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Simultaneous high-performance liquid chromatographic determination of urinary mandelic and phenylglyoxylic acids as indirect evaluation of styrene exposure

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Abstract

Styrene is rapidly metabolised to mandelic acid (MA) and phenylglyoxylic acid (PGA), which are excreted in urine. In this work, we have developed a simple, sensitive and specific high-performance liquid chromatographic method with minor sample preparation procedures for the simultaneous determination of MA and PGA in urine of workers exposed to styrene. Moreover, urine samples from workers of two plastic factories were analysed, styrene exposure levels of the workers were estimated and data obtained from the two factories were compared.

Keywords: Mandelic acid; Phenylglyoxylic acid; Styrene; HPLC; Urine

1. Introduction

Styrene (CAS No. 100-42-5) is a colourless to yellowish, very refractive, oily liquid which on exposure to air or light undergoes polymerisation and oxidation [1]. Commercial production is carried out by reacting benzene with ethylene catalytically, followed by dehydrogenation of the ethylbenzene obtained to styrene [2]. The highest human exposure to styrene occurs during the production of fibreglass reinforced polyester products [3], where unsaturated polyester resins containing about 40% of styrene as a reactive diluent are commonly used. In such factories, during lamination by hand procedures, as much as 10% of the styrene can evaporate into the workroom air.

The two major effects of acute exposure to styrene in humans include irritation (both skin and respiratory tract) and central nervous system depression [4]. Also, change in semen quality and sperm structure [5], increased mutation frequencies at several loci [6,7] and elevated incidence of single-strand breaks, sister chromatid exchanges and chromosome aberrations in peripheral lymphocytes [8–10] have been associated with chronic styrene exposure in the workplace. However, data regarding potential carcinogenicity are inconclusive [11].

Styrene penetrates the organism by inhalation, since skin resorption plays only a minor role in most practical situations [12]. Hepatic metabolisation con-

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stitutes its main way of elimination. Metabolites formed are excreted in urine, with 85% of the absorbed styrene eliminated as mandelic acid (MA) and 10% as phenylglyoxylic acid (PGA) [13]; only less than 2% of the inhaled and retained dose is exhaled as unchanged styrene [4].

Biological monitoring of a population exposed to some toxic or contaminant in the workplace has several advantages over air monitoring reflecting effective internal dose [14]. Moreover, the choice of urine as biological fluid for monitoring makes frequent sample collection possible since the procedure is noninvasive and does not imply damage to the individuals.

Gas chromatographic [15–18], liquid chromatographic [19–25] and supercritical fluid chromatographic [26] methods have been used to quantify the metabolites of styrene, most of them including derivatization [15], liquid–liquid extraction [15,17,24,25] or solid-phase extraction [20,22] procedures in order to obtain concentrations high enough to quantify. This work describes a direct high-performance liquid chromatographic (HPLC) method for the simultaneous determination of the styrene metabolites MA, PGA, hippuric acid (HA) and also of 2-methylhippuric acid (mHA) in human urine. In addition, urine samples from workers of two fibreglass reinforced plastic factories located in A Coruña (Spain) were analysed, and levels of styrene exposure were estimated from urine concentration of its two main metabolites: MA and PGA.

2. Experimental

2.1. Chemicals

Mandelic acid, hippuric acid, 2-methylhippuric acid and benzoylformic acid (phenylglyoxylic acid) were supplied by Sigma (St. Louis, MO, USA). Anhydrous potassium dihydrogenphosphate, acetic acid, 85% orthophosphoric acid, methanol (LiChrosolv grade) and acetonitrile (LiChrosolv grade) were obtained from Merck (Darmstadt, Germany). Milli-Q (Millipore, Bedford, MA, USA) deionised water was used for all aqueous solutions.

2.2. Equipment and chromatographic conditions

The HPLC system consisted of a Model 616 quaternary pump, a Model 600S system controller, a 717 plus autosampler, a temperature controller module and a 996 diode array detector, all from Waters (Milford, MA, USA). Millennium software was used for system and data management. The separation was performed in the isocratic mode at a 1 ml/min flow-rate and 25°C on a Waters Symmetry C_{18} cartridge column (150×3.9 mm) with a Sentry guard column (20×3.9 mm) packed with Symmetry C_{18}. The mobile phase was a mixture of 50 m*M* KH_2PO_4 in 1% acetic acid (adjusted to pH 2.5 with 85% orthophosphoric acid)–acetonitrile (90:10, v/v). The UV–Vis absorbance over the 195–400 nm range was registered and the wavelength used for quantification was 225 nm for all compounds.

