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Note

Determination of methyl ethyl ketone in human urine after derivatization with *o*-nitrophenylhydrazine, using solid-phase extraction and reversed-phase high-performance liquid chromatography and ultraviolet detection

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Methyl ethyl ketone (MEK, 2-butanone) is used extensively as a solvent in the chemical industry. MEK has a good solubility for most organic compounds, evaporates easily and is readily absorbed by the lungs and skin during exposure. MEK and related metabolites have been found in blood and urine of workers and animals after exposure to this solvent [1–6]. The concentration of MEK in urine is thought to be a good parameter for the biological monitoring of MEK exposure.

The analysis of MEK in aqueous solutions such as blood and urine is usually performed with headspace techniques and gas chromatography (GC). Recently we described an additional method for the determination of MEK and its metabolites, using solid-phase extraction and capillary GC [5].

The determination of MEK and related metabolites in urine is also possible with high-performance liquid chromatography (HPLC) [7–9]. For the HPLC method no extraction of the highly water-soluble analytes is necessary, but because direct UV detection of MEK is not sensitive enough, MEK has to be derivatized with compounds such as *o*-nitrophenylhydrazine, a reagent that reacts with carbonyl groups under acidic conditions [10].

Raymer et al. [11] presented an extensive and time-consuming scheme for the preparation of samples prior to derivatization of MEK in order to avoid interference of the sample medium during the analysis.

However, for the determination of MEK on a large scale, a simple and accurate method must be available without time-consuming sample preparation. In the present study, MEK was derivatized in urine samples with *o*-nitrophenylhydrazine, and solid-phase extraction was introduced as a tool for simple sample prep-

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aration. The *o*-nitrophenylhydrazones were determined with isocratic reversed-phase HPLC and UV detection.

EXPERIMENTAL

Chemicals and reagents

The following chemicals were used: chloroform (Uvasol), cyclohexane (Uvasol), methanol (p.a.), acetonitrile (LiChrosolv), MEK (p.a.) (Merck, Amsterdam, The Netherlands), *o*-nitrophenylhydrazine (Sigma, Brunschwig Chemie, Amsterdam, The Netherlands). Solid-phase extraction was accomplished with the SPE-21 system of Baker using 3-ml C_{18} reversed-phase disposable columns (Baker Chemicals, Deventer, The Netherlands). Demineralized and bidistilled water was used for the eluent.

Apparatus

A Perkin-Elmer HPLC combination, consisting of a Series 10 pump and an LC95 detector (Perkin-Elmer, Gouda, The Netherlands), and a Shimadzu CR3A integrator (Shimadzu, Kyoto, Japan) were used. For separation of the *o*-nitrophenylhydrazones a Pecosphere 3CR-C_{18} HPLC column (particle size 3 μm, 83 mm$\times$4.6 mm I.D.) (Perkin-Elmer) was used.

Derivatization of MEK

MEK was derivatized according to the method of Scoggins [10] with some modifications. For the derivatization procedure a 30.9 m*M* solution of *o*-nitrophenylhydrazine in 1 *M* hydrochloric acid was used. The reagent was washed twice with one fifth volume of chloroform to remove significant impurities. Fresh reagent was prepared each day and it was kept in the dark at 4°C because of its sensitivity to light and instability at room temperature (18–20°C).

The *o*-nitrophenylhydrazine reagent (200 μl), 150 μl of urine and 1 ml of cyclohexane were added to a 1.5-ml Eppendorf plastic reaction-tube (Eppendorf, Hamburg, F.R.G.). Samples were shaken thoroughly (60 rpm) overnight at room temperature (18–20°C) in the dark in a test-tube rotator (GFL, Burgwedel, F.R.G.).

Thereafter, the reaction-tubes were centrifuged at room temperature (18–20°C) for 5 min at 10 000 *g* (Eppendorf) to separate the water and the cyclohexane layers. Solid compounds, such as denaturated proteins, were sedimented on the bottom of the reaction tube during the centrifugation. The cyclohexane phase, containing the *o*-nitrophenylhydrazones, was used for further preparation. All samples were derivatized in duplicate.

