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Note

Quantitative analysis of aromatic amines in human urine by gas chromatography—mass spectrometry—selected-ion monitoring

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Epidemiological studies in several countries on workers in the rubber industry point to a high incidence of various types of tumours [1]. Bladder cancer has been associated with exposure to aromatic amines and in particular to 2-naphthylamine. Other types of cancer (lung, stomach, and leukaemia) occurring in excess among rubber workers have not been associated with exposure to specific compounds; but this is explained by the fact that the industrial processes involved use a wide variety of chemicals whose toxicological properties are unknown. Since it is unlikely that toxicological information about these compounds will be available in the near future, a practical approach would be to reduce the exposure of these workers and to measure it.

N-Phenyl-2-naphthylamine (PBNA), N-isopropyl-N′-phenyl-*p*-phenylenediamine (IPPD) and N-(1,3-dimethylbutyl)-N′-phenyl-*p*-phenylenediamine (6PPD) are widely used as antioxidants and antiozonants (generally at levels of 1—2%) in the rubber industry [2] and workers may be exposed to them.

Contrasting results have been reported on the carcinogenicity of PBNA in laboratory animals: oral [3—5], subcutaneous [3,4,6] and aerosol [7] administration to mice resulted in a significantly higher incidence of different tumours; PBNA intragastrically administered for life did not cause neoplastic growth in Sprague—Dawley rats [8] or in Syrian golden hamsters [9]; no bladder tumours were observed in dogs fed orally with PBNA [10]. There are no data in the literature on IPPD and 6PPD carcinogenicity. Hence, it is important to quantify the possible exposure to these chemicals in order to avoid or

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reduce contact with those chemicals for which the toxicological hazard is not yet clear.

One of the simplest and most easily applicable systems is to measure urinary excretion of aromatic amines in exposed individuals. A number of methods have been reported for the determination of aromatic amines in human urine by gas chromatography (GC) [11—13], high-performance liquid chromatography (HPLC) [14] and mass spectrometry (MS) [15]. However, to our knowledge there are no methods to determine PBNA in human urine and only one method reports separation and quantitation of IPPD and 6PPD in this biological fluid [16]; in that method aromatic amines were measured by HPLC with a sensitivity of 50 μg/l.

In order to study the exposure of workers to aromatic amines normally used in the rubber industry, we devised a reliable, specific and highly sensitive analytical method for the determination of trace levels of PBNA, IPPD and 6PPD in human urine using combined gas chromatography—mass spectrometry—selected-ion monitoring (GC—MS—SIM). The application to biological monitoring of rubber industry workers is reported.

EXPERIMENTAL

Chemicals and reagents

PBNA was provided by E. Merck (Darmstadt, F.R.G.); IPPD and 6PPD were obtained from Bayer (Leverkusen, F.R.G.). Reagent-grade sodium hydroxide and ammonium hydroxide were from Farmitalia Carlo Erba (Milan, Italy). Trifluoroacetic anhydride (TFAA) of reagent grade was purchased from Janssen (Beerse, Belgium). Silylation-grade pyridine was obtained from Pierce (Rotterdam, The Netherlands). *n*-Hexane of pesticide analytical grade was supplied by Riedel-de Haen (Hannover, F.R.G.).

Extraction and derivatization of biological samples

In a 40-ml centrifuge tube, 20 ml of human urine were adjusted to pH 11 using 10 *M* sodium hydroxide. The compounds were extracted three times with 10 ml of *n*-hexane by mixing for 10 min on a horizontal reciprocating shaker. After centrifuging at 2000 *g* for 5 min the combined organic extracts were concentrated to 1 ml in a rotating evaporator, transferred to a 10-ml reaction tube and evaporated under vacuum to dryness. The residue was dissolved in 500 μl of *n*-hexane and reacted with 20 μl of TFAA and 20 μl of pyridine at 60°C for 30 min using a thermostated bath. After cooling, 1.5 ml of *n*-hexane and 1 ml of 5% aqueous ammonia solution were added to the sample and the mixture was shaken on a vortex mixer for 1 min. The organic layer was transferred to another 10-ml conical tube and evaporated to dryness under an air stream. The residue was dissolved in 50 μl of *n*-hexane; 4 μl were analysed by GC—MS.