2.3. Standard preparation

Stock solutions of individual standards (1000 mg/l) were made in water (MA and PGA) or in methanol–water (1:1, v/v) (HA and mHA) and stored at 4°C. Standard curves were obtained by analysing appropriate dilutions of stock solutions in the mobile phase. Calibration curves were constructed by plotting the peak height against the concentration of each analyte.

2.4. Urine samples

Urine samples from non-exposed individuals were collected in glass containers for method evaluation. Urine samples from 22 workers of two fibreglass reinforced plastic factories were collected at the end of the workshift on Friday and before starting work on Monday, in 3 different weeks. Samples were stored at −20°C until analysis (less than 1 week). For HPLC analysis urine samples were diluted 10-fold with mobile phase, centrifuged at 2000 rpm for 10 min and an aliquot of 25 μl was injected directly into the chromatographic system. Creatinine was determined by the Jaffé colorimetric method using picric acid (commercial kit from Boehringer Mannheim).

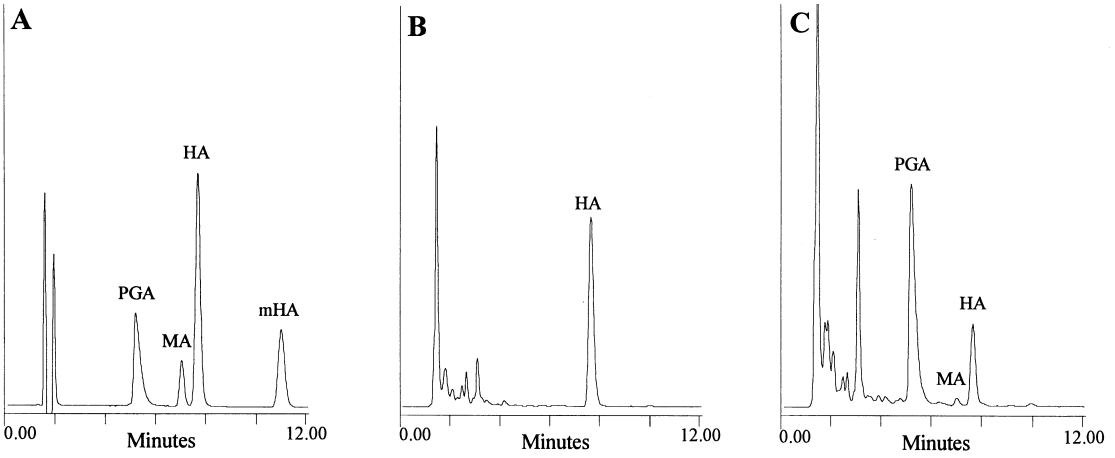

Fig. 1. Chromatograms of a standard mixture of PGA, MA, HA and mHA in mobile phase (A); 10-fold diluted urine from a non-exposed individual (B) and 10-fold diluted urine from a styrene exposed worker (C). Extracted wavelength 225 nm (0.5 AUFS).

2.5. Precision

Within-day precision for each metabolite was determined by analysing five replicates of diluted urine samples spiked at five concentrations for each metabolite. Between-day precision was evaluated by assaying diluted urine samples spiked at five concentrations for each analyte on 3 separate days. Relative standard deviations (RSDs) were calculated from peak height values at each concentration of the analyte.

2.6. Calculations

Styrene exposure levels (C_{exp}) in ppm have been calculated from post-shift urinary concentrations (mg/g creatinine) of MA+PGA (expressed as MA) following the relationships found by Elia et al. [15] [$C_{exp}=10^{1.012\ \log(MA+PGA)-1.24}$] and Ikeda et al. [21] [$C_{exp}=0.051(MA+PGA)-4.8$]. The regression line from Ikeda et al. does not start from the origin of the Cartesian axis, so when the sum MA+PGA was too low giving a negative value for styrene concentration, this was assumed to be 0.

