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Direct analysis of urinary *trans,trans*-muconic acid by coupled column liquid chromatography and spectrophotometric ultraviolet detection: method applicability to human urine

Giorgio Marrubini[a,*], Teresa Coccini[b], Luigi Manzo[a,b]

[a]*Laboratory for Pharmacokinetics and Analytical Toxicology, Clinical Toxicology Division, Department of Internal Medicine, University of Pavia, Via Brodolini 7, 27028 San Martino Siccomario (Pavia), Italy*
[b]*Toxicology Division, Salvatore Maugeri Foundation, Institute of Pavia, Pavia, Italy*

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Abstract

A coupled column liquid chromatographic (LC–LC) method for the direct analysis in human urine of the ring opened benzene metabolite, *trans,trans*-muconic acid (*t,t*-MA) is described. The method was tested using urine samples collected from five refinery workers exposed to low concentrations of airborne benzene (0.2–0.5 ppm), and from non-exposed volunteers. The analytical columns used were of 50×4.6 mm I.D. packed with 3 μm p.s. Microspher C_{18} material as the first column (C-1), and a 100×4.6 mm I.D. column packed with 3 μm p.s. Hypersil ODS material as the second one (C-2). The mobile phases applied consisted, respectively, of methanol–0.074% trifluoroacetic acid (TFA) in water (4:96, v/v) on C-1, and of methanol–0.074% TFA in water (10:90, v/v) on C-2. Under these conditions *t,t*-MA eluted 15 min after injection. The present method, coupling the LC–LC technique with UV detection at 264 nm, permits the quantitation of *t,t*-MA directly in urine at levels as low as 0.05 mg/l. The determination is performed with a sample throughput of 2 h^{-1} requiring only pH adjustment and centrifugation of the sample. Calibration plots of standard additions of *t,t*-MA to pooled urine taken from five non-exposed subjects were linear ($r>0.999$) over a wide concentration range (0.05, 0.1, 0.5, 1.0, 2.0 mg/l). The precision of the method (RSD) was in the range of 0.5 to 3.8%, and the within-session repeatability on workers urine samples (levels 0.06, 0.1, 0.2, 1.0 mg/l) was in the range of 3 to 8%. The present method improves the applicability of routine *t,t*-MA analysis, where it is most desirable that a large number of biological samples can be processed automatically or with minimal human labour, at low cost, and with a convenient turn-around time.

Keywords: Muconic acid; Benzene

1. Introduction

Benzene is an ubiquitous environmental pollutant which is often present at very low concentrations (0.1 ppm or less) in both the working and the general environment. *trans,trans*-Muconic acid (*t,t*-MA, Fig. 1), or *trans,trans*-2,4-hexadienedioic acid (CAS 3588-17-8), is a urinary metabolite of benzene which has been intensively investigated as a biomarker in benzene-exposed workers.

*Corresponding author. Tel.: +39-0382-556-600, +39-0382-566-605; fax: +39-0382-24-605, +39-0382-556-615.
E-mail address: gmarrubini@fsm.it (G. Marrubini).

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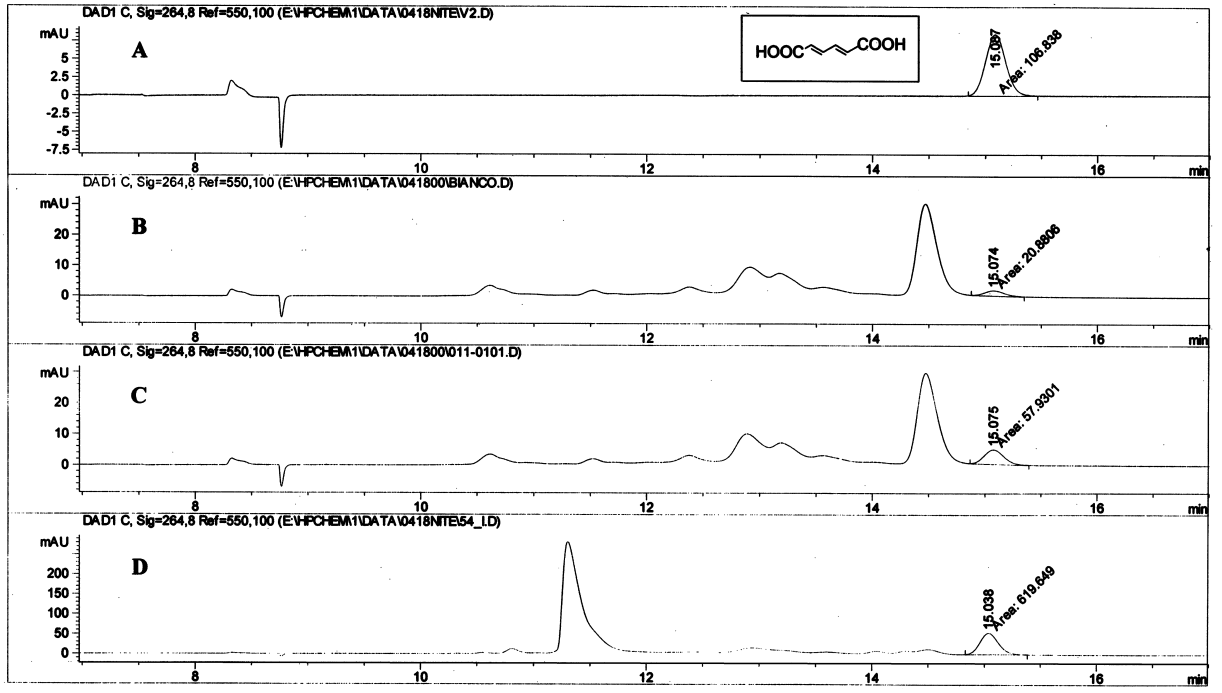