Solid-phase extraction

Because the eluents acetonitrile–water and cyclohexane form two immiscible phases, reversed-phase extraction columns were used to extract the *o*-nitrophenylhydrazones out of the cyclohexane phase.

The disposable columns were conditioned by running through 3 ml of methanol under reduced pressure (13 mbar) and were thereafter equilibrated with 2 ml of

cyclohexane. Then 900 μl of the cyclohexane layer were transferred to the columns under reduced pressure (13 mbar). After loading, the columns were dried for 20 min under maximum underpressure (50 mbar) to evaporate cyclohexane. To collect the *o*-nitrophenylhydrazones, the columns were eluted twice with 500 μl of acetonitrile under reduced pressure (13 mbar).

Chromatography

For the determination of the *o*-nitrophenyldrazones, isocratic reversed-phase liquid chromatography was used, with acetonitrile–water (55:45, v/v) as eluent. The eluent was filtered through a 0.45-μm filter (RC 55, Schleicher and Schuell, Den Bosch, The Netherlands) and degassed with pure helium for 5 min. The flow-rate was 1.5 ml/min at ambient temperature (18–20°C). For analysis, 10 μl of sample were injected in duplicate. The detection wavelength was 254 nm.

Quantification

MEK solutions of 0, 21, 42, 63, 84 and 105 μM (0–7.5 mg/l) in the urine of a subject not exposed to MEK were used as standards for the calibration curve and analysed according to the standard procedure. The concentrations of MEK in urine samples were calculated from the calibration curve, using linear regression and peak area.

RESULTS

Fig. 1 shows the chromatograms of a urine sample of a non-exposed subject, a urine sample spiked with 105 μM MEK and a urine sample of an occupationally exposed subject. Several aldehydes and ketones occurring naturally, such as acetaldehyde, formaldehyde and acetone, were present in the urine of both non-exposed and exposed subjects. The concentration of MEK in the urine of non-exposed subjects (ten men, aged between 20 and 30 years) was less than 1.5 μM (less than 100 μg/l). The chromatogram of urine spiked with MEK shows that MEK was separated into two peaks with retention times of 6.2 and 7.3 min, respectively. The existence of two peaks can be explained by the synthesis of two isomeric hydrazones (the *cis* and the *anti* isomer), as described by Behforouz et al. [12]. The main peak (retention time = 7.3 min) accounted for 81.7 ± 3.2% (S.D.) ($n = 12$) of the total area and was used for the calculations.

The calibration curve is linear in the concentration range from 0 to 105 μM MEK (0–7.5 mg/l MEK). The equation of the calibration is:

$$\text{peak area} = 5451 \times \text{concentration (mg/l)} - 189 \quad (n = 12, r = 0.997).$$

The detection limit (expressed as three times the noise signal) for MEK proved to be 1.5 μM (100 μg/l MEK). The intra-assay precision for a duplicate determination, expressed as a coefficient of variation, was 4.1% ($n = 18$) and was independent of the concentration of MEK in the samples.

The sensitivity (s), expressed as the slope of the calibration curve, varied slightly from day to day: $s = 5520 \pm 263$ (S.D.) ($n = 3$).

