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Fast liquid chromatographic determination of urinary *trans,trans*-muconic acid

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Abstract

trans,trans-Muconic acid (1,3-butadiene-1,4-dicarboxylic acid, MA), a minor urinary metabolite of benzene exposure, was determined, after clean-up by solid-phase anion-exchange chromatography, by reversed-phase HPLC on a C_{18} column (5 × 0.46 cm I.D., 3 μm particle size), using formic acid–tetrahydrofuran–water (14:17:969) as mobile phase and UV detection at 263 nm. The recovery of MA from spiked urine was >95% in the 50–500 μg/l range; the quantification limit was 6 μg/l; day-to-day precision, at 300 μg/l, was C.V.=9.2%; the run time was less than 10 min. Urinary MA excretion was measured in two spot urine samples of 131 benzene environmentally exposed subjects: midday values obtained in non-smokers (mean±S.D.=77±54 μg/l, $n = 82$) were statistically different from those of smokers (169±85 μg/l, $n = 30$) ($P<0.0001$); each group showed a statistically significant increase between MA excretion in midday over morning samples. Moreover, in subjects grouped according to tobacco-smoke exposure level, median values of MA were positively associated with and increased with daily smoking habits.

Keywords: Muconic acid; Benzene

1. Introduction

Urinary *trans,trans*-muconic acid (1,3-butadiene-1,4-dicarboxylic acid, MA), an unsaturated short-chain dicarboxylic acid known to be a minor metabolite of benzene ring cleavage, has recently been proposed as a valuable indicator for the biological monitoring of low-level benzene exposure in substitution of the non-specific phenol [1–7]. Both gas chromatography–mass spectrometry (GC–MS) and high-performance liquid chromatography (HPLC) have been used for MA determination [1,2,8–10]. However, the GC–MS method is time-consuming and tedious with regard to sample purification and derivatization, while HPLC methods suffer some drawbacks, mainly poor sensitivity and very long-lasting elution time.

The aim of this work was to develop a simple HPLC method suitable for routine analyses. The method proposed here, coupling together sample clean-up by solid-phase anion-exchange chromatography and short RP-C_{18} column, allows fast determination of MA in samples containing low concentrations such as from persons exposed to benzene urban pollution [11]. The reliability of the proposed procedure was verified by application in biological

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monitoring of airborne benzene exposure in subjects exposed to urban pollution, passive and active tobacco smoke.

2. Experimental

2.1. Materials

trans,trans-Muconic acid was obtained from Aldrich Italia (Milan, Italy); sodium chloride, methanol, tetrahydrofuran, formic acid and hydrochloric acid (analytical grade) were supplied from C. Erba (Milan, Italy). The octadecyl dimethyl silyl column (5 × 0.46 cm I.D., 3 μm particle size) Supelcosil C_{18}, the octadecyl dimethyl silyl guard column (2 × 0.46 cm I.D., 5 μm particle size) Supelguard C_{18} and solid-phase anion-exchange disposable extraction SAX columns (3-ml sample capacity; packed with 500 mg of strong anion-exchange sorbent, quaternary amine) were purchased from Supelco (Bellefonte, PA, USA). The SAX columns were conditioned by rinsing them with 2.5 ml of methanol and 2.5 ml of water. Used SAX columns were regenerated by eluting through 5 ml of 2 mol/l HCl aqueous solution and rinsing with 15 ml of water; they can be used at least ten times without worsening of performance.

2.2. Instrumentation

A SPE 24-port vacuum manifold (Alltech Italia, Sedriano, Italy) was employed for sample purification. A quaternary solvent-delivery system, PU 4100 liquid chromatograph coupled with a PU 4120 diode array detector was used (Unicam Italia, Monza, Italy). Data acquisition and elaboration were by means of a Unicam 4880 data elaboration system.

2.3. Studied group

Urine samples were obtained from 131 healthy male subjects (95 non-smokers, 36 smokers) environmentally exposed to benzene, working in an urban environment. Information regarding modification and confounding factors (e.g. age, tobacco smoke, alcohol consumption) were recorded by questionnaire. Subjects were divided into five groups according to tobacco-smoke exposure level: level-0, non-smokers not exposed to environmental tobacco smoke (ETS) ($n = 66$); level-1, passive smokers, non-smokers exposed to ETS ($n = 29$); level-2, smokers smoking no more than 10 cigarettes/day ($n = 11$); level-3, smokers smoking between 11 and 20 cigarettes/day ($n = 22$); level-4, smokers smoking more than 20 cigarettes/day ($n = 3$).