2.7. Statistical analysis

The non-parametric Mann–Whitney *U*-test (two-tailed) was applied to evaluate the statistical significance of the difference between groups. Analyses were carried out using the SPSS statistical package, version 9.0.

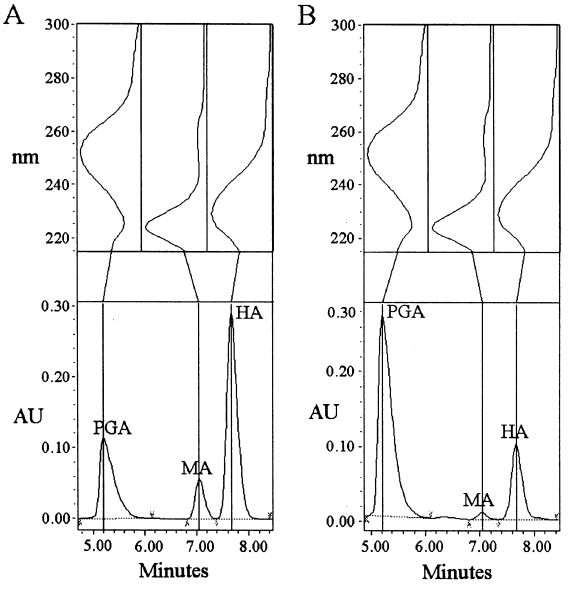

Fig. 2. Chromatograms and peak apex spectra of a standard mixture (A) and 10-fold diluted urine from a worker exposed to styrene (B).

Table 1
Detection and quantification limits in urine samples

Analyte	DL (mg/l), $S/N=3$	QL (mg/l), $S/N=9$
PGA	3.0	9.0
MA	7.6	22.8
HA	1.5	4.5
mHA	1.2	3.6

S/N: Signal-to-noise ratio.

3. Results and discussion

The adjustment of mobile phase pH to 2.5 renders the styrene metabolites in a non-ionised state allowing adequate retention and peak shape in the C_{18} column used. Addition of acetic acid to the mobile phase improved the shape of the PGA peak. Fig. 1 shows a representative chromatogram of a standard mixture (A), 10-fold diluted blank urine (from a non-exposed individual) (B) and 10-fold diluted urine from an exposed worker (C). A good separation was obtained, with all analytes being resolved and showing adequate peak shapes. The retention times were 5.4 min for PGA, 7.1 min for MA, 7.7

Table 2
Within- and between-day precisions of PGA, MA, HA and mHA

	Concentration[a] (mg/l)	Within-day[b] ($n=5$)	Between-day[b] ($n=3$)
PGA	12	1.08	3.40
	30	0.75	1.03
	60	0.11	0.45
	84	0.52	1.23
	120	0.32	1.09
MA	10	0.36	2.62
	26	0.68	1.33
	51	0.20	0.42
	72	0.39	0.43
	102	0.51	0.63
HA	11	0.18	0.26
	27	0.30	0.82
	53	0.20	0.35
	74	0.31	0.27
	106	0.47	0.56
mHA	10	1.55	1.00
	25	0.34	0.73
	50	0.07	0.30
	71	0.34	0.26
	101	0.14	0.57

[a] Concentration of 10-fold diluted urine.
[b] RSD (%).

Table 3
Values of MA and PGA in post-shift urine samples from workers of factory 1 collected on Friday in three different samplings; corresponding values of styrene exposure following Elia et al. [15] and Ikeda et al. [21] and MA/PGA ratio

Sampling	Individual	MA (mg/g creatinine)	PGA (mg/g creatinine)	Exposure [15] (ppm)	Exposure [21] (ppm)	MA/PGA
1	1	44.29	25.44	4.24	0.0	1.74
1	2	197.71	41.56	14.74	7.4	4.76
1	3	93.18	47.52	8.63	2.4	1.96
1	4	129.89	87.9	13.44	6.4	1.48
1	5	197.86	101.05	18.50	10.5	1.96
1	6	43.93	29.29	4.46	0.0	1.50
2	1	262.24	175.11	27.21	17.6	1.50
2	2	91.94	63.61	9.56	3.2	1.45
2	3	125.04	108.75	14.45	7.2	1.15
2	6	365.74	202.39	35.44	24.3	1.81
3	1	46.29	54.89	6.20	0.4	0.84
3	2	166.67	68.18	14.48	7.2	2.44
3	3	86.59	83.97	10.51	4.0	1.03
3	6	547.09	293.74[a]	52.70[b]	38.3	1.86

