Benzyl Alcohol as a Marker of Occupational Exposure to Toluene

Toshio KAWAI1, Tsuneyuki YAMAUCHI1, Yuriko MIYAMA1, Haruhiko SAKURAI2, Hirohiko UKAI3, Shiro TAKADA3, Fumiko OHASHI3 and Masayuki IKEDA3*

1 Osaka Occupational Health Service Center, Japan Industrial Safety and Health Association, Nishi-ku, Osaka 550-0001, Japan
2 Occupational Health Research and Development Center, Japan Industrial Safety and Health Association, Minato-ku, Tokyo 108-0014, Japan
3 Kyoto Industrial Health Association, 67 Nishinokyo-Kitatsuboicho, Nakagyo-ku, Kyoto 604-8472, Japan

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Abstract: Benzyl alcohol (BeOH) is a urinary metabolite of toluene, which has been seldom evaluated for biological monitoring of exposure to this popular solvent. The present study was initiated to develop a practical method for determination of BeOH in urine and to examine if this metabolite can be applied as a marker of occupational exposure to toluene. A practical gas-liquid chromatographic method was successfully developed in the present study with sensitivity low enough for the application (the limit of detection; 5 µg BeOH /l urine with CV=2.7%). Linearity was confirmed up to 10 mg BeOH/l, the highest concentration tested, and the reproducibility was also satisfactory with a coefficient of variation of 2.7% (n=10). A tentative application of the method in a small scale study with 45 male workers [exposed to toluene up to 130 ppm as an 8-h time-weighted average (8-h TWA)] showed that BeOH in the end-of-shift urine samples was proportional to the intensity of exposure to toluene. The calculated regression equation was Y=50+1.7X (r=0.80, p<0.01), where X was toluene in air (in ppm as 8-h TWA) and Y was BeOH in urine (in µg/l of end-of-shift urine). The levels of BeOH in the urine of the non-exposed was about 50 µg/l, and ingestion of benzoate as a preservative in soft drinks did not affect the BeOH level in urine. The findings as a whole suggest that BeOH is a promising candidate for biological monitoring of occupational exposure to toluene.

Key words: Benzyl alcohol, Biological monitoring, Toluene, Occupational exposure, Urinalysis

Introduction

Although the use of toluene appears to have been decreasing in recent years, this solvent is still most popular in various workplaces, especially in the workplaces for printing, adhesive spreading/adhesion, and painting1–4). Accordingly, investigations have been made successfully to develop methods for biological monitoring of exposure to toluene by means of urinalysis. The target analyte in the past was hippuric acid5–8). Toluene levels in workplaces has been gradually decreasing in pace with the reduction in the occupational exposure limit for toluene to 50 ppm as proposed by many organizations9–10). Confounding effects of soft drinks and other foods containing benzoic acid (a food preservative which is converted in vivo to hippuric acid) have also been better elucidated11, 12). These factors have brought up a suspicion that hippuric acid may no longer be the analyte of choice. Accordingly, the attention to target analytes shifted from hippuric acid to other urinary toluene metabolites such as o-cresol13–15) and benzylmercapturic acid16, 17). Urinary level of un-metabolized toluene (excreted possibly by diffusion) was also evaluated as a marker of exposure18–22).
Benzyl alcohol is a well-known intermediate in toluene metabolism\(^2\), but has been seldom examined for the purpose of toluene biological monitoring. A successful trial was made in the present study to show that this metabolite can be measured by means of simple gas-liquid chromatography after acid hydrolysis of urine samples. Preliminary application to a field survey showed that this new analyte in end-of-shift urine is a promising candidate marker of toluene exposure at low levels, e.g., the level well below 50 ppm as a time-weighted average for 8-hour work shift (8-h TWA).

**Materials and methods**

**Ethical issue**

The Ethics Committee of Kyoto Industrial Health Association approved the study protocol, and each of the participants (including five non-exposed controls) agreed to participate in the study.