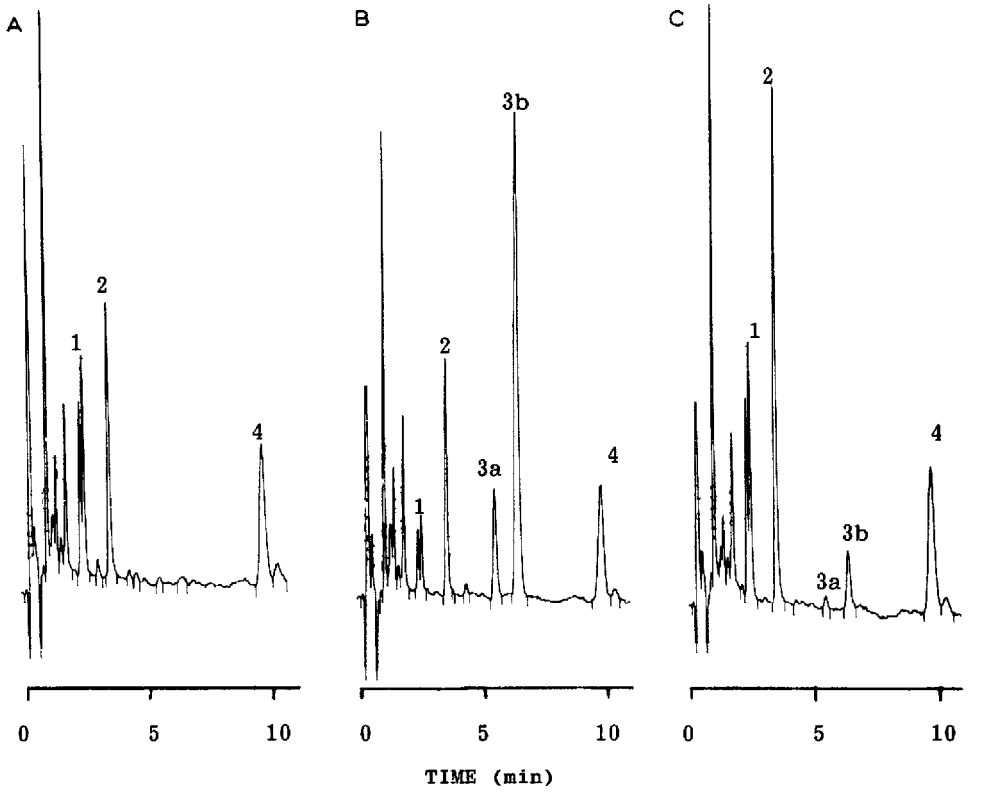

Fig. 1. Chromatograms of (A) urine from a non-exposed subject, (B) the same urine spiked with MEK (7.5 mg/l) and (C) urine from a subject exposed to MEK. Chromatographic conditions and sample preparation are described in Experimental Peaks: 1 = formaldehyde; 2 = acetone; 3a and 3b = two isomeric forms with MEK; 4 = cyclohexanone (internal standard) (full scale = 1 mV)

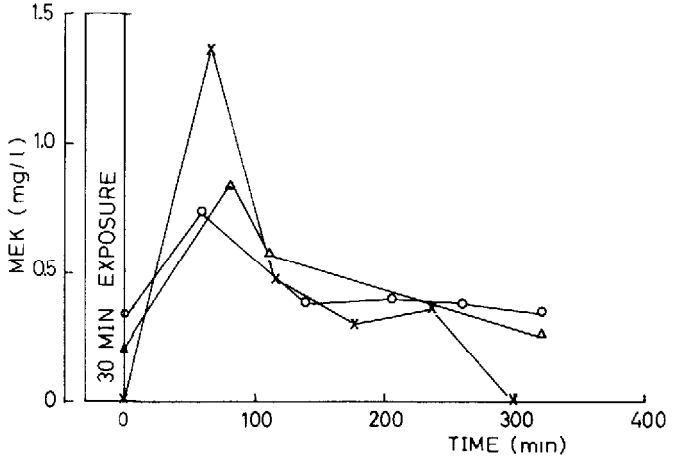

Fig. 2. MEK concentrations in the urine of subjects exposed to 200 ppm MEK for 30 min, up to 5 h after exposure. Values are the means of a duplicate analysis. (×) Subject 1; (○) subject 2; (△) subject 3.

A practical application of the method is shown in Fig. 2, which shows the concentration of MEK in the urine of subjects exposed to 200 ppm MEK (590 mg/m^3) for 30 min, up to 5 h after exposure. The MEK concentration increased during the first hour and returned to normal within a few hours.

DISCUSSION

We have presented a method for the determination of MEK in urine samples without complicated and time-consuming preparation of the urine samples. MEK was derivatized with *o*-nitrophenylhydrazine to obtain the corresponding hydrazones, and the hydrazones formed were extracted continuously with cyclohexane.