[a] Over recommended BEIs.
[b] Over TLV-TWA.

min for HA and 11.1 min for mHA. Analysis of 10-fold diluted blank urine showed the presence of HA, a normal constituent of human urine (from canned foods, fruits and endogenous protein metabolism) and also a minority metabolite of styrene. Under our experimental conditions, peaks from other urine components that could interfere with the compounds of interest were not observed. No differences were found between peak apex spectra from pure standards (Fig. 2A) and from 10-fold diluted urine from exposed individuals (Fig. 2B). Furthermore, purity of sample peaks was confirmed by

Table 4
Values of MA and PGA in post-shift urine samples from workers of factory 2 collected on Friday in three different samplings; corresponding values of styrene exposure following Elia et al. [15] and Ikeda et al. [21], and MA/PGA ratio

Sampling	Individual	MA (mg/g creatinine)	PGA (mg/g creatinine)	Exposure [15] (ppm)	Exposure [21] (ppm)	MA/PGA
1	1	163.17	77.38	14.85	7.5	2.11
1	2	77.75	98.54	10.87	4.3	0.79
1	3	95.14	36.44	8.06	1.9	2.61
1	4	117.98	69.49	11.54	4.8	1.70
1	5	52.95	45.65	6.03	0.3	1.16
1	6	906.88[a]	149.12	66.18[b]	49.2[b]	6.08
1	7	46.51	35.53	5.01	0.0	1.31
1	8	341.07	182.97	32.66	22.0	1.86
1	9	1227.39[a]	333.98[a]	98.41[b]	75.1[b]	3.68
1	10	391.22	185.11	35.94	24.7	2.11
1	11	664.52	268.28[a]	58.49[b]	43.0	2.48
1	12	90.64	43.24	8.20	2.1	2.10
1	13	166.74	55.29	13.68	6.6	3.02
1	14	233.35	77.38	19.22	11.1	3.02
1	15	123.36	65.62	11.63	4.9	1.88
2	1	206.99	60.66	16.52	8.9	3.41
2	2	70.02	64.55	8.26	2.1	1.08
2	3	193.94	39.94	14.40	7.2	4.86
2	4	52.72	26.80	4.84	0.0	1.97
2	5	77.91	52.28	7.98	1.9	1.49
2	6	724.87	146.91	54.53[b]	39.8	4.93
2	7	250.58	94.55	21.38	12.9	2.65
2	9	584.69	209.74	49.70	35.9	2.79
2	10	551.23	193.94	46.58	32.3	2.84
2	11	914.06[a]	308.23[a]	76.85[b]	57.7[b]	2.97
2	12	33.33	18.97	3.17	0.0	1.76
2	13	248.00	98.99	21.50	13.0	2.51
2	14	296.38	131.53	26.59	17.1	2.25
2	15	92.37	52.63	8.90	2.6	1.76
2	16	575.34	193.5	48.07	34.5	2.97
3	2	194.63	107.92	18.73	10.7	1.80
3	3	137.59	62.24	12.30	5.4	2.21
3	6	257.33	76.00	20.63	12.3	3.39
3	10	514.8	166.00	42.50	30.0	3.10
3	11	1370.26[a]	554.91[a]	121.77[b]	93.8[b]	2.47
3	14	159.31	51.47	12.98	6.0	3.10
3	15	96.43	72.75	10.41	3.9	1.33
3	16	92.06	39.11	8.03	1.9	2.35

[a] Over recommended BEIs.
[b] Over TLV-TWA.

assessing spectral homogeneity across the chromatographic peaks.

