Analysis of Nicotine in Archaeological Skeletons from the Early Modern Age and from the Bronze Age

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In the last few years, identifications of drugs in archaeological human remains were reported several times but comments on the reliability of the data were often missing. To obtain valid data, in particular on nicotine residues in ancient bones, two skeletal series were analyzed and environmental influences on the results estimated in an exposure experiment. Bone samples from the early modern age (18th century, Goslar, Germany) and from the Bronze Age (Lichtenstein Cave, Germany) were analyzed for the tobacco alkaloid and its major metabolite, cotinine. In 22 out of 34 femur samples of the Goslar series, trace amounts of nicotine were found, but no cotinine, the major metabolite. Its finding would have proved the use of tobacco in the historic population, whereas the mere discovery of nicotine cannot discriminate between endogenous material and that resulting from a contamination during the sample treatment or museum storage. This points to the necessity of further pilot studies for long-term persistence, since it was not clarified whether insufficient accumulation or poor stability may have prevented the detection of cotinine. The magnitude of a possible nicotine deposition was estimated in a control exposure experiment. A Bronze Age bone sample that definitely contained no nicotine, as proven by pretests, was exposed to environmental tobacco smoke for six weeks and then analyzed. In one part, 11.6 ng g\(^{-1}\) nicotine were estimated and, in another, washed before the analysis, 35.9 ng g\(^{-1}\) were estimated. The higher amount of nicotine can be explained by the fact that tobacco smoke deposits were rinsed from the surface into the inner parts during the washing step. The results highlight the importance of additional analyses for metabolites; new patterns must be created that incorporate all now widespread drugs.

Keywords: Archaeological skeletons; Bronze Age; Contamination; Cotinine; Early modern age; Gas chromatography/mass spectrometry

INTRODUCTION

Investigating archaeological human remains at the molecular level is a method of obtaining knowledge of cultural behaviors of the past. In the last two decades, chemical analyses in particular of lipids and proteins, which have the highest preservation probabilities, have been well established (Eglinton and Logan, 1991; Evershed et al., 1999; Wischmann et al., 2000) and corresponding studies deal with the elucidation of historical food preferences (Morgan et al., 1984; Stott and Evershed, 1996) as well as investigating the decomposition of the bone collagen matrix (Balzer et al., 1997). In addition to interpreting these endogenous biomolecules, active substances from drugs have been analyzed as markers for their use in ancient societies. The presence of cocaine, nicotine and tetrahydrocannabinol (hashish) has been suggested for various tissues of Egyptian mummies (Parshe and Nerlich, 1995). Nicotine was found in 12 of 39 hair samples from adults and children from Egyptian Nubia (Balabanova et al., 1994). Báez et al. (2000) studied hair of Chilean mummies, using enzyme-linked immunosorbent assay (ELISA) and single ion monitoring gas chromatography/mass spectrometry (GC/MS) for traces of cocaine, opiates and cannabis, but revealed exclusively negative results in all 19 adult samples. Nicotine and its main metabolite cotinine was claimed to be detected in 25.9% of 81 prehistoric skeletons from South China (Balabanova et al., 1996). Detection of tobacco alkaloids was achieved by
means of radioimmunoassay (RIA). The antibody used registers nicotine and some of its metabolites; therefore, the results obtained by RIAs represent the total of the alkaloid and its derivatives. These findings concerning both nicotine and cotinine were then claimed to be confirmed by GC/MS, but the total number of specimens giving both compounds was neither revealed nor details of analytical tests given. However, a critical evaluation of data quality is important in this particular case, since the genus *Nicotiana* as a source of nicotine is indigenous to America and was not known in Eurasia before the 16th century, and analytical findings of tobacco residues in older samples had been considered impossible.

The objective of this study was to investigate both a Bronze Age (Lichtenstein Cave, Harz, Germany) and an early modern age (18th century, Goslar, Germany) skeleton series for nicotine, cotinine and other bioactive substances, which would reveal the use of drugs for medical or beneficial purposes. However, this paper is dedicated to problems related to nicotine and cotinine only. Basic aspects of the deposition of active substances from the environment and conditions of skeleton storage led us to conduct a controlling exposure experiment, as described below.

