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Simultaneous determination of mandelic acid enantiomers and phenylglyoxylic acid in urine by high-performance liquid chromatography with precolumn derivatization

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Abstract

A reversed-phase HPLC method for the simultaneous quantitative determination of mandelic acid enantiomers (MA) and phenylglyoxylic acid (PGA) in urine is described. MA and PGA were extracted with ethyl acetate from urine at acidic pH and derivatized with *S*-(−)-1-(1-naphthyl) ethylamine. A ZORBAX SB-C_{18} column (250 mm × 4.6 mm i.d., 5 μm, Agilent, USA) was used with a mobile phase composed of methanol–10 mmol/L phosphate buffer [pH 2.5 (65:35, v/v)] at a flow-rate of 0.8 ml/min. Detection was set at UV wavelength of 254 nm. The mean absolute recoveries were 94.2%, 91.9%, 92.5% and 86.3% for *S*-MA, *R*-MA, PGA and salicylic acid (I.S.), respectively. The intra- and inter-day precisions determined at three different concentrations ranged from 2.8% to 4.8%, 0.7% to 7.7% and 1.3% to 6.8%, respectively. The lower limits of detection for MA enantiomers and PGA in urine were 1 μg/ml and the lower limits of quantification were 5 μg/ml (R.S.D. < 10%, $n = 5$). The method has been applied to determine the urinary excretion of MA enantiomers and PGA from Sprague–Dawley rats after orally administered with styrene.

Keywords: Mandelic acid enantiomers; Phenylglyoxylic acid; Styrene; Indirect enantioseparation; Precolumn derivatization

1. Introduction

Mandelic acid (MA) and phenylglyoxylic acid (PGA) are major urine metabolites of styrene in human which hence are used as biological indicators of occupational exposure to styrene [1]. Styrene is widely used in plastic industry and has been implicated as reproductive toxicant, neurotoxicant and possible carcinogen. In rodents and human, styrene can be metabolized to styrene epoxide (SO) by the microsomal cytochrome P-450 dependent liver monooxygenase and further metabolized to 1,2-phenylethanediol by epoxide hydrolase, then 1,2-phenylethanediol can be metabolized to MA and PGA that are excreted in urine [2]. Recent studies indicated that styrene epoxide is accounted for the styrene toxicity and contribute to the genotoxicity of styrene in human blood lymphocytes [3]. Both styrene epoxide and 1,2-phenylethanediol is chiral and shows stereo-selectivity in metabolism in vivo leading to different urinary excretions of mandelic acid enantiomers and phenylglyoxylic acid [4]. Thus, the investigation of phenylglyoxylic acid and the enantiomeric composition of MA in urine would be helpful to illuminate the toxicity of styrene.

The common strategy for study of MA and PGA were performed in two steps. Firstly, PGA and the total MA were determined. Secondly, the enantiomeric composition of MA was investigated using chiral methods. There have been some reports concerning enantioseparation of MA including GC [5], HPLC/chiral ligand exchange [6], CE [7] and CEC [8]. However, only one paper described the simultaneous enantioselective analysis of MA enantiomers and PGA in urine samples. In that report, GC chiral DEX columns were used but column efficiency deteriorated gradually and the sample preparation was rather tedious as two derivatization procedures were needed [5]. The aim of this study was to develop a HPLC method for the simultaneous analysis of mandelic acid enantiomers and phenylglyoxylic acid in urine samples to investigate the stereoselective aspect of styrene metabolism.

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2. Experiment

2.1. Equipments and reagents

A system of HPLC LC-10A (Shimadzu Corporation, Kyoto, Japan) with a UV–vis detector was used. MA enantiomers were kindly donated by Yiming Fine Chemicals Ltd (Taixing, China), enantiomeric purity >99%. Phenylglyoxylic acid and *S*-(−)-1-(1-naphthyl) ethylamine (*S*-NEA) were purchased from Sigma Chemical Company (St. Louis, MO, USA). 1-Hydroxybenzotriazole (HOBT) was obtained from ACROS ORGANICS (New Jersey, USA). 1-(3-Dimetthylaminopropyl)-3-ethylcarbodiimide HCl (EDC) was purchased from Hangli Fine Chemical Ltd (Chengdu, China). Methanol was of HPLC grade and other chemicals were of analytical grade.

