extraction (ng/mg)	MeOH extraction (ng/mg)
1	Smoker	0.229	0.230
2	Smoker	0.338	0.407
3	Smoker	1.30	0.982
4	Smoker	0.955	1.078
5	Smoker	0.054	0.0
6	Non-smoker	0.0	0.0
7	Smoker	11.5	17.8
8	Non-smoker	0.0	0.0
9	Non-smoker	3.50	0.456
10	Non-smoker	0.0	0.0
11	Non-smoker	0.0	0.0
12	Non-smoker	0.0	0.0
13	Non-smoker	0.0	0.0
14	Non-smoker	0.0	0.0
15	Smoker	3.280	2.986

but is rather due to air oxidation of nicotine adhering to the dog hair. This is made more likely by the fact that the preparation of a small amount of nicotine N-oxide by reaction of nicotine with m-chloroperoxybenzoate produced two peaks for the N-oxide in the same ratio as was observed in the methanol extracts from the dog hair suggesting chemical rather than enzymatic reaction. Table 3 shows the data obtained for samples prepared by methanol extraction and by NaOH digestion without any pre-wash. In most cases there is good agreement between the NaOH extraction process and the methanol extraction indicating that the methanol wash is removing all the nicotine in the hair. The RSD for the repeat determination of methanol in dog 7 using this method was $\pm 1.9\%$. The methanol extraction method in combination with HILIC analysis presents a very convenient method for determining nicotine and its metabolites and rather than pre-washing the hair it might be best to report nicotine levels after 15 min extraction and 2 h extraction. The method would also be compatible with a GC–MS analysis although perhaps a drop of methanolic ammonia might be added prior to the analysis in order to scavenge any anions which might pair with the nicotine. Although Exactive is not capable of MS–MS limits of quantification around 1 ng/ml were obtained for nicotine by using narrow range (0.01 amu) extracted ion chromatograms. The main inconsistency between the methanol extraction and the NaOH extraction in Table 3 is for dog 9, which is a poodle. This dog supposedly belongs to a non-smoker and this might mean that the smoke exposure is old and the nicotine is much more embedded within the hair or heterogeneously distributed within the hair or it may be that methanol extraction is less effective in extracting this type of dog hair.

4. Conclusion

In conclusion extraction of nicotine from dog hair using sonication in methanol in combination with hydrophilic interaction chromatography provides a straightforward method for determining nicotine absorption due to passive smoking in dogs. The problem still remains over whether the nicotine is on the fur or within the fur but in the case of companion animals nicotine will be orally absorbed anyway as a result of self grooming. The determination of levels of the metabolites of nicotine might provide a more definitive answer with regard to systemic absorption, however, it is quite possible that nicotine N-oxide and even cotinine might be formed either during the burning of tobacco or via atmospheric oxidation of nicotine adhering to the fur. Perhaps the most reliable indicator of metabolism observed in the current case is nornicotine N-oxide which is quite clear in a number of samples and which is more likely to be formed via enzyme action, however, nornicotine has been reported, along with cotinine and norcotinine, as a constituent of cigarette smoke [22]. The sample preparation steps in the current case are very simple avoiding potential losses of sample and metabolites, which can occur when digestion in NaOH followed by liquid–liquid extraction or solid phase extraction is used. Since organic solvents are weak solvents in HILIC mode the methanol extract can be injected directly onto the HILIC column without losing sample focusing and thus nicotine release with time can be readily assessed.

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References

[1] P. Jacob, L.S. Yu, M.J. Duan, L. Ramos, O. Yturralde, N.L. Benowitz, J. Chromatogr. B 879 (2011) 267.
[2] K. Rangiah, W.T. Hwang, C. Mesaros, A. Vachani, I.A. Blair, Bioanalysis 3 (2011) 745.
[3] T.R. Church, K.E. Anderson, C. Le, Y. Zhang, D.M. Kampa, A.R. Benoit, A.R. Yoder, S.G. Carmella, S.S. Hecht, Biomarkers 15 (2010) 345.
[4] F. Marclay, M. Saugy, J. Chromatogr. A 1217 (2010) 7528.
[5] H. Llaquet, S. Pichini, X. Joya, E. Papaseit, O. Vall, J. Klein, O. Garcia-Algar, Anal. Bioanal. Chem. 396 (2010) 379.
[6] N.L. Benowitz, K.M. Dains, D. Dempsey, B. Herrera, L. Yu, P. Jacob, Nicotine Tob. Res. 11 (2009) 954.
[7] C.A. Chadwick, B. Keevil, Measurement of cotinine in urine by liquid chromatography tandem mass spectrometry, Ann. Clin. Biochem. 44 (2007) 455.
[8] N. Jacob, C. Berny, J.C. Boyer, B. Capolaghi, G. de l'Homme, G. Desch, D. Garelik, N. Houdret, G. Le Moel, M. Moulsma, E. Plantin-Carrenard, Ann. Biol. Clin. 63 (2005) 397.
[9] H.S. Shin, J.G. Kim, Y.J. Shin, S.H. Jee, J. Chromatog. B 769 (2002) 177.
[10] R. Greaves, L. Trotter, S. Brennecke, E. Janus, Ann. Clin. Biochem. 38 (2001) 333.
[11] S. Pichini, I. Altieri, M. Pellegrini, R. Pacifici, P. Zuccaro, Forensic Sci. Int. 84 (1997) 243.
[12] G.N. Mahoney, W. Al-Delaimy, J. Chromatogr. B 753 (2001) 179.
[13] J.T. Bernert, J.R. Alexander, C.S. Sosnoff, J.E. McGuffey, J. Anal. Toxicol. 35 (2011) 1.
[14] H.J. Ryu, M.W. Seong, M.H. Nam, S.Y. Kong, D.H. Lee, Rapid Commun. Mass Spectrom. 20 (2006) 2781.
[15] T. Chetiyanukornkul, A. Toriba, R. Kizu, K. Kimura, K. Hayakawa, Biomed. Chromatogr. 18 (2004) 655.
[16] C.N. Man, S. Ismail, G.L. Harn, R. Lajis, R. Awang, J. Chromatogr. B 877 (2009) 339.
[17] J.S. Torano, H.J.M. van Kan, Analyst 128 (2003) 838.
[18] A.E. Kalkbrenner, R.W. Hornung, J.T. Bernert, S.K. Hammond, J.M. Braun, B.P. Lanphear, J. Expo. Sci. Environ. Epidemiol. 20 (2010) 615–624.
[19] O.M. Denk, G.G. Skellern, D.G. Watson, J. Pharm. Pharmacol. 54 (2002) 87.
[20] F. Musshoff, B. Madea, Anal. Bioanal. Chem. 388 (2007) 1475.
[21] V. Hill, T. Cairns, M. Schaffer, Forensic Sci. Int. 176 (2008) 23.
[22] L.Q. Sheng, L. Ding, H.W. Tong, G.P. Yong, X.Z. Zhou, S.M. Liu, Chromatographia 62 (2005) 63.