Calibration curves for all compounds were linear over the concentration range of 5–100 mg/l for PGA (r=0.9985), 5–200 mg/l for MA (r=0.9999), 5–100 mg/l for HA (r=0.9998) and 5–100 mg/l for mHA (r=0.9999). Detection and quantification limits in urine samples are reflected in Table 1. Detection limits determined on the basis of a signal-to-noise ratio of 3 ranges from 1.2 to 7.6 mg/l of urine, depending on the compound being studied. Recommended biological exposure indices (BEIs) are 250 mg/l or 240 mg/g creatinine for PGA and 1 g/l or 800 mg/g creatinine for MA [28]. Detection and quantification limits of the present method are far below BEIs for PGA and MA, making it suitable for the quantitative determination of styrene metabolites in urine. Data showing within- and between-day precision of the method for each analyte are summarised in Table 2. The small values of RSD obtained (lower than 2 and 4%, within-day and between-day, respectively) showed that the determination of each analyte in diluted urine samples was highly reproducible with this HPLC procedure.

Several reports describe positive correlations between urinary concentrations of styrene metabolites and styrene concentration in the ambient air. To estimate exposure of the workers from urinary MA and PGA concentrations, the relationships found by Elia et al. [15] and Ikeda et al. [21] were chosen for the following reasons: (1) correlation coefficients (r=0.96, P<0.001 and r=0.86, P<0.01, respectively) are better than others reported [14,27]; (2) they use post-shift urine samples instead of samples collected the next morning, since MA and PGA peak concentration in urine appears at the end of exposure [28]; (3) the sum of MA and PGA concentrations is used, because if differences in the individual rates of metabolisation occur, the sum of both metabolites will be less influenced than individual values [29], and (4) metabolite concentrations are corrected for urinary creatinine in order to eliminate the inter-individual variation in urine production rate. Moreover, it has been shown that creatinine correction significantly improves the correlation with exposure [29].

As reflected in Tables 3 and 4, values obtained by applying the regression line of Elia et al. are very similar but slightly higher than values obtained by applying the one of Ikeda et al. Furthermore, it can be observed that when a urine sample exceeds the recommended BEIs for MA or for PGA the esti-

Table 5
Values of MA and PGA in urine samples from workers of factory 1 collected on Monday morning in three different samplings, showing the break days during the weekend and the reduction % with regard to the amount of MA+PGA (expressed as MA), MA and PGA in urine samples collected on Friday afternoon

Sampling	Individual	Break days	MA (mg/g creatinine)	PGA (mg/g creatinine)	Reduction %		
					MA+PGA	MA	PGA
1	2	2	ND	ND	100.00	100.00	100.00
1	3	2	ND	ND	100.00	100.00	100.00
1	4	2	ND	ND	100.00	100.00	100.00
1	5	2	15.28	15.48	89.69	92.28	84.68
1	6	2	NQ	ND	100.00	100.00	100.00
2	1	2	ND	ND	100.00	100.00	100.00
2	2	2	44.66	ND	71.44	51.42	100.00
2	3	2	26.20	ND	88.86	79.05	100.00
2	6	2	23.74	ND	95.84	93.51	100.00
3	1	2	ND	ND	100.00	100.00	100.00
3	2	2	42.29	ND	82.06	74.63	100.00
3	3	2	ND	ND	100.00	100.00	100.00
3	6	2	76.61	32.02	87.09	86.00	89.10

ND: Not detected.
NQ: Not quantified.

mated value for styrene exposure of that individual usually also exceeds the recommended threshold limit value-time weighted average (TLV-TWA) of 50 ppm [28], demonstrating the suitability of these relationships to make an indirect estimation of styrene exposure from post-shift urinary MA+PGA concentrations.

Statistical analysis of data obtained showed no

Table 6
Values of MA and PGA in urine samples from workers of factory 2 collected on Monday morning in three different samplings, showing the break days during the weekend and the reduction % with regard to the amount of MA+PGA (expressed as MA), MA and PGA in urine samples collected on Friday afternoon

