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Rapid high-performance liquid chromatographic determination of urinary N-(1-methylethyl)-N′-phenyl-1,4-benzenediamine in workers exposed to aromatic amines

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ABSTRACT

A procedure has been developed for determining N-(1-methylethyl)-N′-phenyl-1,4-benzenediamine in urine by using high-performance liquid chromatography. The method uses chloroform extraction for partial clean-up of the urine sample. The separation is carried out on a reversed-phase column using 65 mmol/l aqueous ammonium acetate in acetonitrile (30:70, v/v) as the mobile phase. The column effluent is monitored at 290 nm with an ultraviolet detector. The analyte is separated from other normal urine constituents in less than 4 min. Peak height and concentration are linearly related. Coefficients of variation assessed for within-day reproducibility were 5.9 and 3.7% at concentrations of 22.3 and 92.1 μg/l, respectively. The mean analytical recovery from urine samples spiked with known amounts of amine was 89.7 ± 6.8%. The request of only a small volume of urine and the simple pre-treatment procedure makes it suitable for the routine monitoring of the exposure of rubber vulcanization workers to aromatic amines.

INTRODUCTION

Aromatic amines derived from *p*-phenylenediamine are widely used as anti-oxidants in rubber vulcanization. N-(1-Methylethyl)-N′-phenyl-1,4-benzenediamine (IPPD) is the most commonly used rubber anti-oxidant in Italy. Control of the working environment and biological monitoring for rubber anti-oxidants is mandatory because IPPD is a suspected carcinogen [1] and a potent skin sensitizer [2]. The concentration of IPPD in the urine of workers is an index of exposure to this compound in their workplace [2]. A method has been described for the determination of IPPD in urine based on the solid-phase extraction of the amine from the urine matrix and subsequent analysis by high-performance liquid chromatography (HPLC) of the redissolved extract. As a result of the need for large sample volumes and the complex manipulation required, this procedure is not applied in large-scale routine analyses [3]. This paper describes a simple and rapid HPLC technique for the accurate determination of IPPD in human urine samples. The method is based on a liquid–liquid extraction, followed by back-extraction and HPLC assay.

EXPERIMENTAL

Materials

The IPPD standard was supplied by Bayer (Germany). Chromatography-grade acetonitrile and other reagents were purchased from Merck (Germany). A 2000 mg/l solution of IPPD in acetonitrile was prepared monthly and stored frozen in the dark. The concentrated standard was diluted daily with water to produce 2.0 and 20.0 mg/l standard

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solutions. Non-exposed urine samples supplemented with 20 and 200 μg/l IPPD were used as working standards.

Urine samples

Urine samples were collected at the workplace after the last shift from 62 rubber vulcanization workers on Fridays for one work-month and from 15 non-exposed control subjects. Dark-glass containers were used and the urine samples, stored at 4°C, were analysed within 24 h.

Extraction procedure

A 5.0-ml aliquot of urine was added to 200 mg of sodium chloride and 1.5 ml of chloroform in a PTFE-stoppered glass tube; the mixture was extracted by shaking vigorously for 15 min with an automatic stirrer. The tube was centrifuged for 5 min at 1000 *g* and, after the separation of the two phases, the organic layer was transferred into a glass test vial. The chloroform was evaporated to dryness in a water-bath at 40°C under a weak oxygen-free stream of helium. The residue was dissolved in 0.5 ml of acetonitrile and 20 μl of the resultant solution were injected into the chromatograph. Samples and standards must be handled with extreme care to avoid serious contamination from the IPPD present. The use of very clean glass vials and disposable materials for sampling and processing the urine samples is necessary.

Apparatus and chromatographic conditions

A Perkin-Elmer 620 quaternary pump liquid chromatograph equipped with an octadecylsilyl LiChrospher 100 RP-18 (10 μm) column (250 × 4 mm I.D.) (Merck), connected to a Perisorb RP-18 (30–40 μm) (Merck) guard column (30 × 4 mm I.D.) and a Perkin-Elmer 235 diode-array UV–visible detector set at 290 nm were used for the chromatographic analysis. The detector was operated at 0.02 absorbance units full scale. A Perkin-Elmer ISS-100 auto-sampling injector was used. The chromatographic procedure and data management were controlled by a PC AX2 computer (Epson, Japan) equipped with expert system software (Analyst, Perkin-Elmer, UK); the chromatograms were printed out with an Epson FX-850 printer.

Isocratic elution was performed at a flow-rate of 2.0 ml/min, with a acetonitrile–65 mmol/l ammonium acetate aqueous solution (70:30, v/v), adjusted to pH 6.5 with ammonium hydroxide solution, as the mobile phase.

RESULTS

Separation of IPPD in urine

The extraction of urine with chloroform in the presence of sodium chloride, followed by centrifugation, allowed the optimum separation between the two phases to be obtained. Fig. 1. shows typical chromatograms obtained with samples prepared from urine from a non-exposed control subject, the same urine spiked with 100 μg/l IPPD and a urine sample taken from an exposed subject at the end of a shift. IPPD eluted as a well resolved peak in a blank zone of the chromatogram which usually contains the peaks of highly lypophylic compounds.

