GC Profiles of Volatile Constituents from Human Urine Obtained by Closed Loop Stripping, Purge and Trap Technique and Simultaneous Steam Distillation-Extraction

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Different techniques like "closed loop stripping" [CLSA], "purge and trap" [PTI], and continous steam distillation extraction [SDE] were used to establish GC profiles of major histocompatibility complex-associated volatile constituents of human urine and statistically evaluated for reliability. Of the three methods investigated, PTI appeared to be superior for the detection of very volatile substances, whereas SDE was the most efficient one with respect to yield. A number of short to medium-chain ketones, 4-hydroxy-3-methoxy-styrene, menthol and nicotine were identified in preliminary analyses.

Introduction

Volatile constituents of human urine represent a valuable source of information of biochemical processes inside the organism and at the same time offer a channel for chemical information. Among other things, in urine indications for diseases and metabolic interferences are found. It also serves as a matrix for pharmaceutical metabolites and can be considered as a base of an olfactoric communication system, in terms of sex and kin recognition and as a carrier of the individual odor as well.

The chemosensory identity of humans is determined partly by polymorphic genes of the *major histocompatibility complex MHC* (= *human leucocyte antigene HLA*) (Ferstl *et al.*, 1992; Bestmann *et al.*, 1995). In the course of a programme to explore the chemosensory identity of humans and characteristics of their individual specific odor, the recording of odor profiles from urine and the identification of some of the possibly MHC-associated constituents were achieved. The aim of the present work was the elaboration and evaluation of different techniques for the analysis of volatile urine components. The reliability and accuracy of the urine profiles thus obtained was to be investigated as a function of time, external, and intrinsically uncontrollable conditions.

For this study we generally used three different techniques. Both the "closed loop stripping analysis" [CLSA] (Boland *et al.*, 1984) and the "purge and trap injection" [PTI] (Chrompack, Germany) technique are dynamic head space methods, which principally differ in the way of enriching the volatiles. With the CLSA technique, as it was used for the analysis of insect pheromones (Bestmann, Erler and Vostrowsky, 1988; Vostrowsky, Erler and Bestmann, 1989), the volatiles are adsorbed on a charcoal filter, whereas with the PTI technique they are collected in a cool trap. Simultaneous steam distillation and extraction [SDE], a standardized technique for the preparation of essential oils from plants (Schultz *et al.*, 1977) was used as the third method for isolating urine odorants.

Materials and Methods

Closed loop stripping analysis (CLSA)

A constant air stream (1.5 l/min) is continuously flushed through 20 ml urine samples in a closed
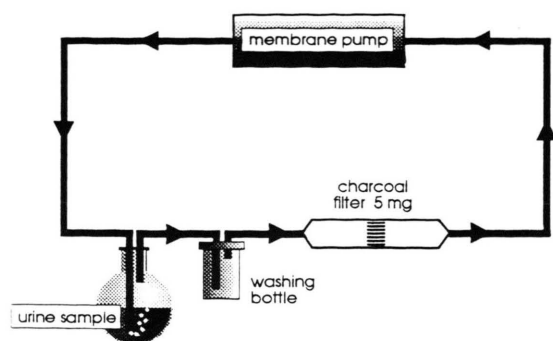
Fig. 1. Closed loop stripping [CLSA] system for urine volatiles analyses.

loop for 5 h by means of a membrane pump (Fig. 1). The volatile fragrance compounds are carried along with the air and then adsorbed onto an activated charcoal filter (5 mg, Fisons Scientific Equipment, Mainz-Kastel, Germany). Subsequently, the filter cartridge is replaced, the adsorbates are desorbed by repeated extractions with 20 μl portions of CS_2 and the combined CS_2 solutions finally analyzed by GC.

Purge and trap injection (PTI)

To determine the volatile constituents of urine the TCT/PTI injector CP 4001 (Chrompack GmbH, Frankfurt, Germany), attached to a CP 9001 gas chromatograph, was used. 10 ml/min of He were flushed through a 20 ml urine sample over a period of 30 min, ridded of water by cooling
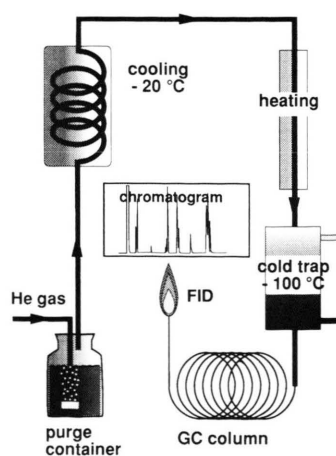
to -20°C, and the volatile components finally trapped at -100°C in a CP-Sil 5CB wide bore (Chrompack) precolumn (Fig. 2). After 30 min the substances were thermodesorbed by heating to 200°C and the volatiles investigated by GC.

