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# Capillary gas chromatographic method for mandelic and phenylglyoxylic acids in urine

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**Summary.** In support of an occupational investigation of styrene exposure, a capillary gas chromatographic method was developed for the quantitation of the styrene metabolites mandelic and phenylglyoxylic acids. The method was based on that of Guillemin and Bauer [7], in which phenylglyoxylic acid was converted to mandelic acid by reduction before instrumental analysis. The earlier method had to be modified for use with capillary columns; the resulting method was sensitive, selective and reproducible. The detection limit was approximately 0.001 mg/ml urine. Approximately < 5% relative precision was achieved in the range of 0.05–2 mg/ml urine. Mandelic acid was resolved from other components of urine and from by-products of derivatization.

**Key words:** Styrene – Urinary metabolites – Gas chromatography – Occupational health

## Introduction

Workers in the reinforced plastics industry are often exposed to styrene vapor and liquid. Biological monitoring of workers has been promulgated as a method of exposure assessment of styrene. The end-of-day concentrations of mandelic acid or phenylglyoxylic acid (PGA) in urine are measures of workplace exposure to styrene, and the biological exposure indices are 1 g/l and 250 mg/l, respectively [1]. Sollenberg et al. [12] recently suggested that the sum of mandelic acid and PGA is the best predictor of absorbed dose.

As part of a health effects assessment of workers in the reinforced plastics industry, a method was needed for the quantitation of the styrene metabolites mandelic acid and PGA in urine. High-pressure liquid chromatography has been used to quantitate the metabolites [4, 10]; thin-layer chromatography has also been used [6]. Our review of the literature showed that two types of gas chromatographic methods have been used successfully; both required derivatization of PGA and mandelic acid since these metabolites were too polar to chromatograph without derivatization. Under carefully controlled conditions, diazomethane methylated PGA and mandelic acid [2, 3, 5, 9]. Alternatively, PGA was reduced to mandelic acid using acidic zinc prior to formation of the trimethylsilyl derivatives and chromatography [7]. Low-resolution packed columns were used in each of these gas chromatographic methods.

Bauer and Guillemin [2] considered methods using derivatization with diazomethane to be unsuitable for routine monitoring since diazomethane readily formed multiple derivatization products with PGA. Because of this observation and the instability of diazomethane, we decided to use the reduction method [7] to quantitate PGA. However, we found that the reduction method developed for packed-column gas chromatography was not directly transferable to capillary gas chromatography. Solvents and reagents used for the derivatization of mandelic acid were not optimal for the latter technique. Therefore, we altered the method of Guillemin and Bauer [7] to make it compatible with automated capillary gas chromatography. In addition, we found improvements in sample preparation that increased precision.

## Materials and methods

*Materials.* β-Phenylpropionic acid (K & K Laboratories, Plainview, N.Y.) was purified through acid and base partitioning and, finally, through recrystallization from chloroform. This was necessary to remove an interfering contaminant. Isooctane was obtained from Burdick and Jackson (Muskegon, Mich.). Ethyl acetate was of reagent grade (J.T. Baker, Phillipsburg, N.J.). Other chemicals were obtained from Aldrich (Milwaukee, Wi.). Dry ethyl acetate was produced by running small volumes through an activated alumina column; dried ethyl acetate was stored over anhydrous sodium sulfate. Granular zinc (20-mesh) was purified prior to use [11].