2.4. Procedure

2.4.1. Urinary sample collection, storage and processing

Urine spot samples were collected from each subject twice a day, at 07:00 h (second urination, workshift beginning) and 5 h later, at 13:00 h (before lunch interval). As soon as possible duplicate 3-ml aliquots were separated and stored in polyethylene tubes at −18°C until analysis.

Before analysis, frozen samples were conditioned at 37°C for 15 min, with frequent stirring, and then centrifuged at 2000 *g* for 5 min.

2.4.2. Preparation of standard solution

MA stock standard solution (40 mg/l) was prepared by dissolving 10 mg of pure compound in 10 ml of methanol and diluting to 250 ml with deionized water; 1-ml aliquots of this solution, stored at −18°C, were stable for at least 6 months. Working standards for calibration were prepared daily by diluting stock solution with mobile phase to obtain concentrations of 200, 400 and 800 μg/l, respectively.

2.4.3. Solid-phase extraction

The SAX extraction columns were loaded with 2 ml of urine and then washed sequentially with 2.0 ml of water, 1.0 ml of 0.1 *M* aqueous sodium chloride solution and 2.0 ml of water. All effluents were discarded. The purified isolate containing MA was then collected from the SAX column by elution with 1.0 ml of 2% (v/v) aqueous formic acid solution in a 2-ml polyethylene test tube. No correction of final eluate volume was done. The eluate was thoroughly mixed before injection into the HPLC.

2.4.4. Chromatographic conditions

Samples (40 μl) were injected into HPLC system and eluted isocratically using a mobile phase consisting of formic acid–tetrahydrofuran–water (14:17:969, v/v). At the beginning of the chromatographic run the flow-rate was set at 1 ml/min and then increased to 3 ml/min from 4 min onward; after 8 min total run time the system was allowed to re-equilibrate for 2 min at 1 ml/min. The MA determination was performed recording UV response of eluate at 263 nm. The concentrations of real samples were determined by external standard method using MA aqueous solutions as reference.

3. Results

3.1. Chromatographic separation

The chromatograms shown in Fig. 1 correspond to (A) 106 μg/l MA aqueous sample, (B) and (C) urine samples of a non-smoker and a smoker,