The Goslar series was characterized by a special feature: eight male individuals showed significant round gaps in their sets of teeth, which are presumed to be pipe gaps resulting from tobacco smoking (Fig. 1). In at least some of these particular specimens detectable remnants of nicotine were expected. The tests were complemented by analyzing the bone samples for the major metabolite, cotinine. The concentration of this compound in body fluids has been proven to be the best indicator to categorize smokers and non-smokers accurately (Jarvis et al., 1987). We conducted this by using GC/MS, which has a clear advantage over RIAs, since it permits discrimination between a compound and its structurally similar derivatives. The sensitivities of both techniques are of the same order of magnitude, but RIAs pose problems in analyzing excavated materials, since antibodies will very likely cross-react with other compounds coming from the complex soil organic matter and having characteristic structural features like those of the target compounds.

The bones of the Goslar series had been wrapped in polyethylene bags and were stored in a storeroom, allowing unhindered exchange with the surrounding air. For other, unconnected investigations, the bones had been exposed directly to the environment. These circumstances indicated the need to conduct a control experiment to investigate the effects of sample exposure to environmental tobacco smoke. For this, a femur was chosen from the Lichtenstein Cave that had been dated to the Bronze Age, when tobacco consumption was unknown outside America. This bone came from an almost inaccessible site, where it had survived during the last 3000 years protected by a gypsum coating (Flindt, 1996). It had been stored in a locked cold room immediately after its recovery and had been proven free of nicotine residues. A sample from this femur was exposed to sidestream smoke for six weeks; then the nicotine uptake in the inner layers of the compact bone was determined. Additionally, eight modern (control) femur samples, including three definitely from smokers, were analyzed to estimate the nicotine concentration ranges that could be expected in the bone matrix.

**RESULTS**

The Bronze Age and early modern age bone samples were first screened for active substances from drugs. Results were negative for both groups analyzed. Then using the selected ion monitoring GC/MS mode for nicotine and cotinine, trace amounts of nicotine were found in 22 of 34 femur samples of the Goslar series as well as in calculus deposits scraped from teeth of an individual showing a presumed
substance amounts from sample contamination and concerning maximum amounts to be expected from recent bones. In the first test, a piece of femur from a Bronze Age individual was exposed to an undefined amount of tobacco smoke in an office room for six weeks. The previous absence of nicotine in this sample had been proven before. After the exposure time, one half of the sample was prepared for chemical analysis, including washing the outer surfaces with bidistilled water, whereas the other half was not subjected to this procedure. Table II gives the nicotine concentrations in both sample parts remaining after removing layers of about 1.5 mm from all surfaces. In the central regions of the two differently-cleaned parts, nicotine content was determined: the inner region of the washed half had received three times as much nicotine.

Subsequently, to estimate the maximum expectable alkaloid concentrations in historical bone samples, eight recent femur samples were analyzed with the same method. Three of these bone samples definitely came from smokers. In seven specimens, nicotine was estimated in trace amounts of 8–12 ng·g⁻¹; only in one bone from a smoker was a higher amount of 17 ng·g⁻¹ determined. In these cases also, the major metabolite cotinine was not detected.

### DISCUSSION

Nicotine was found in most of the samples from the Goslar series. It was reliably identified by the respective single ion chromatograms and fits well with the findings that eight male individuals in this study population had teeth with presumed pipe gaps. Tobacco spread from the beginning of the 16th century and was consumed in large quantities in Europe in the following centuries. In Germany, tobacco smoking spread rapidly from the outbreak of the Thirty Years War, when it was introduced by soldiers from neighboring countries (Rien and Dorén, 1985).

Nicotine was identified in specimens from both sexes and in all age groups. Therefore, not only active, but also passive smoking must be considered as a possible cause for a deposit in bones. Other possibilities for an intake of nicotine must also be

### TABLE 1 Nicotine findings in femora from the Goslar series

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of samples analyzed</th>
<th>Positive nicotine findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female adults</td>
<td>12</td>
<td>7</td>
</tr>
<tr>
<td>Male adults</td>
<td>16</td>
<td>13</td>
</tr>
<tr>
<td>Adults, sex not identified</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Infants</td>
<td>5</td>
<td>2</td>
</tr>
</tbody>
</table>