Instrumentation

An LKB 2091 gas chromatograph, low-resolution mass spectrometer, equipped with an LKB 2130 computer data processing system, was used. A 2 m × 2 mm I.D. silanized glass column packed with 3% OV-1 on Gas Chrom

Q (80—100 mesh) (Supelco, Bellefonte, PA, U.S.A.) was used. Column and injector port temperatures were 265°C and 290°C, respectively. Helium was used as carrier gas and the column head pressure was 2.5 bars. The mass spectrometer was operated in the electron-impact (EI) mode with the following conditions: electron energy 70 eV, trap current 50 μA, ion source temperature 250°C. SIM chromatograms were obtained by monitoring the ions at *m/z* 315, 376/418, 376/460 for PBNA, IPPD, 6PPD, respectively.

Calculations

Since early attempts to synthesize deuterium-labelled aromatic amines to be used as internal standard failed, all measurements were made by comparison of the areas of unknown samples with areas of reference mixtures containing known amounts of PBNA, IPPD and 6PPD. Reference mixtures were injected every three samples.

Calibration curves were constructed by derivatizing known amounts (0.1, 0.2, 0.4, 1, 2 ng) of aromatic amines as described above. Extraction and clean-up efficiency were evaluated by adding known amounts (2—80 ng) of standards PBNA, IPPD and 6PPD to 20 ml of blank urine samples which were then processed as described above. For routine analysis, blank and spiked urine samples were processed together with each batch of samples.

Human studies

Urine specimens (200 ml) were collected from 21 workers in a rubber factory, who were likely to have been exposed to aromatic amines by inhalation or skin contact, and kept frozen (−20°C) until analysed.

RESULTS AND DISCUSSION

The mass spectra of the three underivatized amines gave intense peaks which could be used for SIM detection at *m/z* 219 $(M)^+$ for PBNA, *m/z* 211 $(M-15)^+$ and 226 $(M)^+$ for IPPD, *m/z* 211 $(M-57)^+$ and 268 $(M)^+$ for 6PPD as shown in Fig. 1. However, the attempt to use these for SIM analyses failed because aromatic amines gave broad GC peaks because of absorption on the GC columns and interference from urine components.

To improve the GC behaviour of the compounds to be analysed derivatization was decided upon. Trifluoroacetyl (TFA) derivatives were found to be suitable for GC—MS analysis. Derivatization was quantitative: PBNA gave rise to a monoderivative while IPPD and 6PPD reacted with two TFAA molecules. All the derivatives produced sharp and symmetrical GC peaks. The mass spectra of the three derivatives are shown in Fig. 2. The retention times and masses of the most abundant ions in each spectrum are listed in Table I together with their abundance ratios relative to the most intense ion.

Multiple-ion detection was performed by monitoring the ion intensities at *m/z* 315 for PBNA, at *m/z* 376 and 418 for IPPD, at *m/z* 376 and 460 for 6PPD. Typical SIM chromatograms are shown in Fig. 3; there were no interfering peaks from amine-free urine.