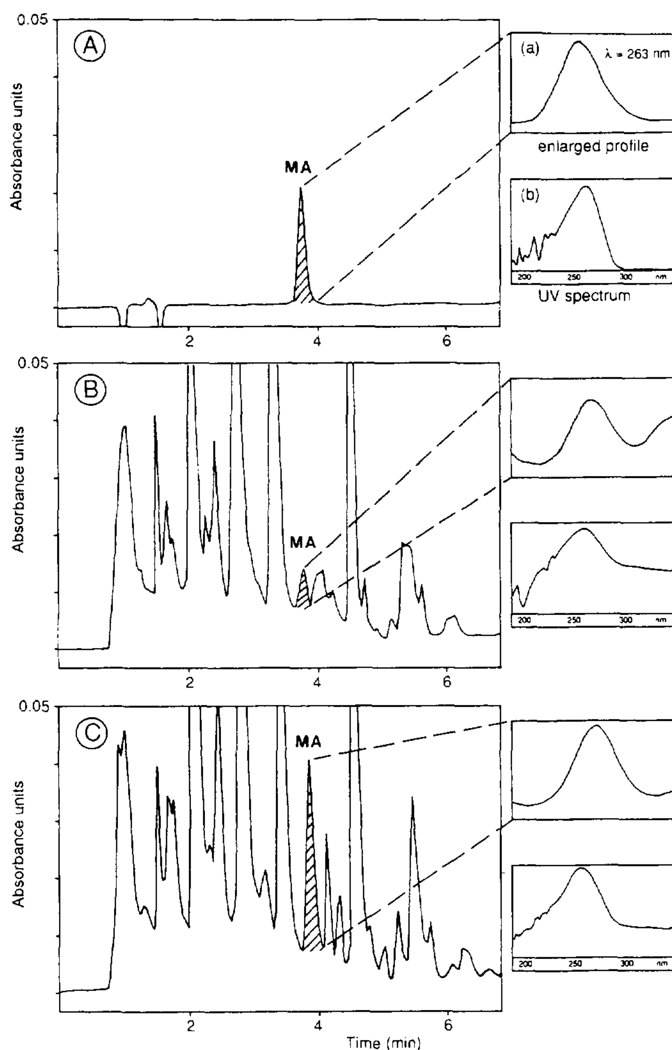

Fig. 1. Representative chromatograms of MA solutions: (A) aqueous standard solution, MA=106 μg/l; (B) non-smoker urine, MA=29 μg/l; (C) smoker urine, MA=165 μg/l. In insets (a) there are enlarged views of MA peak and in insets (b) diode array spectra are presented. The shaded areas are MA peak integrated areas.

respectively. MA is eluted after 3.8 min and the analysis is completed in 8 min. The day-to-day precision of MA retention time, determined on ten analytical series in 2 months, gave a relative standard deviation (R.S.D.) < 2%. As can be best appreciated from an enlarged view of MA peak chromatographic profiles (insets a in Fig. 1A–C), MA is well separated from other urinary components and the absence of interferents is confirmed by the good correspondence between urine MA peak diode array spectra and that of the pure compound (compare inset b of Fig. 1A with those of Fig. 1B and C).

3.2. Calibration, recovery, and reproducibility

The calibration curve ($y = -0.64 + 0.58x$) was linear for concentrations of MA up to 2000 μg/l. The limit of detection (Lc) and the limit of quantitation (Lq) were obtained from spiked urine calibration curves (four different added MA concentrations in the range 12.5–500 μg/l, six determinations for each point), by use of the intercept (a) and the intercept standard error S.E.(a) of the regression line between MA concentrations versus signals [12]. The limit of detection, calculated from $y-a=3\text{S.E.}(a)$, was Lc=1.9 μg/l, and the limit of quantitation, calculated from $y-a=10\text{S.E.}(a)$, was Lq=6.2 μg/l. Analytical recovery was assessed by adding different amounts of MA (in the range 50–500 μg/l) to urine ($n=9$) containing low levels (10–30 μg/l) of endogenous metabolite. The results were expressed as a percentage of the values obtained by direct injection of standard MA solution used for spiking. The analytical percent recovery was in any case higher than 95%. The within-assay precision of the method, determined by repeated analysis of two urine samples containing, respectively, about 30 μg/l and 300 μg/l metabolism-derived MA, gave a R.S.D. of 8.1% for the low-level sample and 3.4% for the high-level one ($n = 10$). The same samples were used to assess day-to-day precision over 2 months, giving a R.S.D. of 14.9% and 9.2%, respectively ($n = 6$).

3.3. Stability of MA in urine

MA was found to be stable in urine for at least 4 months when stored at −18°C without any chemical preservative, while the same samples stored at room temperature were stable for a week.

3.4. Urinary concentrations of muconic acid

Detectable amounts of MA were found in all but two analyzed samples. Values obtained in the studied group, with subjects classified according to tobacco smoking status, are reported in Table 1. As the large inter-individual variability observed in MA excretion

Table 1
Muconic acid concentration in spot urine samples of a group of benzene environmentally exposed subjects

	Urinary muconic acid concentration							
	Before workshift				After 5-h workshift			
	Non-smokers		Smokers[a]		Non-smokers		Smokers[b]	
	μg/l	μg/g creatinine	μg/l	μg/g creatinine	μg/l	μg/g creatinine	μg/l	μg/g creatinine
Mean	65	32	113	47	77	50	169	100
Standard deviation	56	30	68	33	54	36	85	45
Median	46	22	110	40	69	42	185	104
Geometric mean	46	23	88	36	58	38	142	89
Minimum value	<6[c]	<6[c]	<6[c]	8	<6[c]	<6[c]	17	34
Maximum value	256	147	265	141	242	192	382	171
n	90	84	28	28	82	81	30	30

Values observed in samples collected before workshift (07:00 h) and after 5-h shift (13:00 h) are reported.
[a]Daily smoked cigarette mean number: 17 cigarettes/day.
[b]Mean number of cigarettes smoked during the monitored time interval: 5 cigarettes/5 h.
[c]Analytical method quantitation limit.