Accuracy and precision

The analytical recovery of IPPD, determined by analysing urine samples spiked with known amount of amine, ranged between 83.5 and 102.0% (Table I). The first extraction step with chloroform was able to separate most of the amine from the urine; further extraction did not improve the recovery yield. Recoveries were not affected by variations of the pH of the urine sample within the range 2–12.

IPPD shows a typical UV absorption spectrum with a maximum at 290 nm. To investigate the specificity of the method, the IPPD peaks in urine samples from 20 workers were examined on-line by UV spectroscopy and compared with those of the synthetic compound. The spectra were the same, which indicates that IPPD was well separated from other components in urine. The reproducibility has been tested with replicated intra-assay determinations to give coefficients of variation (C.V.) of 5.9 and 3.7% at IPPD concentrations of 22.3 and 92.1 μg/l, respectively.

The detector response at 290 nm was linearly related to the concentration of IPPD in urine over the range 0–300 μg/l. By using a calibration graph, the concentration of IPPD in urine samples was easily determined after measurement of its peak height. The sensitivity was 5 μg/l at a signal-to-noise ratio of 3:1.

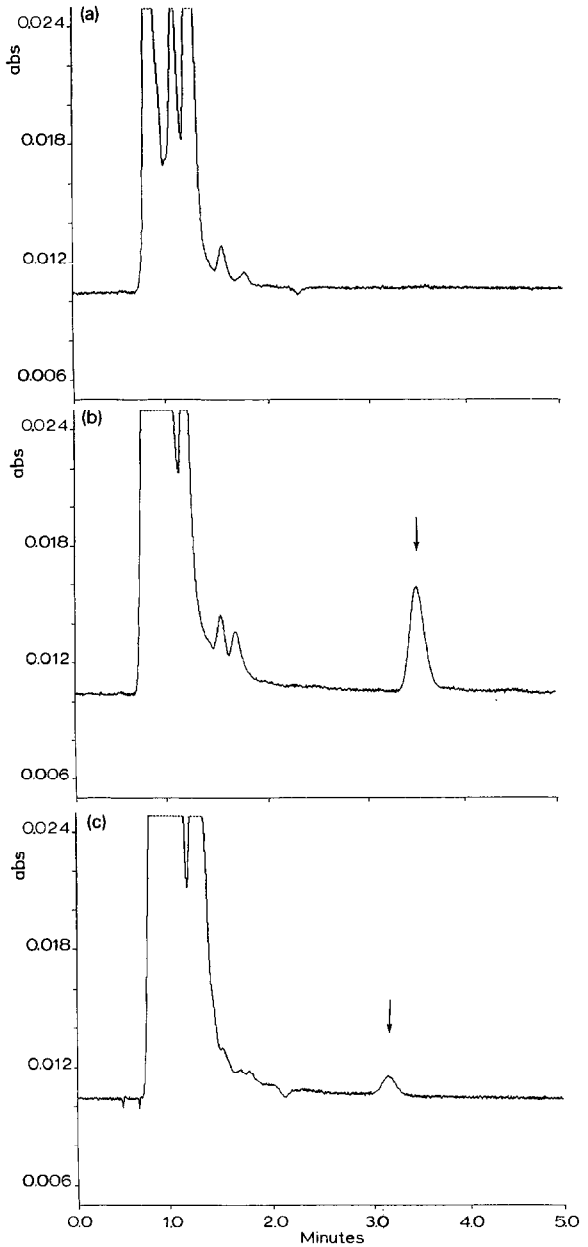

Fig. 1. Chromatographic traces of (a) urine from a non-exposed control subject, (b) the same urine supplemented with 200 μg/l IPPD and (c) a urine sample collected from an exposed subject at the end of a shift (35 μg/l).

Biological results

Concentrations of IPPD in urine samples from 15 non-exposed control subjects were less than 5 μg/l. The concentrations measured in urine samples collected from 62 rubber vulcanization subjects after the end of a shift ranged from 4.3 to 174 μg/l (29.3 ± 32.5 μg/l, mean ± 1 S.D.). These results confirm the significant absorption of IPPD during the working week.

TABLE I

ANALYTICAL RECOVERY OF IPPD ADDED TO A POOLED URINE SAMPLE FROM A NON-EXPOSED SUBJECT

Means (± S.D.) from ten assays are shown. The data fit the line $IPPD_{found}$ = (0.86 ± 0.02) $IPPD_{added}$ + (2.12 ± 2.05), with a standard error of the estimate, $s_{y\,x}$ = 1.4, and a correlation coefficient r = 0.995.

Added (μg/l)	Found (μg/l)	Recovery (%)	C.V. (%)
–	< 5.0	–	–
20.0	18.8 ± 1.5	94.1 ± 7.6	8.1
100.0	88.5 ± 5.9	88.5 ± 5.9	6.7
200.0	173.0 ± 9.4	86.5 ± 4.7	5.4

CONCLUSIONS

A liquid–liquid extraction procedure, followed by HPLC separation, has been developed for the determination of IPPD in urine samples. The isocratic chromatographic step requires 4 min to be completed and a simple mobile phase composition. The extraction is easy to perform and relatively fast; twenty batched samples can be processed within 1 h. No improvement of the recovery yields was obtained either by increasing the volume ratio of chloroform to urine or further extracting or varying the pH of the urine samples.

The method described requires small amounts of urine, is sensitive, accurate and suitable for large-scale routine analyses, and its application could be extended to other similar aromatic amines.

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