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Note

Determination of mandelic and phenylglyoxylic acids in rat urine by high-performance liquid chromatography and by isotachophoresis

JAN SOLLENBERG*

National Board of Occupational Safety and Health, S-171 84 Solna (Sweden)

and

ANTHONY W. SMALLWOOD and LARRY K. LOWRY

Division of Biomedical and Behavioral Science, National Institute for Occupational Safety and Health, 4676 Columbia Parkway, Cincinnati, OH 45226 (U.S.A.)

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The metabolism of ethylbenzene in the rat has been well characterized following inhalation exposure [1]. The major metabolites have been identified as 1-phenylethanol, ω-hydroxyacetophenone, mandelic acid (MA), phenylglyoxylic acid (PGA), hippuric acid, and phenylaceturic acid [2]. In humans, the major urinary metabolites have been identified as MA and PGA, and these metabolites have been suggested for monitoring occupational exposure to ethylbenzene [3, 4]. Several methods have been used to determine MA and PGA in urine including gas chromatography (GC) [5], high-performance liquid chromatography (HPLC) [6], and isotachophoresis (ITP) [7].

The purpose of this study was to evaluate HPLC and ITP methods for the analysis of MA and PGA in rat urine as a part of a biotransformation study of ethylbenzene.

EXPERIMENTAL

Four male Sprague-Dawley rats weighing between 190 and 210 g were given single oral doses of 350 mg of ethylbenzene in corn oil per kilogram of body weight (0.1% of the reported LD-50). Rats were individually housed in metab-

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olism cages and had access to standard laboratory chow and tap water after dosing. Urine samples were collected as voided, diluted with distilled water, and filtered with 0.45-μm filters to remove particulates. The filtered samples were frozen at −20°C until analysis.

A 0.5-ml volume of each filtered urine sample was extracted twice with 5 ml portions of diethyl ether. The combined ether extracts were evaporated to dryness under a gentle stream of nitrogen. The residue was dissolved in 0.2 ml of distilled water. Each extract was analyzed by both ITP and HPLC.

ITP was performed as described by Sollenberg and Baldesten [7], but the leading ion and pH were modified to give optimum separation of PGA and MA from interfering substances in these rat urine samples. The leading electrolyte was 5 mmol/l trichloroacetic acid in 0.4% hydroxypropylmethylcellulose adjusted to pH 3.35 with beta-alanine. The terminating electrolyte was 5 mmol/l caproic acid. The capillary length was 330 mm. Samples were analyzed on an LKB 2127 Tachophor with an initial current of 200 μA followed by 100 μA prior to detection of the zones, and the UV detection wavelength was 254 nm. The instrument was calibrated with aqueous standards. MA was obtained from Eastman Kodak (Rochester, NY, U.S.A.). PGA was obtained from Aldrich (Milwaukee, WI, U.S.A.).

A new HPLC procedure was developed for the combined analysis of MA and PGA. Two 250 × 4.5 mm I.D. HPLC columns were connected in series: the first column was a Vydac cation exchange column, and the second was a Waters μ-Bondapak C_{18} reversed-phase column. The particle size was 10 μm in both columns. Analysis was done on a Varian 8500 dual-pump HPLC equipped with a Waters U6K injector and a Varian Varichrome UV detector set at 210 nm. Peak heights were measured with a Hewlett-Packard Model 3390 integrator. A 20-μl volume of sample was injected and the following gradient program started: 100% "A" (50 ml of acetonitrile, 0.2 ml of concentrated sulfuric acid diluted to 1.0 l with distilled water) for 5 min, increase "B" (250 ml of acetonitrile, 0.2 ml of concentrated sulfuric acid diluted to 1.0 l with distilled water) by 10% per min for 10 min, hold at 100% "B" for 10 min, then decrease "B" by 10% per min for 10 min. The total flow-rate was 3 ml/min and the columns were at room temperature. Under these conditions, approximate retention times of 3.0 min and 3.9 min were observed for PGA and MA, respectively. This procedure was also capable of separating other ethylbenzene metabolites and their polar conjugates, but these were not determined in this study.

RESULTS AND DISCUSSION

The recovery of MA and PGA from rat urine was determined by analysis of pooled rat urine samples with MA or PGA added at 0.5, 2.0, 5.0 and 7.0 mmol/l. Duplicate urine samples were analyzed by HPLC using aqueous standards. Recoveries were calculated at each concentration and averaged 93.7% (S.D. = 12.1%) for MA and 100.6% (S.D. = 11.9%) for PGA. Recoveries were consistent over the range 0.5—7.0 mmol/l.