Sampling	Individual	Break days	MA (mg/g creatinine)	PGA (mg/g creatinine)	Reduction%		
					MA+PGA	MA	PGA
1	2	1	7.08	11.13	89.66	90.89	88.71
1	4	1	17.38	12.67	83.96	85.27	81.77
1	5	1	NQ	10.55	89.22	100.00	76.89
1	9	1	15.22	9.71	98.40	98.76	97.09
1	11	1	25.13	24.78	94.63	96.22	90.76
1	13	1	NQ	4.94	97.76	100.00	91.07
1	14	1	9.36	7.68	94.50	95.99	90.07
1	15	1	12.33	12.92	86.61	90.00	80.31
2	2	1	8.29	14.58	82.97	88.16	77.41
2	5	1	15.55	20.33	72.38	80.04	61.11
2	6	1	156.45	56.06	75.59	78.42	61.84
2	11	1	70.09	55.03	89.74	92.33	82.15
2	12	1	NQ	10.28	80.19	100.00	45.81
2	13	1	14.03	15.90	91.35	94.34	83.94
2	14	1	45.40	23.96	83.78	84.68	81.78
2	15	1	22.04	25.23	67.33	76.14	52.06
3	2	1	NQ	9.44	96.85	100.00	91.25
3	3	1	NQ	16.18	91.83	100.00	74.00
3	14	1	NQ	7.50	96.41	100.00	85.43
3	15	1	15.94	16.47	80.82	83.47	77.36
1	1	2	22.44	9.21	86.85	86.25	88.10
1	3	2	24.13	8.04	75.56	74.64	77.94
1	6	2	27.63	14.00	96.05	96.95	90.61
1	7	2	NQ	8.77	89.24	100.00	75.32
1	10	2	29.60	18.28	91.69	92.43	90.12
1	12	2	NQ	4.37	96.70	100.00	89.89
2	1	2	94.85	34.15	51.78	54.18	43.70
2	3	2	NQ	13.13	94.33	100.00	67.13
2	4	2	14.24	11.08	68.12	72.99	58.66
2	7	2	23.76	23.76	86.19	90.52	74.87
2	9	2	21.73	28.28	93.68	96.28	86.52
2	10	2	92.30	54.94	80.21	83.26	71.67
2	16	2	46.98	29.24	90.07	91.83	84.89
3	6	2	33.00	17.82	84.73	87.18	76.55
3	10	2	142.33	76.55	67.81	72.35	53.89
3	11	2	40.79	35.35	96.04	97.02	93.63
3	16	2	24.21	12.36	72.11	73.70	68.40

NQ: Not quantified.

difference in styrene exposure between factories 1 and 2. It has been described that MA/PGA ratio increases as a direct function of solvent exposure [21,29,30], so the significant difference ($P \leq 0.005$) found in that ratio between the two factories (mean±SE: 1.82±0.25 for factory 1 and 2.52±0.17 for factory 2) could indicate some distinction between both exposures not reflected in the statistical analysis of estimated styrene levels.

The reduction % of MA and of the sum MA+PGA in urine samples collected on Monday morning, with regard to those collected on Friday afternoon, did not show any difference if the individuals had had 1 (38 h) or 2 (62 h) break days during the weekend (Tables 5 and 6). Nevertheless there was a significant increase ($P=0.061$) in reduction % of PGA in individuals with 1 break day in relation to individuals with 2 break days. This may be due to the excretion kinetics of the metabolites, since MA and the sum MA+PGA present a diphasic elimination with two half-lives [28,30], so their excretion curves exhibit an undoubtedly biexponential shape, and their urinary levels decrease fast during the first 24 h and then much slower. However, the excretion curve of PGA is almost linear, characterised by a unique half-life, and its urinary concentrations decline in a constant way, being this decline slower than the previous ones in the first 24 h and then faster, making it possible to find differences between the workers who had not been exposed for 1 or 2 days.

The reduction % of the sum MA+PGA was compared between factories taking into account only the individuals with 2 break days. It was observed that this parameter is significantly lower ($P<0.05$) in factory 2 than in factory 1 (83.60±3.07 and 93.46±2.53%, respectively), probably because of an accumulation of styrene in fatty tissues [4,13] as a consequence of chronic exposure to apparent higher levels. We do not think this may be due to saturation in metabolisation enzymes, since it has been described for exposure levels between 100 and 200 ppm [13,31], not usual in the studied factories.

4. Conclusion

The chromatographic method here presented is simple, sensitive and specific, allowing the simultaneous measurement of PGA and MA in urine samples in less than 15 min and without needing expensive and time consuming sample preparation procedures. In addition, this methodology has shown to be suitable to make an indirect estimation of styrene exposure in workers of fibreglass reinforced plastic factories.

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