2.2. Chromatographic conditions

Chromatography was performed on a ZORBAX SB-C_{18} column (250 mm × 4.6 mm, i.d., 5 μm, Agilent, USA) with a C_{18} guard column (20 mm × 4.6 mm, i.d., 5 μm). The mobile phase was consisted of methanol–phosphate buffer (pH 2.5) (65:35, v/v). The flow-rate was set at 0.8 ml/min. Detection was set at UV wavelength of 254 nm. The column temperature was maintained at room temperature. The injected volume was 20 μl.

2.3. Preparation of standard solutions

The stock solutions of *RS*-MA, *R*-MA, *S*-MA, PGA and salicylic acid (1 mg/ml) were prepared in methanol and stored at 4 °C before use.

2.4. Sample preparation

Urine sample was diluted five-fold with distilled water, prior to extraction. To 100 μl diluted urine sample, 10 μl of salicylic acid (I.S.) solution and 20 μl of 5 mol/L hydrochloric acid solution were added. The mixed sample was then extracted with 0.5 ml ethyl acetate. After vortexing for 3 min, sample was centrifuged at 10,000 × *g* for 5 min. The organic layer was transferred to another tube and evaporated to dryness under nitrogen. To the residue, the following were added in order: 100 μl of HOBT (containing 2% pyridine, v/v), EDC and *S*-NEA solutions (10 mg/l, all prepared freshly in methylene dichloride). The mixture was vortexed gently and allowed to stay at 40 °C for 1 h. The solution was evaporated to dryness under nitrogen. The residue was reconstituted with 200 μl of the mobile phase. An aliquot of 20 μl of the resulting solution was injected into the LC system for analysis.

2.5. Preparation of calibration curves

Blank rat urine was spiked with various amounts of MA and PGA, final concentrations were 5, 25, 50, 125 and 250 μg/ml. The samples were treated and assayed as described above. Calibrations were performed by a least-squares linear regression of peak area ratios (PAR) of each analyte to internal standard versus the corresponding standard concentration. The slope, intercept and correlation coefficient (r^2) were calculated using Microsoft Excel software.

2.6. Sensitivity

The lower limit of quantification (LLOQ) was defined as the lowest compound concentration that could be determined with acceptable precision (i.e. R.S.D. ≤ 20%) and accuracy (i.e. recovery of 100 ± 20%). The limit of detection was defined as the amount that could be detected with a signal-to-noise ratio of 3.

2.7. Assay precision accuracy and absolute recovery

Quality control (QC) samples were prepared by spiking blank rat urine with various amounts of MA and PGA, final concentrations were 5, 25 and 125 μg/ml. The intra-day precision was determined by analyzing each QC sample ($n = 5$) on the same day. The inter-day precision was determined by analyzing each QC sample over a period of 5 days. The R.S.D. was used as an index of precision. Accuracy was calculated by comparing the calculated concentrations of QC samples with their nominal values. The absolute recoveries were determined by spiking 100 μl aliquots of blank urine with various amounts of each analyte (5, 25 and 125 μg/ml) and 10 μl solution of internal standard. The samples were prepared as described in Section 2.4. In another set of tubes, equivalent amounts of each analyte and the internal standard were added and followed by the derivatization procedure. Recovery was assessed by comparing the chromatographic peak area of each analyte for the extracted urine standard to those obtained from equivalent amounts of standard with the same concentration ($n = 5$).

2.8. Stability

The stability of *R*-MA, *S*-MA, PGA and salicylic acid in methanol were evaluated over 7 days. The stability of *R*-MA, *S*-MA and PGA in urine stored at −20 °C was evaluated at two concentration levels (25 and 250 μg/ml). The short-term storage stability at room temperature and after three freeze–thaw cycles was investigated. The samples were analyzed after 1 month to investigate the long-term storage stability. The stability was assessed by comparing the mean value of each analyte with the initial one and expressed as a percentage of the initial value. The stability of amides in mobile phase at room temperature was assessed by injecting a sample at different time intervals.