Simultaneous steam distillation and extraction (SDE)

20 ml of urine were diluted with 1 l of H_2O and subjected to steam distillation in a glass apparatus according to Nickerson and Likens (1966) over a period of 5 h (Fig. 3). The steam distillate was simultaneously extracted with 5 ml pentane and the resulting pentane solution dried over $MgSO_4$, filtered and analyzed by GC and GC-MS.

GC analyses

CLSA and SDE: Hewlett Packard 5890A gas chromatograph, 25 m FSCC SE 52, 0.25 mm x 0.28 μ, temperature program 4 min 50°C, 50–260°C, 5°/min, hold; injector 220°C, det. FID 260°C, 1 ml N_2/min. –

PTI: Chrompack CP9001 chromatograph, 25 m FSCC SE52, 0.25 mm x 0.28 μ, temp. progr. 4 min 40°C, 40–200°C, 10°/min, hold; Inj. 220°C, det. FID 260°C, 0.5 ml He/min.

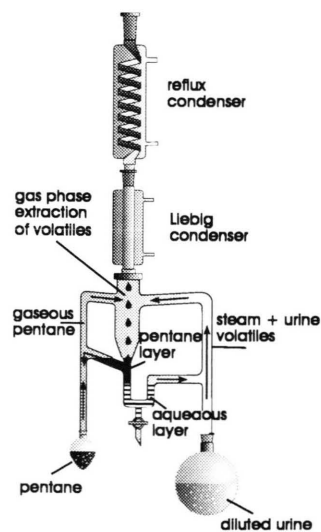Fig. 2. Purge and trap injection [PTI] technique for urine analysis.

Fig. 3. Simultaneous steam distillation and extraction [SDE] according to Nickerson and Likens (1966).

GC-MS analyses

GC-MS combination Finnigan MAT90 coupled to a Varian GC6000 and data system, 25 m FSCC SE52, 4 min 50°C, 50–260°C, 5°/min, hold; Inj. 220°C, transfer line 280°C, 1 ml He/min, 70 eV EI spectra, 1 s/scan.

Results and Discussion

To prove the reproducibility of the CLSA technique for this special purpose, four different urine samples were selected, each divided into two equal parts, and both of these fractions stripped under the same conditions and consequently analyzed by GC. The chromatograms thus obtained were statistically evaluated; each of the sample pairs was correlated by regression analysis, using the program SPSS 5 (Windows), showing eventually an excellent congruency (correlation factors 0.995–0.998).

However, to study the individual urine profile of a particular test person, it is important to consider its temporary differences, caused by different nourishment, state of health, physiological status, *etc.* Thus, from a (female) proband urine samples were collected in two weeks and six weeks intervals and analyzed by CLSA. The chromatograms of the different samples corresponded relatively well with respect to their peak composition and differed in peak intensities only.

This led to the assumption that samples taken at different times from the same individual test person could be combined ("*pooled*") and the corresponding *pooled* samples treated like the *authentic* samples. To proof this, three different urine samples [P1, P2 and P3] taken at different times from a (male) test person were collected and two or three of these *authentic* samples mixed together [pool P1/2, pool P1/3, pool P2/3 and pool P1/2/3, respectively]. The GC profiles of the *pooled* samples were recorded by CLSA technique and compared with the original samples, their conformity is given in Table I.

Comparing the three methods CLSA, PTI and SDE, each one has its advantages as well as disadvantages. Choosing a method may be a matter of what the objective of the respective investigation is and which instrumental equipment available. CLSA offers the advantage of a manyfold and optionally long lasting gas extraction and does not

Table I. Statistical correlation between the CLSA-GC profiles of *pooled* and *authentic* samples. System conditions: 20 ml urine, flow rate 1.5 l/min, 5 mg charcoal, purge time 5 h, solvent CS_2.