*Sample preparation.* In a 10 ml screw-cap test tube, 50 µl internal standard solution was added to 2 ml urine, which was then acidi-

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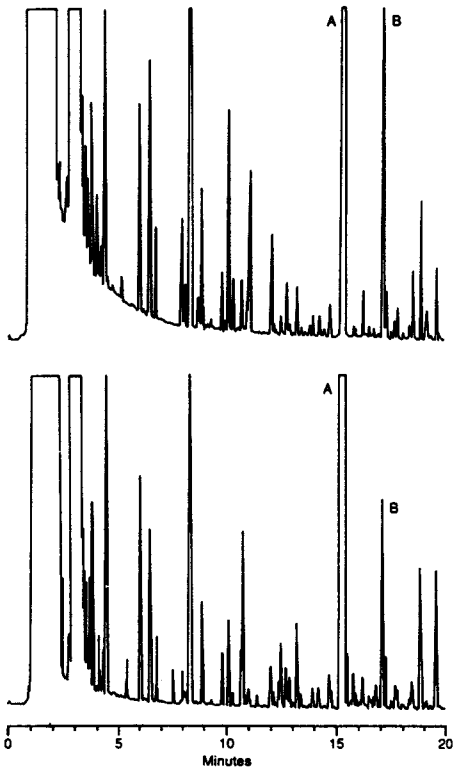


**Fig. 1.** Tracings of representative chromatograms. *Top panel:* chromatogram of blank urine spiked with internal standard (0.5 mg/ml urine) and mandelic acid (0.05 mg/ml). *Bottom panel:* chromatogram of a urine sample from a worker who was exposed to styrene. The concentration of mandelic acid in this sample was 0.005 mg/ml. *A*, Internal standard β-phenylpropionic acid; *B*, mandelic acid

fied with 0.5 ml 10 *N* sulfuric acid. The internal standard was β-phenylpropionic acid dissolved in acetonitrile (20 mg/ml). Approximately 700 mg granular zinc was added to the urine samples and the test tubes were closed with Teflon-lined caps. The samples were then vortexed for 1 min every 5 min over a 30-min period. During this time, PGA was reduced to mandelic acid. Next, 2 ml ethyl acetate and approximately 3 g salt were added to each tube. The tubes were recapped and vortexed for 5 min. The samples were then centrifuged at 1,000 *g* for 5 min and the layer of ethyl acetate was transferred to a 3-ml conical vial. The ethyl acetate was completely evaporated in a vacuum using a SpeedVac concentrator (Savant Instruments, Farmingdale, N.Y.). A black viscous residue usually formed at the bottom of the vial; this residue was dissolved in 100 µl dry ethyl acetate, following which 100 µl BSTFA (*N,O-bis*-(trimethylsilyl)-trifluoroacetamide; Supelco, Bellefonte, Pa.) was added. The vials were sealed with a Teflon-lined cap and heated to 60°C for 30 min; after they had cooled to room temperature, 1 ml isooctane was added and the mixture was transferred to a gas chromatographic vial for analysis.

Urine samples were obtained from workers in three reinforced-fiberglass products plants. These work sites included a truck-manufacturing plant, a plastic pipe- and tank-manufacturing plant, and a plastic grate-manufacturing plant. Liquid styrene was used as the bonding agent at each plant. The samples included 18 truck assembly workers who had not been exposed to styrene or other solvents.

*Instrument parameters.* A gas chromatograph (Hewlett-Packard, model 5840A; Avondale, Pa. with an autosampler and a capillary inlet system was used in the splitless mode for the analyses. Hydrogen carrier gas had a linear velocity of 40 cm/s. Nitrogen make-up gas flow to the flame ionization detector was 30 ml/min. The inlet purge was activated at 0.45 min with a split ratio of 40. The detector temperature was 300°C and the injector temperature was 200°C. The initial oven temperature was 50°C for 1 min, followed by a 5°C/min ramp; at 20 min, the rate was increased to 15°C/min. The final oven temperature was 250°C, which was held for 5 min. A DB-5 bonded-phase fused-silica capillary column (30 m long × 0.32 mm inside diameter, 0.25-mm film thickness; J & W Scientific, Folsom, Calif.) was used. The injection volume was 1 µl. Figure 1 shows representative chromatograms of a spiked urine sample and a urine sample from a worker with low exposure to styrene.