Peak areas as a function of the amounts of amines injected were linear in the range 0.1—2 ng as shown by linear regression analysis correlation coefficients of

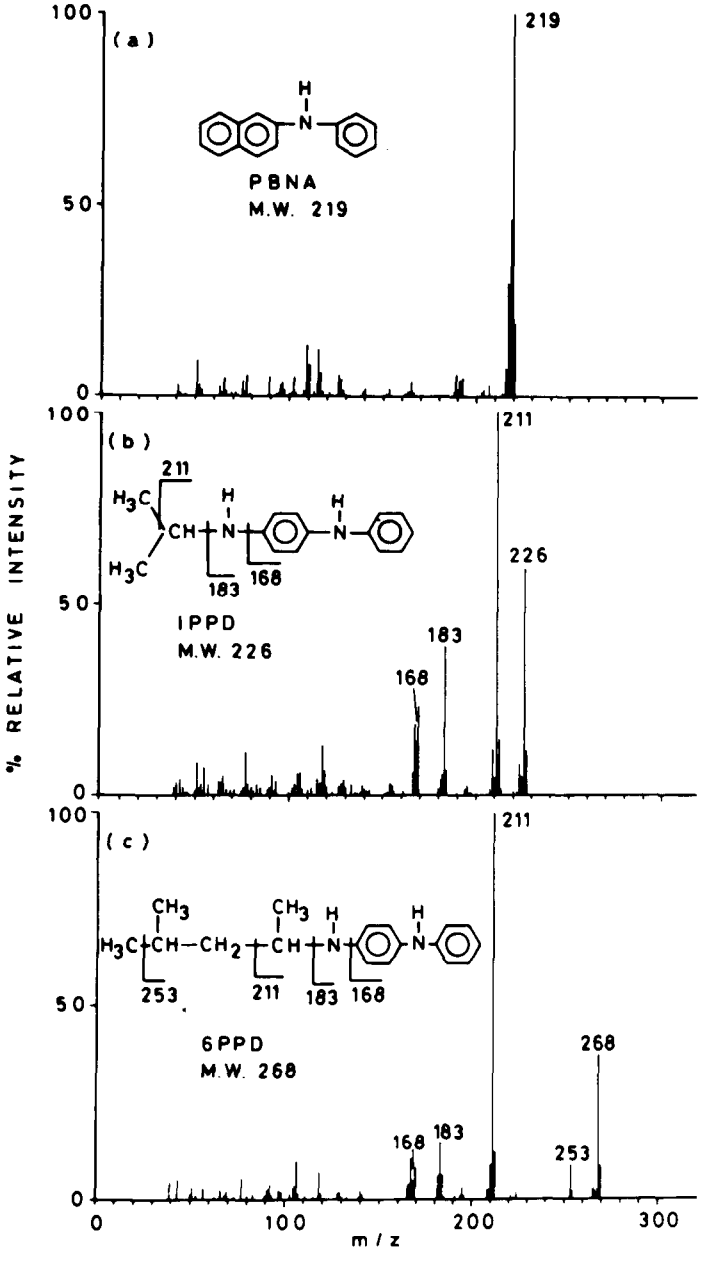

Fig. 1. Mass spectra of PBNA (a), IPPD (b) and 6PPD (c).

0.9999, 0.9991 and 0.9989 for PBNA, IPPD (*m/z* 418) and 6PPD (*m/z* 376), respectively.

The minimum detectable amount of aromatic amines was calculated to be 0.1 μg/l of urine with a signal-to-noise ratio $>3:1$. Mean recovery values and standard errors, in the range of concentrations in urine from 0.1 ng/ml to 4 ng/ml, were 86±3%, 77±5% and 70±5% for PBNA, IPPD and 6PPD, respectively.