2.9. Assay of MA enantiomers and PGA in rat urine

Twelve male Sprague–Dawley rats (obtained from Laboratory Animal Center of Zhejiang University, Hangzhou, China), 200 ± 10 g weight, were fasted overnight before treatment. Styrene was administrated orally (mixed with tea oil) at 10, 50 and 100 mg/kg to three groups ($n = 4$), respectively. Rats were kept in stainless steel metabolic cages. Food was not allowed and water was free accessed throughout the experiment. Urine

Table 1
The calculated contents of MA enantiomers and PGA in two urine samples with different dilution ratios (mg)

Sample no.	Dilution ratio	*S*-MA	*R*-MA	R.S.D. (%)	PGA	R.S.D. (%)
1	1 to 5	N.D.	5.916	4.9	3.072	4.9
	1 to 10		5.368		3.138	
	1 to 20		5.592		2.857	
2	1 to 5		5.761	3.8	2.247	6.9
	1 to 10		5.429		2.396	
	1 to 20		5.841		2.087	

N.D.: not detected.

samples were collected for 24 h, then centrifuged at 3000 × *g* for 10 min and stored at −20 °C until analysis. The concentrations of MA and PGA in rat urine were calculated from the regression equations described in Section 2.5. The contents of MA and PGA in rat urine were calculated as follows:

$$\text{content} = \text{mean calculated concentration} \times \text{volume of urine} \times \text{dilution ratio}$$

3. Results and discussion

3.1. Chromatography

S-(−)-1-(1-Naphthyl) ethylamine has been successfully applied to the enantioseparation of some 2-arylpropionic acid non-steroidal anti-inflammatory drugs and mandelic acid using EDC and HOBT as coupling agents [9]. PGA is an acidic compound possessing a carboxyl group in its structure. It could also react with *S*-(−)-1-(1-naphthyl) ethylamine, thus allowing determination of three metabolites (*S*-MA, *R*-MA and PGA) in one run. Another advantage is that using the described detected wavelength, the interference of endogenous compounds are greatly reduced while it would be severe at 225 nm, the UV wavelength used for direct analysis of MA and PGA.

3.2. The effect of dilution on the quantification

Urine samples were diluted before extraction because the concentrations of MA and PGA in urine samples were too high to be accurately determined. Two urine samples of the highest dose group were diluted as 1:5, 1:10 and 1:20. The diluted samples were treated as described in Section 2.4. As can be seen in Table 1, the R.S.D. of final contents of analytes were less than 5% which indicated that the derivatization procedures were complete and quantitatively. The dilution ratio was set at 1:5 in the following analysis, considering the sensibility of the method.

3.3. The effects of reaction time and amounts of derivatizing agents on the derivatization

Two diluted urine samples as in Section 3.2 were used to investigate the effects of reaction time and amounts of coupling agents on the derivatization. The extracted samples were added with 100 μl of HOBT, EDC and *S*-NEA (10 mg/ml). Derivatization were carried out at 40 °C for 0.5, 1, 2, 3, 4, 5 and 24 h, respectively. After 1 h, no significant increases of peak area of each analyte were observed, so the reaction time was set at 1 h in the following urine sample analysis. The extracted samples were also added with different concentrations of HOBT, EDC and *S*-NEA (1, 2.5, 5 and 10 mg/ml), respectively. As can be seen from Fig. 1, no significant increases of peak areas of MA and PGA were observed when the concentrations of HOBT, EDC and S-NEA were above 5 mg/ml. To ensure the completeness of derivatization, 10 mg/ml coupling agents were used in the urine sample analysis.

3.4. Selectivity

The typical chromatograms for rat urine sample are shown in Fig. 2. No interference peak was detected in the eluting time of MA and PGA.