**Reagents**

Authentic benzyl alcohol (to be abbreviated as BeOH; purity 99%) was obtained from Kanto Chemicals (Tokyo, Japan). cis-3-Methylcyclohexanol [purity 95%; to be used as an internal standard (IS)], and diisopropyl ether (purity 99%) were purchased from Wako Pure Chemicals (Osaka, Japan).

**Toluene-exposed workers and urine samples**

Stored urine samples were employed for development and validation of the present method. Spot urine samples were collected at the end of an 8-h work-shift, and kept at \(-30^\circ\)C until analyzed. In practice, urine samples from 45 solvent (including toluene)-exposed male workers in two plants were employed. Among them, 23 men in Plant A were engaged in printing work, whereas remaining 22 men in Plant B produced adhesive tapes. They were equipped with diffusive samplers for an entire shift of 8 hours, and the solvent components adsorbed in the exposed carbon cloth were analyzed on a DB-WAX capillary column\(^2\). The solvent concentrations are summarized in Table 1. Five non-exposed controls (all men) were office workers in the same plants.

**Volunteers for the study on possible excretion of benzyl alcohol after ingestion of benzoate**

Five adult subjects with no occupational exposure to toluene volunteered to participate in the study and submitted informed consents.

**Standard method of urinalysis for benzyl alcohol, as established in the present study**

An aliquot (5 ml) of urine (or authentic BeOH dissolved in water) was added 0.5 ml of 36% hydrochloric acid (HCl) in a screw-capped 10-ml tube (with a Teflon septum) and heated in a boiling water bath for 30 min to hydrolyze BeOH conjugates (assumedly sulfate and glucuronide by analogy to 1-butanol\(^2\)). After cooling to room temperature, 2 ml of diisopropyl ether (containing 9.5 mg cis 3-methylcyclohexanol/l ether) and 2 g of sodium chloride (NaCl) were added, and each tube was shaken vigorously for 30 sec.

After spinning at 1,870 \(\times\) g for 10 min for separation of the two layers, 2 \(\mu\)l of the ether layer was taken to be introduced to GC for quantification of BeOH.

The GC system (Agilent 6890N Network system) used was a product of Agilent Technologies (Wilmington, DE, U.S.A.), equipped with a flame-ionization detector (FID), a DB-1 capillary column (60 m in length, 530 \(\mu\)m in inner diameter and 1.5 \(\mu\)m in film thickness; a product of J&W, Folsom, CA, U.S.A.), and an auto-injector (Agilent 7683 series). The system was operated in a splitless mode. Helium (as a carrier gas) was allowed to flow through the column at 18 ml/min, and hydrogen was supplied to the detector at 40.0 ml/min. The detector was heated at 250\(^\circ\)C. The column temperature was maintained at 40\(^\circ\)C for 7 min, elevated at a rate of 5\(^\circ\)/min to 120\(^\circ\)C to stay there for 2 min, and elevated at a rate of 40\(^\circ\)/min to 250\(^\circ\)C to stay there for 2 min before brought down to 40\(^\circ\)C, so that one sample was analyzed in about 30 min.

**Other methods of determinations**

Hippuric acid (HA) was measured after Kawai et al.\(^\)\(^2\). The same procedures were applied also to the determination of benzoic acid in urine; the retention time was about 9.0 min for benzoic acid, and about 3.6 min for hippuric acid.

Creatinine (CR) and specific gravity (SG) were measured by colorimetry and refractometry, respectively. The SG was expressed in terms of factor G which is defined as factor G = (SG–1.000) \(\times\) 1,000. In some instances, urinary metabolite concentrations were corrected for CR\(^2\) or a SG of 1.016\(^\)\(^2\).