Aqueous standards analyzed by HPLC show a within-day R.S.D. of 0.31% for MA and 1.57% for PGA. Day-to-day R.S.D. values for the same aqueous

standards were 2.28% for MA and 2.01% for PGA. The analysis of a limited number of rat urine extracts showed similar variation. An aqueous standard of MA was analyzed by ITP on ten occasions and a PGA standard on five occasions during a 5-week period. The R.S.D. values were 4.3% and 1.8%, respectively.

Fig. 1 shows the comparison of HPLC and ITP for MA in 59 rat urine samples over a concentration range of 0.01—1.6 mmol/l. The correlation coefficient of 0.982 indicates that the two methods give similar results. The

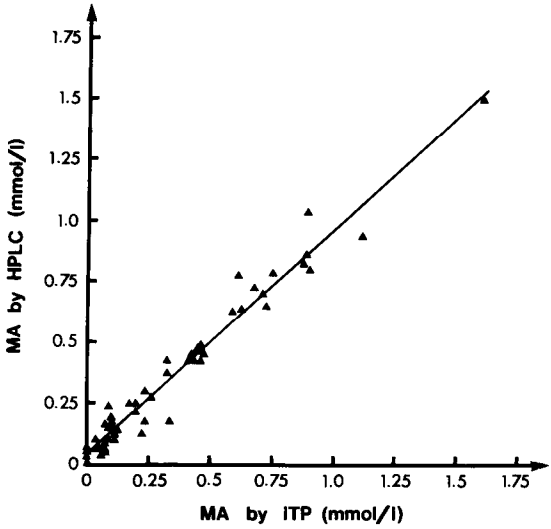

Fig. 1. Linear regression plot of MA (mmol/l) by HPLC vs. ITP. Specimens were from rats dosed with 0.1% of the LD-50 for ethylbenzene. The regression equation is HPLC = 0.0037 + 0.930 (ITP) with a correlation coefficient, $r = 0.982$.

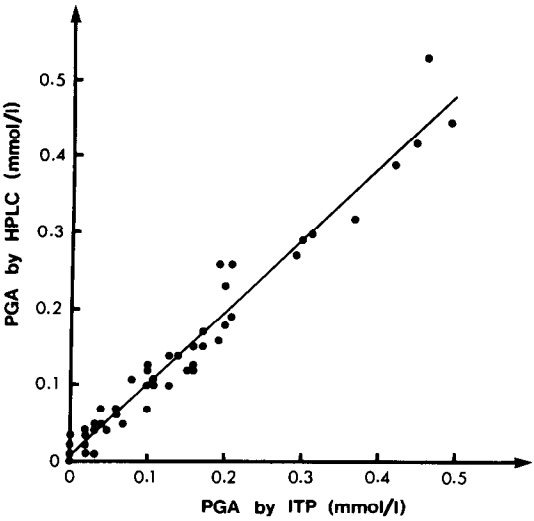

Fig. 2. Linear regression plot of PGA (mmol/l) by HPLC vs. ITP. Specimens were as described in Fig. 1. The regression equation is HPLC = 0.0039 + 0.958 (ITP) with a correlation coefficient, $r = 0.979$.

estimated lower limit of quantitation, defined as twice the lower limit of detection for MA in rat urine by HPLC, is 0.01 mmol/l. The corresponding limit for ITP is 0.04 mmol/l.

Fig. 2 shows the comparison of HPLC and ITP for PGA in the same 59 rat urine samples over a concentration range of 0.01—0.53 mmol/l. The correlation coefficient of 0.979 again indicates that the two methods give similar results. The estimated lower limits of quantitation for PGA in rat urine by HPLC is 0.01 mmol/l. The corresponding limit for ITP is 0.02 mmol/l.

There is no significant difference between the results obtained by the two methods for MA ($P = 0.075$) and PGA ($P = 0.55$) according to the paired t-test. These calculations are based on results from all urine samples. The two procedures used here produce comparable data. The HPLC method is more sensitive for these analytes, although the ITP method is more rapid. Both methods have sufficient sensitivity to be used interchangeably depending on the availability of equipment.

The excretion of MA and PGA in rat urine begins with the first voiding after dosing, reaches a peak concentration in ca. 15—19 h, and returns to undetectable levels by 48 h.

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