3.5. Calibration curves

The calibration curves were constructed by plotting peak areas ratios of *S*-MA, *R*-MA and PGA to internal standard against their concentrations, respectively. Calibration curves of *S*-MA, *R*-MA and PGA in spiked urine were linear over the concentration ranges studied. The regression equations for *S*-MA, *R*-MA and PGA, were $y = 0.0072x - 0.0118$ ($r^2 = 0.9992 \pm 0.0015$; $n = 5$), $y = 0.0096x + 0.0002$ ($r^2 = 0.9993 \pm 0.0015$; $n = 5$), and $y = 0.027x - 0.0756$ ($r^2 = 0.9997 \pm 0.0018$; $n = 5$), respectively.

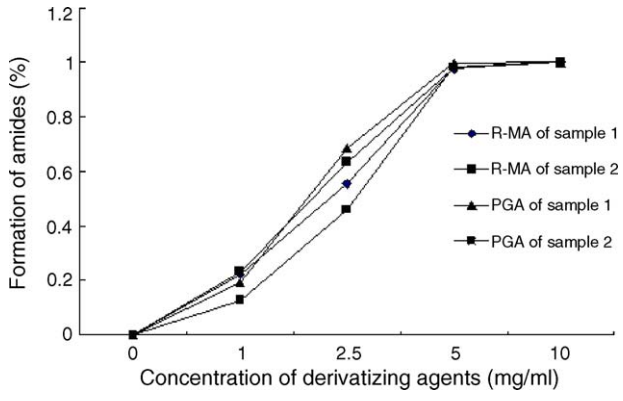

Fig. 1. The effects of amounts of derivatizing agents on the derivatization.