### TABLE 2 Uptake of nicotine in Bronze Age bone during six weeks of exposure to environmental tobacco smoke (n = 4)

<table>
<thead>
<tr>
<th>Treatment of sample</th>
<th>Concentration of bone nicotine after exposure (ng·g⁻¹)</th>
<th>Standard deviation (ng·g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No washing of outer surface</td>
<td>11.6</td>
<td>2.0</td>
</tr>
<tr>
<td>After washing of outer surface</td>
<td>35.9</td>
<td>2.0</td>
</tr>
</tbody>
</table>
taken into account, and two reasons other than smoking are conceivable for these findings. First, nicotine could have been taken in from indigenous sources other than species of the genus *Nicotiana*, or an uptake could have taken place after the recovery of the respective skeletons. Though nicotine is detected as a side alkaloid in plants from the family Solanaceae as well as in other plants (e.g. sour cherry and tea leaves), the concentrations are not sufficient to explain these findings in ancient bones (Davis et al., 1991). However, it cannot be excluded that the early modern age skeletons from the Goslar series were contaminated post excavation during their archaeological processing and storage. It has to be considered that the bone samples may already have been investigated, under conditions that did not avoid the possible intake of volatile organic compounds from the surrounding air. So the noticeable round gaps in the dentitions of eight individuals from the Goslar series are not sufficient to prove tobacco use. They could stem from the smoking of other substances instead, such as cherry leaves, woodruff or nettle, which was not uncommon.

While analysis of the parent compound cannot distinguish between an endogenous origin or such a contamination, the analysis of metabolites could make this distinction. In forensic chemistry, the smoking of tobacco is regarded as an indicator of an inclination to drug abuse, and smoking is considered to be certain if higher than background concentrations of the major product in nicotine metabolism, cotinine, are measured in body fluids. Cotinine has a half-life 10 times that of its physiological precursor and is found in the body fluids of smokers. The compound has been shown to be superior to other biomarker compounds, though in a clinical study 7 out of 90 active smokers were classified as non-smokers on the basis of a cotinine analysis (Jarvis et al., 1987). However, the degradation product of nicotine was not found in any of the bone samples analyzed, independent of their origin. This lack of cotinine detection as well as basic considerations about the reliability of the experimental data led to additional control experiments to verify the results of nicotine analysis. The intensity of an environmental nicotine intake in the inner regions of a femur compact bone was estimated in an exposure experiment, and expectable amounts of nicotine in bone were estimated by investigating recent bone samples, including those from known smokers.

At the beginning of our experiments, we had expected that remains from the processing of the materials would be removed by washing the bones and removing their surface layers. The exposure experiment tested whether it was possible to remove all residues left by smoking during the processing of the ancient materials. To estimate the influence of nicotine contamination from environmental tobacco smoke, a nicotine-free Bronze Age bone sample from the Lichtenstein Cave was exposed to tobacco smoke in an office room for six weeks. After exposure, concentrations of 11.6 and 35.9 ng g⁻¹ were measured in the inner regions of the compact bone. Greater amounts in the bone pieces that had been previously washed can be explained in the following way: During the tobacco smoke exposure, nicotine was deposited on the accessible surface, and the alkaloid was rinsed into the inner regions by washing the material. Molecules from environmental tobacco smoke first interfere with substances on the materials' surface layers. These residues can be removed only by cutting off sufficiently thick layers. However, our experiment shows that remaining deposits affect the analysis of endogenous substances. Therefore, contact of samples with environmental tobacco smoke must be excluded in preparation for chemical analysis. This is shown when comparing the results from exposed bones without previous washing with those of recent samples. With 11.6 ng g⁻¹ nicotine, concentrations in the exposed samples are in the same order of magnitude as those in most of the recent samples and as those in the quantifiable archaeological bones. The results show that a clear distinction between findings from ancient smokers and non-smokers is not possible if the samples have been affected by environmental tobacco smoke over an undefined period after excavation. In particular, the recent bone samples, including those of known smokers, were analyzed for cotinine, with negative results. There are two possible reasons for this. Either the accumulated amounts of this compound are too small, or low stability precludes its detection.

Cotinine is characterized by a higher detection limit of 0.05 ng μL⁻¹, as compared with the unchanged alkaloid (0.02 ng μL⁻¹), and is thus less sensitively analyzed in our procedure, which includes an alkaline organic extraction followed by a GC/MS analysis. There are few references from the forensic sciences concerning the accumulation of cotinine in mammalian organs. From investigations of human hair, it is known that cotinine accumulates significantly less than its parent compound. In recent hair samples from smokers, cotinine is often detected only in small concentrations near its detection limit or even not at all (Haley and Hoffmann, 1985; Kintz, 1992). On the other hand, though the measurement of cotinine has been used widely as a biomarker of nicotine intake, it does not represent the total amount of nicotine. Benowitz et al. (1994) found that the major metabolite in urine accounts for less than 15% of the total systemic dose of nicotine, and they identified various presumably less stable metabolites. By means of their determination, a considerable interindividual variability in the pattern of metabolism was shown. A valid percentage
of the dose of nicotine metabolized to cotinine can therefore hardly be estimated, and so it is possible that remaining cotinine concentrations are generally under the detection limit in bones.