Fig. 1. LC–LC chromatograms of human urine and chemical structure of *trans,trans*-muconic acid (in the box). Selectivity attained using modified conditions described in the Experimental section. Injection volume 100 μl. Transfer volume 0.45 ml. (A) 0.1 mg/l *t,t*-MA in 0.01 *M* aqueous hydrochloric acid; (B) pooled urine of healthy volunteers, non-smokers, non-professionally exposed to benzene. *t,t*-MA concentration found is <0.05 mg/l; (C) Same sample of LC–LC chromatogram B spiked with 0.05 mg/l of *t,t*-MA. (D) Refinery worker exposed to 0.5 ppm of benzene in air. Post shift urine sample coded 54. *t,t*-MA concentration found is 1.0 mg/l.

A number of studies have indicated a statistical correlation between urinary *t,t*-MA levels and airborne concentrations of benzene in workplaces [1–10]. Correlations are reported to hold for benzene concentrations in air ranging from as low as 0.01–0.025 ppm [1,11] to levels exceeding 25–30 ppm [8,10]. However, at low benzene air concentrations *t,t*-MA analysis has been reported to pose problems of both specificity and sensitivity [9]. Regarding specificity, questions were raised by the finding that *t,t*-MA can also be observed in urine as a metabolite of sorbic acid (E200) and potassium sorbate (E202) which are widely used preservatives in industrial food production. Recent research demonstrated that the daily diet can be a major confounding factor when *t,t*-MA is used for biological monitoring of benzene at concentrations as low as 0.1–0.5 ppm [12–14]. Despite the difficulties in the interpretation of data obtained from urinary concentration of *t,t*-MA following exposure to low benzene concentrations in air, either in the non-occupational setting [11] or on workplace [4,6,9], this biomarker has been tested in several studies to investigate relationships between benzene exposure and health effect. Epidemiologic studies confirmed that wide individual variations in benzene metabolism exist based also on genetic factors [15]. Still, the collection of data on urinary metabolites requires analysts to test large number of samples during time and labour consuming procedures in which sample handling and pretreatment can be a cause of further variation of the results. Therefore, in the study of *t,t*-MA for assessing low-level benzene exposure, it has become clear that accuracy is a critical factor. The determination of *t,t*-MA in urine has been carried out since 1985 using three techniques: reversed-phase high-performance liquid chromatography (HPLC) and UV detection [16–24] or mass spectrometry (MS) [25], and gas chromatography (GC) combined with mass spectrometric detection [5,8]. HPLC and GC when

applied to the separation of *t,t*-MA, as reviewed [26], require enrichment and clean-up steps, performed either by solid-phase extraction on strong anion-exchange (SAX) sorbents, or by liquid–liquid extraction using diethyl ether [26] or ethyl acetate [8]. GC, in particular requires a time-consuming laborious sample preparation. The biological sample must be extracted, and solvent evaporation and derivatization are needed before injection because of the very polar and non-volatile nature of *t,t*-MA. Internal standardisation is also routinely used in GC–MS in order to control the quality of analytical data [8,26]. By contrast, internal standardisation was not applied in most studies determining *t,t*-MA by HPLC–UV, despite the inherent variability and lack of specificity of such methods. This was also emphasised by two papers [18,22] which reported that the performance of one of the most commonly used HPLC–UV methods for the analysis of *t,t*-MA [17] could be improved if internal standardisation is used.