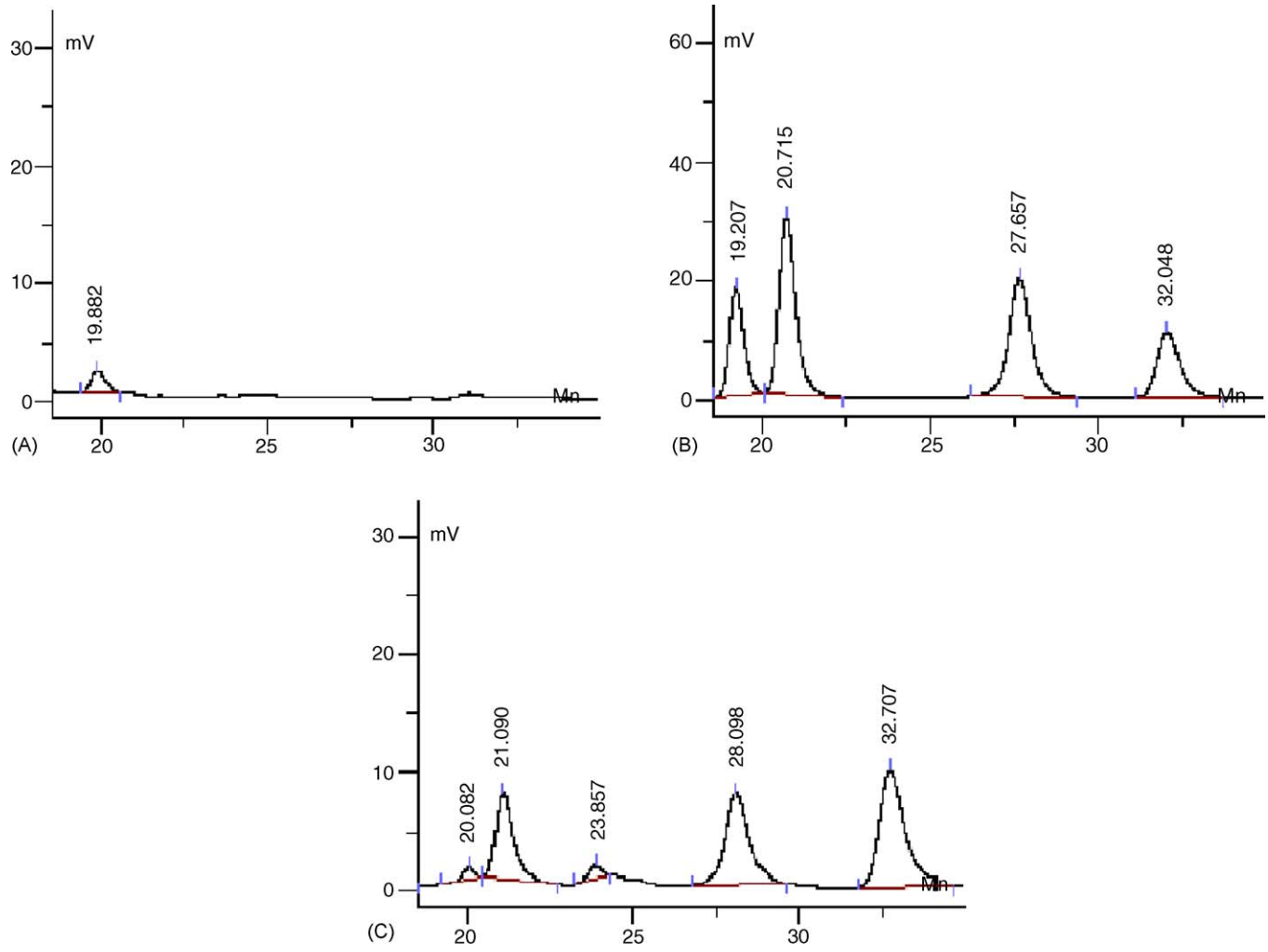

Fig. 2. HPLC chromatograms of blank urine (A), standards of *S*-MA, *R*-MA PGA and salicylic acid–naphthylethylamide derivatives (B) and urine after styrene treatment (C). The retention times of *S*-MA derivative, *R*-MA derivative, PGA derivative and salicylic acid derivative are 19.2, 20.7, 27.7 and 32 min, respectively.

3.6. Sensitivity

The blank urine spiked with lower concentrations of *R*-MA, *S*-MA and PGA was prepared according to the procedure described in Section 2.4. The lower limit of detection (LLOD) and the lower limit of quantization (LLOQ) were measured based on S/N ≥ 3 and S/N ≥ 10, respectively. The results showed LLOD was 1 μg/ml and LLOQ was 5 μg/ml.

Table 2
Intra- and inter-day precisions and accuracy of MA enantiomers and PGA in spiked urine (mean ± S.D., $n = 5$)

Compound	Spiked concentration (μg/ml)	R.S.D. (%)		Absolute recovery (%)	Accuracy (%)
		Intra-day	Inter-day		
S-MA	5	2.8	4.8	90.1 ± 2.2	99.7
	25	1.4	3.3	95.8 ± 3.9	102.2
	125	1.5	3.2	96.8 ± 3.1	106.1
R-MA	5	7.4	7.7	89.1 ± 2.9	100.8
	25	2.3	3.4	93.1 ± 6.4	98.7
	125	0.7	3.9	93.5 ± 3.6	103.4
PGA	5	2.5	6.8	86.2 ± 3.1	108.0
	25	1.3	4.1	95.0 ± 8.1	100.4
	125	1.8	4.5	96.3 ± 2.5	103.1

Table 3
Stability of *R*-MA, *S*-MA, PGA in methanol and urine ($\bar{x} \pm$ S.D., $n = 3$)

Compound	Concentration (μg/ml)	Percentage of initial value			
		Room temperature	Methanol	Three freeze–thaw cycles	−20 °C for 1 month
S-MA	25	98.7 ± 7.4	97.6 ± 7.5	96.8 ± 4.4	97.1 ± 8.8
	250	96.3 ± 5.3	101.3 ± 4.9	98.0 ± 5.8	103.2 ± 3.2
R-MA	25	95.0 ± 4.5	93.5 ± 6.3	104.5 ± 6.1	102.8 ± 6.0
	250	97.3 ± 6.3	102.4 ± 2.4	96.1 ± 4.6	93.7 ± 9.9
PGA	25	94.2 ± 7.8	97.3 ± 6.1	98.3 ± 3.6	97.3 ± 6.9
	250	96.2 ± 5.2	96.7 ± 3.5	95.5 ± 8.4	98.2 ± 7.2