Pooled samples compared with *authentic* samples	Correlation coefficient
P1 + P2 *vs.* P1/2	0.9964
P1 + P3 *vs.* P1/3	0.9854
P2 + P3 *vs.* P2/3	0.9978
P1 + P2 + P3 *vs.* P1/2/3	0.9988

P1, P2, P3 *authentic* urine samples samples, P1/2, P2/3, P1/3 *pooled* samples from P1 and P2 ......, P1/2/3 *pooled* from urins sample 1 and 2 and 3.

exert any temperature stress on the probe. The charcoal extraction volume can be minimized to a few microliters and the samples obtained may directly be subjected to gas chromatography, thus avoiding additional dilution and contamination from solvents. It is a particularly gentle technique, and although the overall yield of volatiles is rather low, it gives rise to proper odor profile recordings (Fig. 4a). The distribution of substances is sufficiently uniform up to C_{20}-compounds, higher boiling and less volatile constituents are detected only after longer extraction periods. As a consequence, a stripping time of 3–5h appears to be optimum. Variation of the stripping flow is not realistic, since it is determined by the gas restriction of the microfilters and the membrane pump used. Due to its excellent solving properties and its low boiling point CS_2 is recommended as the extracting solvent, a conclusion drawn from many CLSA studies. Conflicting superposition of the solvent peak with those of early eluting substances can be avoided by using high boiling solvents. A major disadvantage of CLSA is that specific adsorption of some substances on the relatively large surface area of the system can arise, thus resulting in a suppression of certain compound classes, e.g. polar alcohols, *etc.* Due to this fact, recovery experiments with long chain C_{12} to C_{18} alcohols, acetates and carboxylic esters were performed resulting in total recovery rates of 50–70%; the percent recovery of adsorbed chemicals from the charcoal was 98% (Bestmann, Erler and Vostrowsky, 1988).

The PTI technique seems to be superior for the detection of extremely volatile substances, as it can be seen for the case of acetone in the chromatogram in Fig. 4b. No superposition of a solvent
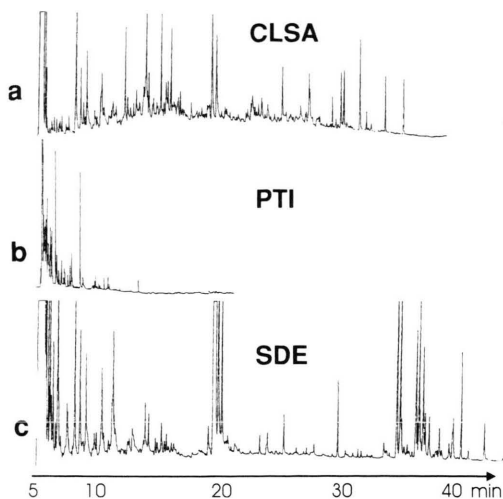
Fig. 4. GC profiles of urine volatiles provided by **a** CLSA analysis, **b** "purge and trap injection" [PTI] and **c** "simultaneous steam distillation and extraction" [SDE].

peak occurs, any dilution and introduction of impurities by the solvent is excluded, and no additional transfer steps are necessary. The yield is certainly the lowest one of the three methods and insufficient for a comprehensive subsequent spectroscopical analysis, yet enough for a qualitative to semiquantitative profile analysis. Imploying inert gases like N_2 or He for the extraction step, the entire process is the gentlest and most careful one of the three under comparison. Compared with the conventional static headspace technique, GC with TPI injection provides a completely integrated system, thus reducing the need for sample handling and transfer processing. The most serious disadvantage is that only components up to a molecular size of about C_{12} can be detected in urine.

Steam distillation, especially in combination with a simultaneous solvent extraction, is the clas-

sical method of isolating aroma and fragrance volatiles and it is mainly used for the preparation of essential oils from plants and fruits. We found it highly suitable for the detection of urine constituents. From the three methods, it gave the highest yields (Fig. 4c), particularly high boiling fractions could be obtained upon prolonged distillation time. This brings up the major disadvantage of the method, i.e. the heating to 100°C, which might give rise to the formation of artefacts and degradation products, thus falsifying the GC profiles. This has to be expected especially with thermolabile and biological material. Nevertheless, because of its excellent yield, the SDE technique appears to be the most suitable one, if subsequent spectroscopic identification with GC-MS and GC-FTIR is necessary. So for a swift acquisition of GC profiles we would eventually recommend the PTI technique as the method of choice. If a more extensive analysis is requested we would propose to first correlate the PTI or CLSA chromatogram, respectively, with the one obtained from SDE distillation products. These SDE samples might be used for subsequent spectroscopic investigations as well.

Even with the aim of merely establishing GC profiles, preliminary GC and GC-MS analyses were carried out to identifiy some of the more pronounced constituents. Among others, acetone, 2-pentanone, 4-heptanone, 2-heptanone, 2-octanone, 2-nonanone, 4-hydroxy-3-methoxystyrene, menthol and nicotine, all of them known urine constituents, were characterized by mass spectrometry and retention behavior.

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