Table 2
Muconic acid concentration in spot urine samples of subjects classified according to tobacco-smoke exposure level (level-0, non-smokers without ETS; level-1, non-smokers with ETS; level-2, smokers smoking no more than ten cigarettes/day; level-3, smokers smoking between 11 and 20 cigarettes/day; level-4, smokers smoking more than 20 cigarettes/day)

	Urinary muconic acid concentration (μg/l)									
	Before workshift					After 5-h workshift				
	0[a]	1	2	3	4	0	1	2	3	4
Mean	54	78	92	107	145	76	77	137	168	300
Standard deviation	42	77	47	58	59	49	60	76	71	80
Median	43	52	95	111	172	71	52	121	192	296
Geometric mean	42	49	80	84	136	61	53	117	147	293
Minimum value	<6[b]	<6[b]	29	<6[b]	80	<6[b]	<6[b]	35	17	223
Maximum value	252	324	177	225	184	216	242	265	276	382
n	63	27	8	17	3	60	22	8	19	3

Values observed in samples collected before workshift (07:00 h) and after 5-h shift (13:00 h) are reported.
[a]Exposure levels.
[b]Analytical method quantitation limit.

was not substantially reduced by urinary creatinine correction, all statistical inferences on groups were done with MA untransformed concentration values (μg/l). The comparison between MA values in smokers and non-smokers showed a statistically significant difference both for morning and before-lunch samples ($P<0.0001$). Moreover, in each group there was an appreciable statistically significant median increase in MA midday in respect to morning excretion (non-smokers: $P<0.02$; smokers: $P<0.01$); This difference was even more significant when individual paired data were considered ($P<0.0001$). In subjects grouped according to tobacco-smoke exposure level, median values of MA excretion were associated with self-reported daily smoked cigarette number and increased with the intensity of tobacco consumption, both in morning and before-lunch samples (Table 2 and Fig. 2A and B).

4. Discussion

Urinary MA SAX purification and chromatographic separation on reversed-phase C_{18} column have been previously described by other authors [9,10]: however, in our hands these systems gave poor performances either with regard to the quality of chromatograms, sensibility and retention-time reproducibility. To improve the reliability of the determination, we studied extensively both the anion-exchange purification procedure and HPLC conditions. A major advance in sample purification was achieved by the use of aqueous sodium chloride solution in the washing step and of diluted formic acid solution to subsequently elute MA from the SAX column. This procedure gives a clear eluate, that, on injection, does not perturb the chromatographic front and provides enhanced stability of the chromatographic profile. Moreover, the parameters of analytical quality appeared not to be influenced by the sample matrix, allowing the adoption of aqueous standard solutions for calibration; this practice helps to reduce the health risk posed by the use of potentially infectious biological fluids. The retention time of the analyte was reproducible with a C.V. of less than 2% for between-day analysis; moreover, the reduced presence of interferents allows a significant sensitivity increase. Also the mobile phase composition contributed to the improvement of the chromatographic elution pattern: the mixture of formic acid and tetrahydrofuran helps to remove some interferents that, otherwise, run very close to MA, as suggested by the sufficient grade purity of urinary MA chromatographic peaks at concentration as low as 10 μg/l. To allow reduction in waste-solvent volumes and disposal cost, it was possible to recycle the same volume of mobile phase (200 ml) for a week (100 analyses performed), observing only a minor drift of the baseline, easily compensated for by zeroing the system of the detector. The reliability