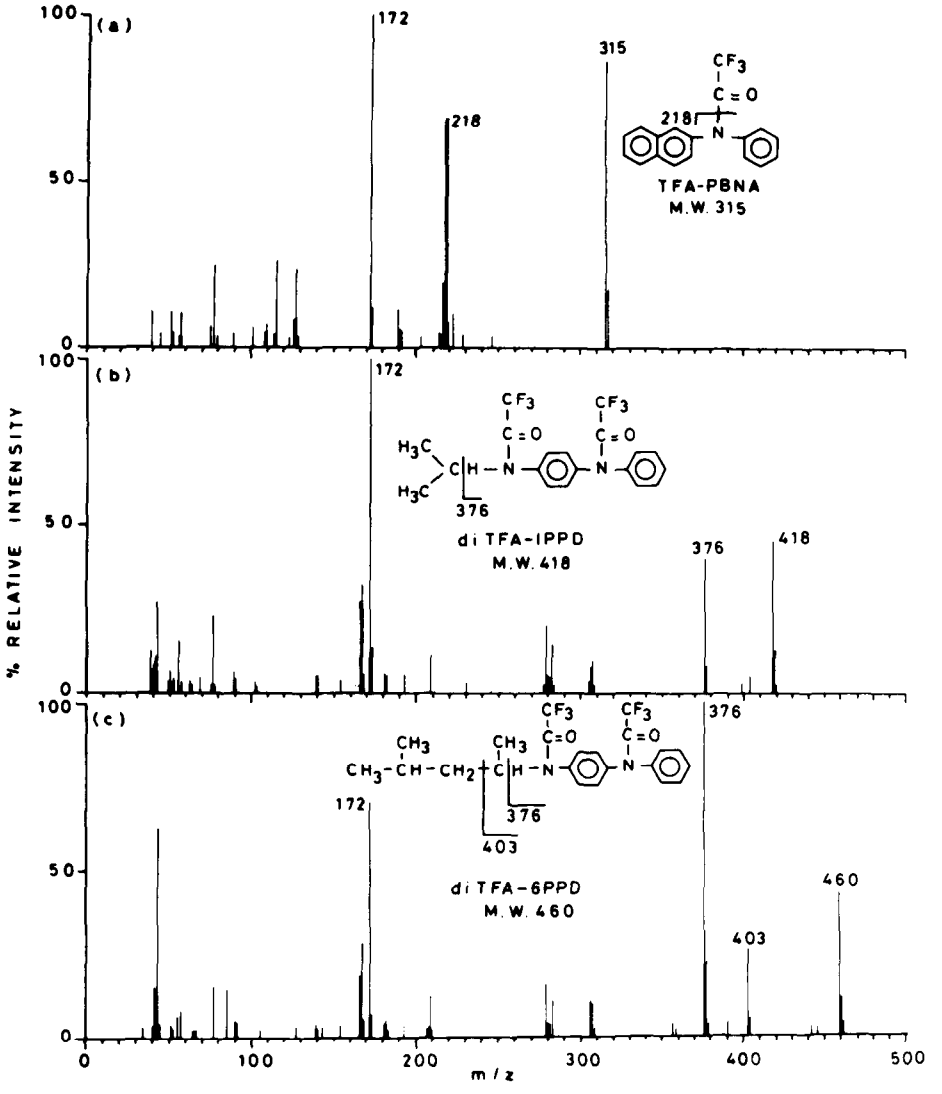

Fig. 2. Mass spectra of TFA derivatives of PBNA (a), IPPD (b) and 6PPD (c).

TABLE I

RETENTION TIME OF TFA AROMATIC AMINE DERIVATIVES, MASSES AND ABUNDANCE RATIOS OF CHARACTERISTIC IONS

Compound	Retention time (min)	Ions, *m/z* (abundance, %)
diTFA-IPPD	1.6	172(100), 418(46), 376(40), 167(32)
TFA-PBNA	2.0	172(100), 315(86), 217(69), 218(68)
diTFA-6PPD	2.4	376(100), 172(70), 43(63), 460(42)

The SIM method was applied to the determination of urine levels of PBNA, IPPD and 6PPD in a group of 21 workers in the rubber industry. Four urine samples were collected during a working week from each worker. About 70%

of the urine samples analysed contained at least one of the aromatic amines studied. As reported in Fig. 4, among the positive samples more than three-quarters presented values ranging from 0.1 to 0.3 μg/l; the highest value detected was 1.3 μg/l and there was no correlation between the presence of the three amines in the same subject. Air samples collected in the work area showed concentrations of 0.01—1 μg/m^3 for each of the three amines.