**Statistical analysis**

As the distribution patterns of BeOH and other parameters were unknown, non-parametric Mann-Whitney test was employed for examination of statistical significance of the difference in addition to parametric unpaired \(t\)-test. Possible difference in slopes and intercepts were examined after Ichihara\(^2\).
Results

Development of analytical conditions

In order to examine hydrolysis conditions, various amounts of 36% HCl (0.1, 0.25, 0.5, 0.75 and 1.0 ml) was added to 5 ml portions of 10 urine samples (obtained from 10 individuals) containing 29–273 µg BeOH and 28 to 1,435 mg hippuric acid/l urine. After adjustment of the total volume to 6 ml by the addition of water, the samples (thus 50 samples in total) were heated in a boiling water bath for 30 min. The mean BeOH detected (10 measurements per concentration) was 93, 116, 139, 134 and 139 µg/l with 0.1, 0.25, 0.5, 0.75, and 1.0 ml HCl, respectively. When the value with 0.5 ml HCl was taken as 100, the relative amount of BeOH was 66.9, 83.5, 100, 96.4 and 99.7, respectively. The changes suggested that a substantial fraction of BeOH in urine is excreted into urine as conjugated, and that 0.5 ml 36% HCl is sufficient for hydrolysis of the conjugates in a 5-ml urine sample.

It was thought possible that the extraction rate might be different when BeOH is extracted from urine as compared with the rate from water. Accordingly, a known amount of BeOH (0.5 ml of 5,170 µg BeOH/l water) was added to either water (Series A) or urine samples (5.0 ml each) from 10 individuals (Series B). In the third series (Series C), the same urine samples were added 0.5 ml water for adjustment of urine volume (thus 5.5 ml as a final volume). The analysis of the three series of samples was conducted for BeOH, and the increments in BeOH of Series B samples over Series C samples were calculated. When compared with BeOH dissolved in diisopropyl ether, the rate of BeOH extraction from water (Series A) was 93.7%, whereas that from urine (i.e., the increment of Series B over Series C) was 90.3% (p>0.10 for the difference). Thus, no effect of urine on the BeOH extraction rate was detected.

It is apparent that the hydrolysis of conjugated BeOH to its free form is in presence of much more hippuric acid. Although the chances should be very remote, possible conversion of hippuric acid to BeOH under hydrolysis conditions was examined. For this purpose, 1 ml of dense hippuric acid solution (4.2 g/l) was added to 5 ml each of urine samples from 10 individuals. Analysis after hydrolysis did not show any increase in BeOH peak for each urine sample. It was also found that, under the hydrolysis conditions, about 6 to 8% of hippuric acid in urine was hydrolyzed to benzoic acid.

When the water phase (containing 1,000 µg each of BeOH and o-cresol/l) was saturated with NaCl by addition of 2 g NaCl to 5 ml water, the salt addition resulted in an increase in the extraction rate by 114, 134, and 98% for the internal standard (IS), BeOH and o-cresol (p<0.01, <0.01 and <0.05 by Mann-Whitney test and p<0.01, <0.01 and >0.05 by unpaired t-test). Thus, the ratio of BeOH/IS also increased significantly (117%: p<0.01 by both Mann-Whitney test and unpaired t-test). The observation suggested that the addition of NaCl at saturating concentrations improves the analysis.

The proportionality of the ratio in the peak area of BeOH over the IS was tested under the conditions thus established. For this purpose, authentic BeOH (liquid) was diluted with water to obtain solutions at the concentrations of 0, 5, 14, 43, 128, 383, 1149, 3447, and 10340 µg BeOH/l, and the preparations were subjected to the analyses. The BeOH/IS ratio was linearly related to up to the highest BeOH concentration tested with a regression line of Y=4+0.25X (r=0.99, p<0.01), where X was BeOH (in µg/l), and Y was the BeOH/IS ratio (in dimensionless arbitrary unit). When urine samples from 5 non-exposed men were pooled and employed to dilute liquid BeOH in place of water, the regression equation thus obtained had the same slope and a slightly larger intercept on the vertical axis. The increment in the intercept should be attributable to the presence of endogenous BeOH in the urine of the non-exposed subjects, as to be discussed later.