*Calibration.* With each group of samples, a blank containing only the internal standard and a set of spiked urine standards were analyzed. Spiking solutions of mandelic acid in acetonitrile were made at concentrations of 0.4, 1, 2, 4, 10, 20 and 80 mg/ml; in addition, 2-ml aliquots of urine from an unexposed individual (typically, a laboratory worker) were spiked with 50 µl spiking solution. The final concentrations of mandelic acid in urine were 0.001, 0.025, 0.05, 0.1, 0.25, 0.5, and 2 mg/ml, respectively. The blank was spiked with 50 µl of acetonitrile containing the internal standard. The blank and spiked urine were taken through the assay along with the samples. The response curve was determined by linear regression of concentration versus the area ratio of analyte to internal standard. The assay measured the sum of the concentrations of PGA and mandelic acid in the urine.

## Results and discussion

During development of the assay, substantial variation between replicate analyses of urine from styrene-exposed workers was observed (Fig. 2). The large variation was caused by the poor solubility in the derivatization solution of the residue from the solvent evaporation; analyte was apparently entrapped to varying degrees in the insoluble residue. The solubility of the residue was evaluated in several solvents including pyridine, the solvent previously used by Guillemin and Bauer [7]. Dry ethyl acetate was the optimal solvent for dissolving the residue. When the residue was dissolved in ethyl acetate prior to derivatization, the average coefficient of varia-

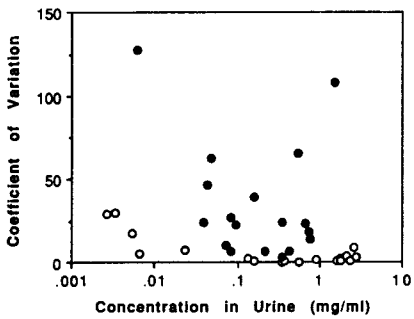


**Fig. 2.** Replicate measurements of total mandelic acid and PGA were performed on urine from workers in the reinforced plastic industry. Some workers did not have known contact with styrene; these individuals had concentrations of mandelic and PGA amounting to < 0.03 mg/ml. (●), Analyses done without redissolving the extraction residue prior to derivatization, as was done in the procedure developed by Guillemin and Bauer [7]; (○) analyses made using the developed procedure, which incorporated redissolving the extraction residue. Variation was reduced by redissolving the extraction residue in dry extraction solvent prior to derivatization

**Table 1.** Recovery and coefficient of variation (CV) from replicate analyses of urine spiked with either PGA or mandelic acid at various concentrations

| Spiked concentration (mg/ml) | Measured concentration (mg/ml) | Spike (%) | CV (%) |
|---|---|---|---|
| *PGA:* | | | |
| 0.01 | 0.016 | 157 | 27 |
| 0.025 | 0.028 | 112 | 2.1 |
| 0.05 | 0.055 | 109 | 1.9 |
| 0.1 | 0.103 | 103 | 6 |
| 0.5 | 0.547 | 109 | 1 |
| 1 | 1.055 | 105 | 1.7 |
| 2 | 2.045 | 102 | 1.6 |
| *Mandelic acid:* | | | |
| 0 | 0.001 | – | 570 |
| 0.01 | 0.011 | 109 | 51 |
| 0.025 | 0.022 | 87 | 14 |
| 0.05 | 0.047 | 93 | 4.1 |
| 0.1 | 0.094 | 94 | 3.1 |
| 0.25 | 0.256 | 102 | 2.1 |
| 0.5 | 0.507 | 101 | 1 |
| 2 | 1.998 | 100 | 2.6 |

tion was 7%. When the residue was derivatized with BSTFA without first dissolving the residue in ethyl acetate, the average coefficient of variation was 33%. In addition, the quantitation range with a coefficient of variation of $<10\%$ was extended from a lower limit of approximately 0.1 mg/ml in urine to approximately 0.01 mg/ml (Fig. 2).