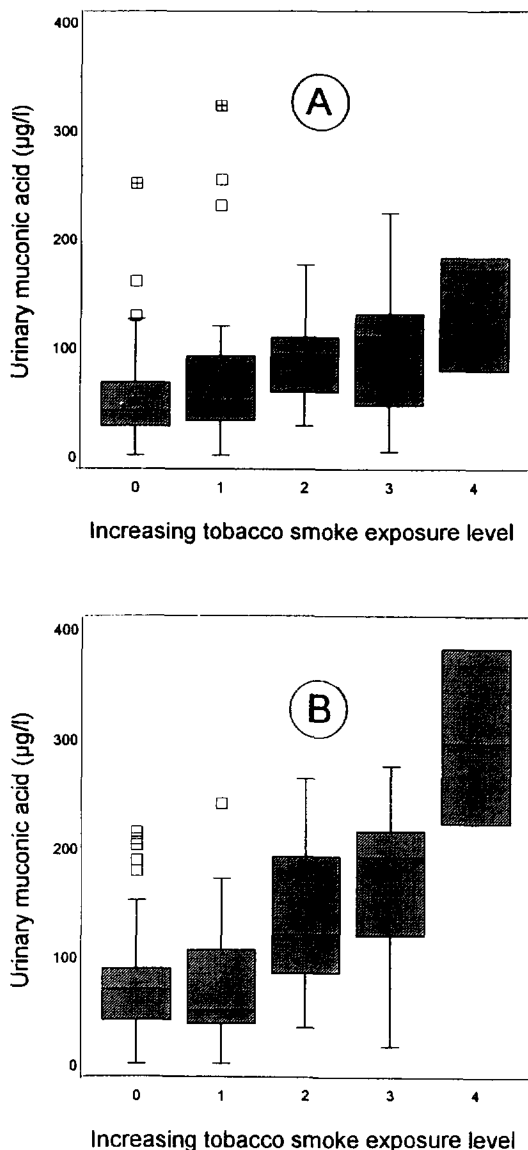

Fig. 2. Concentrations of muconic acid (μg/l), determined in urine samples collected (A) before workshift and (B) after 5-h workshift, in subjects classified according to tobacco-smoke exposure level. The plotted data are divided into four areas of frequency. The box encloses the middle 50%. The horizontal line inside the box represents the median. The lower vertical line extends from the first quartile to the smallest data point within 1.5 interquartile ranges from the first quartile, the other whisker extends from the third quartile to the largest data point within 1.5 interquartile ranges from the third quartile. Data values that fall beyond the whiskers but within 3 interquartile ranges (suspected outliers, □) and those falling outside that interval (outliers, □ with + inside) are plotted as individual points. Mean values are indicated by the + symbol.

of the proposed procedure for the biological monitoring of exposure to airborne benzene is confirmed by the results obtained from the studied group: MA excretion values obtained here are in good agreement with those reported by other authors on similar population groups [5,7,10]. Moreover the sensitivity of the method demonstrated not only a significant difference in MA excretion between smokers and non-smokers both at morning and midday (Table 1), but also evidence of individual increased excretion at midday with respect to morning values in subjects classified according to cigarette consumption (Table 2). The negligible influence of environmental tobacco-smoke exposure on MA excretion of passive smokers (Table 2 and Fig. 2) could be at least partially ascribed to the short exposure time considered (5-h workshift); such an hypothesis is supported also by the lack of difference observed between blood benzene concentrations of non-smokers without ETS (level-0, blood benzene geometric mean: 0.28 μg/l, $n = 61$) and non-smokers passively exposed to ETS (level-1, blood benzene: 0.28 μg/l, $n = 24$) (samples collected at 13:00 h), while in smokers a positive association between tobacco-smoke exposure level and blood benzene was evidenced (level-2, blood benzene: 0.39 μg/l, $n = 10$; level-3, blood benzene: 0.42 μg/l, $n = 17$; level-4, blood benzene: 0.53 μg/l, $n = 3$) [11]. With regard to the number of suspected outliers present in the level-0 group both at morning and before lunch, the existence of an unidentified environmental benzene exposure could not be ruled out. For example, the measurement of environmental benzene exposure by personal sampling in the level-0 group, showed three people, in the midday suspected outliers, having significant higher airborne benzene exposure in respect to the group mean value (airborne benzene individual values: 68, 111 and 123 μg/m^3; level-0 mean value: 42 μg/m^3, $n = 47$). These differences have been tentatively associated with the job position of those subjects (cyclist traffic wardens). Other authors reported a significant amount of outliers in their studies: one of the explanations, apart from alimentary interference from sorbic acid [9] not investigated in the present study, hypothesized the existence of different phenotypes in benzene-metabolizing capacity [13].

The results of the present study confirm the

suggestion given by other authors about the usefulness of MA as a promising biomarker for risk assessment of low-concentration benzene exposure and the proposed method represents an improvement in reliability and performance of analytical procedure that allows the study of urban benzene exposure.

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