Typical chromatograms are shown in Fig. 1 for over-all...
visual understanding of the results of analysis with the authentic BeOH (Fig. 1a) and BeOH in the urine of a toluene-exposed worker (Fig. 1b). A portion of the latter chromatogram in the vicinity of the peak for BeOH is expanded in Fig. 1c for clear presentation of the peak. The low limit of detection (LOD) was 5 \( \mu g \) BeOH/l, when a P/N ratio of 3 was taken. When a urine sample containing 2,300 \( \mu g \) BeOH/l was analyzed for 10 times, the coefficient of variation (i.e., ASD/AM) was 2.7%.

**Application of the procedures to the urine samples from factory workers**

A preliminary study was conducted to examine if BeOH in urine would increase as a function of occupational exposure to toluene. The results are summarized in Table 2. It is clear from the table that BeOH in end-of-shift urine samples increased as a function of 8-h TWA intensity of toluene exposure, and that the correlation was statistically significant \((p<0.01)\). The cases were presented as a scatter diagram in Fig. 2 for visual understanding of the correlation. Further comparison between the workers in Factory A and B showed that the slope of the regression line with observed (uncorrected) values of BeOH was significantly \((p<0.05)\) steeper in Factory B (with lower toluene exposure) than that in Factory A (with higher toluene exposure). The difference was insignificant however when BeOH was corrected for CR or SG (i.e., BeOH\(_{cr}\) or BeOH\(_{sg}\)), suggesting that the difference may not necessarily be meaningful.

**BeOH levels in urine of the non-exposed subjects**

The analysis of the 10 urine samples from 5 concurrent controls and 5 volunteers revealed that, although at low levels, BeOH was present in urine samples from subjects who were not exposed to toluene. The AM \( \pm \) ASD of observed values (i.e., BeOH\(_{ob}\) were 61 \( \pm \) 36 \( \mu g/l \), and GM and GSD were 53 \( \mu g/l \) and 1.16.

**No effect of soft drink ingestion on BeOH in urine**

Some soft drinks contain substantial amount of benzoate\(^{11,12}\) as a preservative. It was thought possible that the benzoate ingested as a preservative in the drinks might be reduced to benzyl alcohol by intestinal flora in the gastro-intestinal tract, which then will be absorbed and excreted into urine. In order to rule out this possibility, each of five adult subjects...
ingested a bottle of benzoate-containing soft drink (138 mg sodium benzoate in 500 ml) and urinary excretion of BeOH was followed for 4 hours in parallel with that of HA. The results are summarized in Table 3. In two hours after the ingestion, a substantial decrease in urine density was observed (possibly due to intake of a bulk of water) as expressed by the reduction in CR and SG. Resultingly, the BeOH as observed (or BeOH\textsubscript{ob}) was reduced to the level below the 0-time level (i.e., pre-ingestion level) at 1 or 2 h after the ingestion. HA tended to increase over the 0-time level by 3-fold but the increase was not statistically significant (p>0.05) due to wide inter-individual variation. When BeOH and HA were corrected for CR, there was a significant (p<0.01) increase in HA\textsubscript{cr} at 1 and 2 h after the ingestion, whereas BeOH\textsubscript{cr} stayed unchanged (p≥0.05). Correction for SG also gave exactly the same results, i.e., significant increase in HA\textsubscript{sg}, but no increase in BeOH\textsubscript{sg}. It was therefore clear that BeOH level did not increase during the 4 h period after the ingestion irrespective of urine density correction, whereas that of HA increased within 1 h after ingestion followed by quick return to the pre-ingestion level by 2 h.

### Discussion

A practical FID-GC method for quantification of BeOH in urine was successfully developed in the present study with sufficient accuracy (the limit of detection; 5 µg/l urine) and precision (the coefficient of variation; 2.7%; n=10). The linearity range is up the maximum concentration tested (i.e., 10 mg BeOH/l), which is sufficiently wide. Diisopropyl ether was selected based on previous experiences with phenolic metabolites\textsuperscript{28}. When the method was applied to the end-of-shift urine samples from toluene-exposed workers in a preliminary small-scale field survey, a close correlation of BeOH in urine was observed with 8-h TWA intensity of vapor exposure to toluene (Table 2). It was also made clear that the ingestion of benzoate-containing soft drinks does
Table 3. Changes in CR, SG and metabolite levels after ingestion sodium benzonate