The quantitation range for the assay was limited on the upper end by detector linearity and column loading. The upper limit for the assay was between 2 and 4 mg/ml. At a spiked-urine concentration of 2 mg/ml, PGA and mandelic acid had a coefficient of variation of 1.6% and 2.6%, respectively (Table 1). The quantitation range for the assay was limited on the lower end by increasing variability. Below a urinary concentration of approximetely 0.01 mg/ml, the coefficient of variation was $>10\%$ for both PGA and mandelic acid.

The detection limit was approximately 0.1 μg/ml, which was lower than the typical concentration of mandelic acid found in persons not occupationally exposed to styrene. The background concentration of mandelic acid in persons with no known occupational exposure to styrene ranged from 0.001 to 0.038 mg/ml ($n = 18$, with 16 repeat samples). Figure 1 shows a chromatogram from an analysis in this range. For concentrations of $>0.01$ mg/ml, the recoveries of mandelic acid and PGA ranged from 87% to 112% (Table 1). PGA recoveries exhibited no trend with concentration; mandelic acid had slightly lower recoveries at lower concentrations.

Standard curves based on spiked-urine calibrants were linear ($r^2 = 0.999$; replicate of three analyses per spiked concentration). The slopes of the two curves were slightly different (1.719 for mandelic acid and 1.853 for PGA) and were not parallel by statistical test ($P < 0.05$). Both compounds were subjected to the same analytical procedure during preparation of the standard curves. Guillemin and Bauer [7] observed that calibration curves for PGA and mandelic acid had the same slopes with their procedure, although no statistical results were reported. Since mandelic acid was the chemical quantitated, regardless of whether mandelic acid or PGA was spiked, the calibration curves theoretically should have been parallel. The slight difference in the curves did not have an effect on the quantitation and was probably a result of an unaccounted impurity in the mandelic acid.

Method parameters were investigated for their influence on recovery. The derivatization of mandelic acid (2 mg/ml) and the internal standard (0.5 mg/ml) was complete after 30 min. The reduction of PGA to mandelic acid was rapid; the amount of mandelic acid formed from a solution of 2 mg/ml PGA was constant after 30 min. Although the presence of light has been reported to destroy PGA [7, 8], we did not observe diminished recovery of PGA when the assay was performed using fluorescent room lighting.

The choice of solvent for the standards was important. We found that after storage for 1 week, PGA reacted with methanol to form 2-phenyl-2,2-dimethoxyacetophenone. The amount of acetophenone increased as the amount of PGA decreased over time. During storage in methanolic solution, mandelic acid formed the methyl ester. Methyl mandelate was found in methanol solutions of mandelic acid after only 1 day. Because of these undesirable reactions in methanol, the standards were prepared in acetonitrile. Impurities were identified by gas chromatography/mass spectrometry.

No analyte or internal standard was lost via the vacuum evaporation process as long as the vacuum was not maintained for $>20$ min after the sample had become dry; after 20 min, loss of the internal standard became noticeable. The amount of BSTFA in the reaction was optimal at 100 μl. Isooctane was used to dilute the derivatization mixture; this dilution was necessary to prevent overloading of the gas chromatograph and also had the desirable effect of precipitating unwanted reaction products of the derivatization. The trimethylsylil derivatives were relatively stable in isooctane when refrigerated in sealed vials. Analyses could be repeated on the stored samples.

As with the method of Guillemin and Bauer [7], PGA was determined by the difference between the amount of mandelic acid and the total quantity of PGA and mandelic acid. Mandelic acid was quantitated by carrying out the procedure without the reduction step. Performing the reduction step gave the total amount of mandelic acid and PGA.

We modified the method of Guillemin and Bauer [7] for use with modern capillary gas chromatography. The present method was used for analysis of human urine samples from workers exposed to styrene. BSTFA, a derivatization reagent more amenable to capillary gas chromatography, was used instead of BSA-pyridine [7]. The precision and sensitivity were improved by the use of dry extraction solvent as the derivatization solvent.

The sensitivity of the method was sufficient to detect mandelic acid in the urine of individuals with no known exposure to styrene.

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