In conclusion, the GC—MS—SIM assay described is convenient for detecting and quantifying PBNA, IPPD and 6PPD in human urine with high sensitivity (0.1 μg/l) and lack of interference.

Nevertheless, the information obtained by application of this method is useful at the present time only for qualitative evaluation of workers' exposure to aromatic amines. Studies on laboratory animals [17—19] and humans

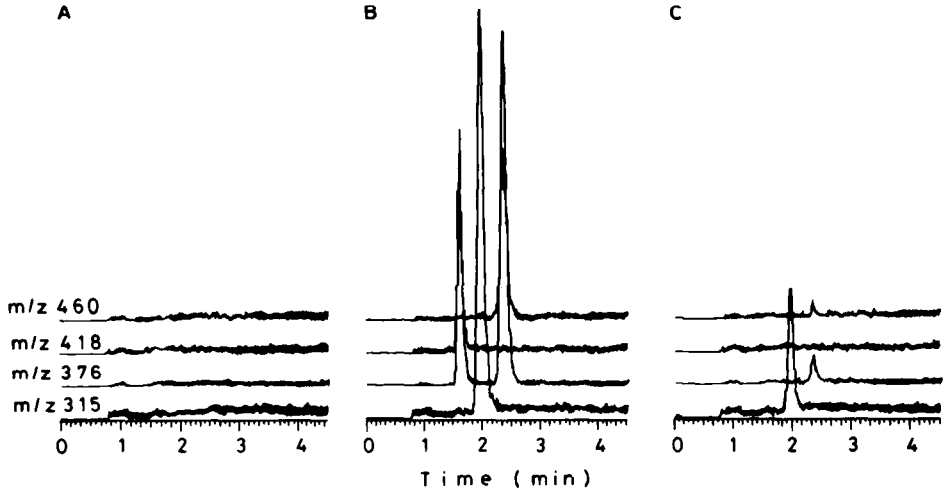

Fig. 3. Mass fragmentograms of selected ions of PBNA (*m/z* 315), IPPD (*m/z* 376, 418) and 6PPD (*m/z* 376, 460). (A) Blank urine sample; (B) standard aromatic amine mixture (2 ng each); (C) urine sample.

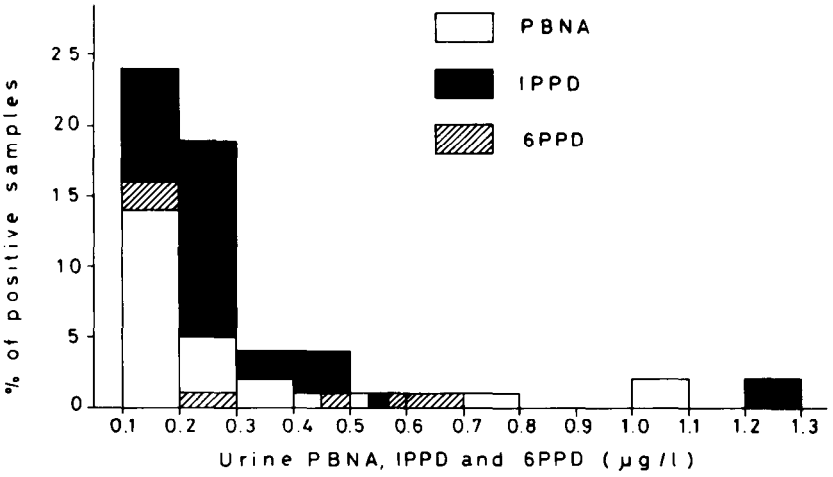

Fig. 4. Distribution of PBNA-, IPPD- and 6PPD-positive samples as a function of urine concentration.

[20,21] have shown that PBNA [18—21] and IPPD [17] are rapidly metabolized; so, in fact, determination of their urinary excretion may not give the best quantitative indicator of exposure. Experiments are in progress to determine whether these compounds are metabolized to products that may better reflect human contamination.

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