<table>
<thead>
<tr>
<th>Correction for</th>
<th>Marker</th>
<th>Nuit</th>
<th>Parameter</th>
<th>Time after ingestion (h)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>None</td>
<td>CR</td>
<td>g/l</td>
<td>AM</td>
<td>1.32</td>
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<td></td>
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<tr>
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<td></td>
<td></td>
<td>CV</td>
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<td></td>
<td>p</td>
<td>*</td>
</tr>
<tr>
<td>None</td>
<td>SG</td>
<td>G</td>
<td>AM</td>
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<tr>
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<td></td>
<td>CV</td>
<td>0.25</td>
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<td></td>
<td>p</td>
<td>*</td>
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<td>None</td>
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<td>µg/l</td>
<td>AM</td>
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<td></td>
<td>ASD</td>
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<td></td>
<td>CV</td>
<td>0.35</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>p</td>
<td>**</td>
</tr>
<tr>
<td>HA</td>
<td>mg/l</td>
<td>AM</td>
<td>300</td>
<td>908</td>
</tr>
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<td></td>
<td></td>
<td>ASD</td>
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</tr>
<tr>
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<td></td>
<td></td>
<td>CV</td>
<td>0.35</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>p</td>
<td>ns</td>
</tr>
<tr>
<td>CR</td>
<td>BeOH</td>
<td>µg/g cr</td>
<td>AM</td>
<td>58.8</td>
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<td>p</td>
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<td>AM</td>
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<td>CV</td>
<td>0.66</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p</td>
<td>**</td>
</tr>
<tr>
<td>SG</td>
<td>BeOH</td>
<td>µg/l</td>
<td>AM</td>
<td>58.9</td>
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<td></td>
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<td>ns</td>
</tr>
<tr>
<td>HA</td>
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<td>AM</td>
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<tr>
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<td></td>
<td>CV</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p</td>
<td>**</td>
</tr>
</tbody>
</table>

Five adult subjects ingested 500 ml of soft drink (containing 138 mg sodium benzoate) at time 0, and urinary excretion of BeOH and HA were followed up for 4 h.

**, * and ns stand for p<0.01, <0.05 and ≥0.05, respectively.

not elevate BeOH level in urine, contrary to the case of huppuric acid (Table 3). The results as a whole suggest that BeOH in urine is a promising marker of occupational exposure to toluene. The validation apparently deserves further confirmation through larger scale factory surveys.

With two assumptions that the rate of absorption of toluene in the lungs is about 50%\(^{28}\) and that the respiration rate is 10 l/min\(^{30}\), exposure to toluene at 50 ppm will result in an absorption of 940 µg toluene /min, when calculated as 50 ppm (or 188 mg/m\(^3\)) × 10 \times 10^{-3} \mathrm{m}^3/\mathrm{min} \times 50/100. According to the calculated regression line in Table 2, toluene excreted into urine as BeOH at the end of an 8-h toluene exposure at 50 ppm will be 85 µg/l \([= 1.7 \times 50]\) or 85 ng/min, assuming that urine out-put volume is 1 ml/min\(^{30}\). As the molecular weight of toluene and BeOH is 92.14 and 108.14, respectively, 85 ng BeOH/min is equivalent to 72.4 ng toluene/min. In other words, less than 0.01% \([0.01 \%(0.0724/940) \times 100]\) of toluene absorbed will be excreted into urine.
as BeOH. Although this estimation is based on the cross-sectional exposure-excretion balance at the end of the work shift, and should be taken as very preliminary, it is quite probable that BeOH is a minor urinary metabolite of toluene.

A field survey is currently in progress in which the scale is expanded not only in the number of toluene-exposed workers to be examined but in the analyte items. It is expected that a conclusive answer should be available to the questions on the most reliable marker and the analytical method of choice to be applied for biological monitoring of occupational exposure to toluene.

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