For several reasons cyclohexane is an ideal extraction solvent. Firstly, it has poor selectivity for *o*-nitrophenylhydrazine and good selectivity for the relatively more apolar *o*-nitrophenylhydrazones. Secondly, proteins and salts in the urine are insoluble in the cyclohexane phase, and are therefore simply removed after the derivatization procedure by centrifugation. Sample preparation with gel exclusion and ion-exchange chromatography, as described by Raymer et al. [11], is not necessary when cyclohexane is used as the extraction solvent. With this procedure, derivatization is completed after ca. 7 h. The derivatization is carried out overnight for reasons of economy.

The yield of the *o*-nitrophenylhydrazones of MEK is a maximum at 20°C [10]. The sensitivity of our method decreased when the volume of the headspace in the reaction tube increased. Therefore, reaction tubes with a minimal headspace were used for the derivatization procedure. Two isomeric hydrazones (*cis* and *anti*) were formed from MEK. For the two isomers a ratio of 1:5 is calculated from the peak areas. This ratio is also found in experiments of Behforouz et al. [12]. The reproducibility of duplicate derivatizations is adversely influenced when the samples are exposed to light; for this reason the whole procedure was done in the dark.

Solid-phase extraction is a powerful tool when a change in solvent or selective separation is necessary for further analysis. Under our conditions, C_{18} reversed-phase columns gave the best results. The *o*-nitrophenylhydrazones were quantitatively loaded on these columns and could be eluted with 100% acetonitrile. The recovery was 100%, although it might be possible to concentrate the analytes several times. High-molecular-mass hydrazones with a relatively high affinity for reversed-phase materials were almost completely removed from the sample because they were not eluted from the SPE columns with 100% acetonitrile.

Separation of different *o*-nitrophenylhydrazones with reversed-phase liquid chromatography can be manipulated easily by changing the ratio water to acetonitrile. The retention time of the hydrazones decreased with higher concentrations of acetonitrile. Separation was achieved with a high-speed reversed-phase column packed with a solid phase containing endcapped residual polar groups. This resulted in very good separation conditions, and at least 200 injections of urine samples could be carried out without regeneration; when necessary, regen-

eration of the analytical column was complete with 100% acetonitrile. With normal reversed-phase materials, regeneration is necessary after ca. 100 injections.

An internal standard (cyclohexanone) was added to correct for differences in the procedure. The method proved to be so reproducible that correction for the internal standard did not improve the results.

Recently we published an article on the determination of MEK and related metabolites, using capillary GC [5]. However, the advantages of the HPLC method described in this paper are that only small amounts of sample and chemicals are needed for analysis, that large numbers of samples can be derivatized at the same time and that most other low-molecular-mass ketones and aldehydes in urine, which react with *o*-nitrophenylhydrazine, can be analysed as well.

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REFERENCES

1 G.D. Vincenzo, C.J. Kaplan and J. Dedinas, Toxicol. Appl. Pharmacol., 36 (1976) 511.
2 F.K Dietz and G.J. Traiger, Toxicology, 14 (1979) 209.
3 L. Perbellini, F. Brugnone, P. Nozzo, V. Cocheo and D. Caretta, Int. Arch. Occup. Environ. Health, 54 (1984) 73.
4 L.L. Needham, R.H. Hill, Jr., D.L. Orti, M.E. Felver and J.A. Liddle, J. Chromatogr., 233 (1982) 9.
5 S. Kežić and A.C. Monster, J. Chromatogr., 428 (1988) 275.
6 J. Liira, V. Riihimaki and P. Pfaffli, Int. Arch. Occup. Environ Health, 60 (1988) 195.
7 S. Selim, J. Chromatogr., 136 (1977) 271.
8 F. Lipari and S.J. Swarin, J. Chromatogr., 247 (1982) 297.
9 L.J. Papa and L.P. Turner, J. Chromatogr Sci., 10 (1972) 744.
10 M.W. Scoggins, Anal. Chem., 13 (1973) 2204.
11 J. Raymer, M.L. Holland, D.P. Wiester and M. Novotny, Anal. Chem , 56 (1984) 962.
12 M. Behforouz, J.L. Bolan and M.S. Flynt, J. Org. Chem., 50 (1985) 1186.