Table 4
The contents of MA enantiomers and PGA in urine samples (mg, $\bar{x} \pm$ S.D., $n = 3$)

Dose (mg/kg)	Sample number	*S*-MA	*R*-MA	PGA
100	1	N.D.	5.761 ± 0.119	2.247 ± 0.024
	2		5.765 ± 0.217	2.231 ± 0.036
	3		5.916 ± 0.143	3.068 ± 0.054
	4		3.886 ± 0.170	2.779 ± 0.047
50	1		3.066 ± 0.183	1.453 ± 0.076
	2		3.314 ± 0.192	0.528 ± 0.032
	3		3.496 ± 0.179	0.924 ± 0.044
	4		2.308 ± 0.131	0.408 ± 0.035
10	1		0.939 ± 0.066	0.243 ± 0.012
	2		1.511 ± 0.059	0.266 ± 0.008
	3		1.087 ± 0.073	0.271 ± 0.011
	4		0.698 ± 0.054	0.199 ± 0.006

N.D.: not detected.

3.7. Precision, accuracy and absolute recovery

The precision of the assay was determined by calculating the intra- and inter-day variations at three concentrations (5, 25 and 125 μg/ml) in five replicates. As shown in Table 2, the intra- and inter-day R.S.D. were less than 8%. The accuracy was found to be in the range of 99.7–106.1% for *S*-MA, 98.7–103.4% for *R*-MA and 100.4–108% for PGA at three concentration levels, respectively. The absolute recoveries of *S*-MA, *R*-MA, PGA and salicylic acid were not less than 80%.

3.8. Stability of MA and PGA

The stability of *S*-MA, *R*-MA and PGA under various conditions is described in Table 3. The R.S.D. of peak areas of each analyte were less than 10% which indicated that they were stable under the conditions tested. No racemizations of MA were observed in methanol and in rat urine. Salicylic acid was stable in methanol solution for at least 5 days. Amides were stable in mobile phase at least for 10 h.

3.9. Assay of MA enantiomers and PGA in rat urine

After a single oral administration of 10, 50 and 100 mg/kg styrene to male SD rats, the urine concentrations of MA enantiomers and PGA were determined using the described method. The contents of *S*-MA, *R*-MA and PGA are shown in Table 4. *S*-MA was not detected in all urine samples, which indicated that a strong stereo-selective metabolism for MA had occurred in SD rats. The excretion of MA enantiomers showed great difference with the previous study which reported that a small excess of *R*-MA in workers exposed to air containing styrene and almost racemic MA in orally styrene-dosed Wistar rats [10]. Some factors can influence the metabolism of styrene such as age, sex, pregnancy or drinking alcohol probably via the change of specific P450 isozyme composition or the total content of P450 [11,12]. Linhart et al. [4] reported that the ratio of MA enantiomers depends on the stereoselectivity of the formation of MA via styrene oxide as well as on the stereoselectivity of its further oxidation to phenylglyoxylic acid. Watabe et al. [13] preferred the latter explanation. In our unpublished study, the urine excretion of PGA by SD rats orally dosed with *S*-MA was about four times more than those dosed with *R*-MA. Though the oxidation to phenylglyoxylic acid plays a great role in the metabolism of MA, further studies are still required to understand the stereochemical aspects of biotransformation of styrene.

4. Conclusions

In conclusion, a simple, selective and sensitive HPLC method for the simultaneous quantization of MA enantiomers and phenylglyoxylic acid in rat urine was developed. The assay had been successfully applied to study the metabolism of styrene in SD rats and the results indicated that PGA and *R*-MA were major metabolites in SD rat urine.

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