The possible persistence of cotinine in ancient bones can hardly be estimated, because there are no data available on its stability in dried materials. For untreated frozen brain and plasma, poor stabilities and significant losses within one week have been reported (Deutsch et al., 1992), whereas Bernert et al. (1997) did not find any evidence of instability of serum pools during storage at −60°C after more than four years. However, Balabanova et al. (1996) stated that both nicotine and cotinine could be identified in ancient bones, even though they presented neither quantitative results nor the number of specimens giving positive findings. This points to the necessity for further pilot studies of the long-term stability of compounds such as the ones discussed here.

In the case of such widespread volatile organic compounds, which can form a deposit and interfere with endogenous constituents, only the evidence of metabolized residues can be used to unmistakably determine which substances were used in ancient times. Therefore, new strategies must be created that incorporate all drugs and their persistent metabolites.

MATERIALS AND METHODS

Sampling and Solvent Extraction

Skeleton materials (femora) from the early modern age (18th century, Goslar, Germany) and from the Bronze Age (Lichtenstein Cave, Harz, Germany) were screened for alkaloids and, in particular, for nicotine and cotinine. The bones from the Goslar series had been washed at the excavation site, wrapped in polyethylene bags and kept in a storeroom at room temperature for about six years. The bones from the Lichtenstein Cave were stored in an airtight cold room at −18°C immediately after their recovery.

Prior to chemical analysis, the surfaces of the samples were removed, and then the pieces were ground to a fine powder using a ball mill. For each extraction, 1−1.5 g of sample was placed with 8.0 ml acetone/triethylamine (99:1, v:v) in a 15 ml Pyrex centrifuge tube, which was then tightly closed using a screw cap with a Teflon/silicone septum. The slurry was treated in a mechanical shaker for 15 h and then centrifuged (3000g, 20 min). A 6 ml aliquot of the supernatant was removed with a graduated pipette and concentrated in a vacuum concentrator. After adding 6.0 ml of acetone to the residue, mechanical agitation followed for 5 min; then the solutions were combined and again concentrated. This extraction step was repeated. After changing the solvent to toluene, N-ethylmornicotine (2.5 mg μl−1 in toluene) was added as an internal standard for quantitative analysis. Additionally, unfortified control samples were analyzed to estimate analytical interference.

The skeleton material used in the exposure experiment was a femur from the Bronze Age (Lichtenstein Cave). This test material was chosen because it dated from pre-Columbian times and therefore cannot contain endogenous nicotine. The bone survived in an inaccessible cave where the temperature did not exceed 8°C during the last 3000 years. Increased protection was given by a sintered gypsum layer that had formed on site. During recovery, all excavators were equipped with face masks and gloves to avoid contamination. Then the material was stored in a closed cold-storage chamber so that a contamination with tobacco alkaloids can be excluded. A 6.7 cm long half-cylinder was sawed from the diaphysis, and the inner surface as well as all cut ends were sealed with a layer of Parafilm and an additional layer of aluminum foil. The remaining uncovered outer surface was exposed to environmental tobacco smoke for six weeks. For analysis, the piece of bone was cut in half and the outer surface of one piece was washed with bidistilled water. For both pieces of bone, all surfaces were removed by sawing away layers of about 1.5 mm. Then the materials were ground to a fine powder and analyzed as described above. Additionally, bone samples from eight recent individuals were prepared in the same way and were also analyzed for nicotine. Three of these samples were from known smokers, whereas the use of tobacco products was not substantiated in the remaining cases. All solvents used were of at least analytical grade.

Gas Chromatography/Mass Spectrometry (GC/MS)

GC/MS analysis was performed on a Finnigan MAT model GCQ gas chromatographic/mass spectrometric system equipped with the Finnigan MAT GCQ data processing and instrument control software, Rev. 2.2. Separations were achieved on a fused-silica capillary column (15 m × 0.25 mm ID) coated with a 0.25 μm film of HP-5 (5% phenyl polysiloxane, Hewlett Packard). Injection port temperature was 250°C; transfer line temperature was 300°C. Injections were conducted in the splitless mode with the inlet liner purged 1.0 min after injection; the splitting ratio was then about 1:30. Helium carrier gas flow rate was 1.2 ml min−1. The temperature programme was: 1 min hold at 50°C, then 50−150°C at 20°C min−1 and 150−290°C at 30°C min−1, followed by a 10 min hold. The MS was operated in the SIM mode; detection was performed by using the m/z 84, 133 and 161 ions for nicotine, m/z 98, 147 and 176 ions for
cotinine and m/z 130, 161 and 175 ions for the internal standard N-ethylnornicotine. The electron ionization energy was 70 eV.

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References