One pioneering work reporting the direct injection of solvent-diluted urine in a HPLC system was published [27], but the method was unsuitable for monitoring individual exposure to benzene concentrations lower than 6–7 ppm because poor selectivity and sensitivity were achievable. On the contrary, outstanding selectivity and sensitivity (detection limit at 3 μg/l) were obtained by large volume injection of 200 μl of off-line SAX aqueous extract with pre-column switching [21].

Two-dimensional liquid chromatography, by a combination of a reversed-phase and ionic chromatography, and UV detection at fixed wavelength was used for the direct determination of *t,t*-MA in urine of exposed workers. One recently published method [23] reported very low detection limit (calculated at 4 μg/l) and outstanding features of specificity, repeatability, and automation which prove that two-dimensional liquid chromatography is a useful technique for direct determination of *t,t*-MA in human urine.

Actually, coupled column reversed-phase HPLC (LC–LC) is a very adaptable strategy for the direct analysis of polar molecules in biological fluids, as indicated by recent studies [28,29] including also the analysis of *t,t*-MA in rat urine [24]. In this work we report the applicability of the direct determination of *t,t*-MA in human urine by reversed-phase coupled column liquid chromatography and UV detection (LC–LC–UV).

2. Experimental

2.1. Chemicals

trans,trans-Muconic acid (*t,t*-MA, 98% purity) was from Sigma–Aldrich (Milan, Italy). Water and methanol both HPLC-grade were from BDH (Milan, Italy). Analytical grade (99% purity) *N,N*-dimethylformamide, pure (99% purity) trifluoroacetic acid (TFA) and concentrated (37%, w/w) hydrochloric acid (HCl) were also from BDH.

A stock solution (500 μg/ml) of *t,t*-MA was prepared in *N,N*-dimethylformamide–methanol (1:10, v/v) and stored at −20°C. For spiking or LC analysis the stock solution was diluted in a solution of 0.01 *M* aqueous HCl.

The mobile phase (M-1) applied on the first column (C-1) consisted of TFA–methanol–water (0.074:4:96, v/v). On the second column (C-2), a mobile phase (M-2) of TFA–methanol–water (0.074:10:90, v/v) was used. A rinsing mobile phase (M-R) consisting of TFA–methanol–water (0.074:25:75, v/v) was applied on C-1 in between every analysis of urine.

2.2. Human samples and measurement of benzene in air

Five urine samples were chosen from those collected and stored during a 1998 campaign of biological monitoring involving 68 male workers. All subjects were employed in maintenance operations of tanks in two Italian refinery plants. The workers were monitored for personal exposure to environmental benzene and provided urine in polythene bottles before and after the 8-h work shift for the determination of *t,t*-MA.

Personal monitoring for measuring time weighted average airborne benzene concentration was accomplished with gas diffusion badges (Radiello, Fondazione Salvatore Maugeri, Padua, Italy). The diffusion badges were always positioned in the breathing area of the worker. Benzene determination was performed according to the standard method pub-

lished by the National Institute for Occupational Safety and Health (NIOSH) by desorption with carbon disulfide and subsequent GC with flame ionisation detection (FID) shortly after sampling [30]. By the applied method, the quantitation of airborne benzene was possible at levels as low as 0.05 ppm.

Blank urine samples were collected from volunteers selected in a control population of healthy subjects, non-smokers, and non-professionally exposed to benzene.

2.3. Rat urine samples and measurement of benzene in air

For comparison purposes, rat urine samples were also examined. Urine was obtained from adult male Sprague–Dawley rats (200–250 g) which were randomly divided into groups and housed in polycarbonate cages containing hardwood chip bedding and filter cups. The animals were maintained on a 12-h light–dark cycle at 20–22°C with relative humidity of 55±5%. Groups of rats (six rats per group) were exposed to concentrations of benzene in air ranging from 0.1 to 2 ppm for 6 h. A separate group (control) was exposed to circulating air. Benzene concentration in the chamber air was monitored during the experiments by an automatic benzene, toluene, and xylene portable gas chromatograph equipped with a flame ionization detector BTX61M (Analysis Automation Trading, Marghera, Venezia, Italy). The limit of quantitation for benzene was at 0.01 ppm. Urine was collected in metabolic cages during the 6-h period of benzene exposure as well as after 18 and 42 h in the post-exposure period. All samples were stored at −80°C until analysis.

2.4. Apparatus

The LC–LC–UV apparatus is illustrated in Fig. 2. It consisted of a series 1100 Hewlett-Packard (Waldbrun, Germany) system including a Model G1311A quaternary gradient pump (GP), a Model 1310A isocratic pump (IP), a Model G131A ALS

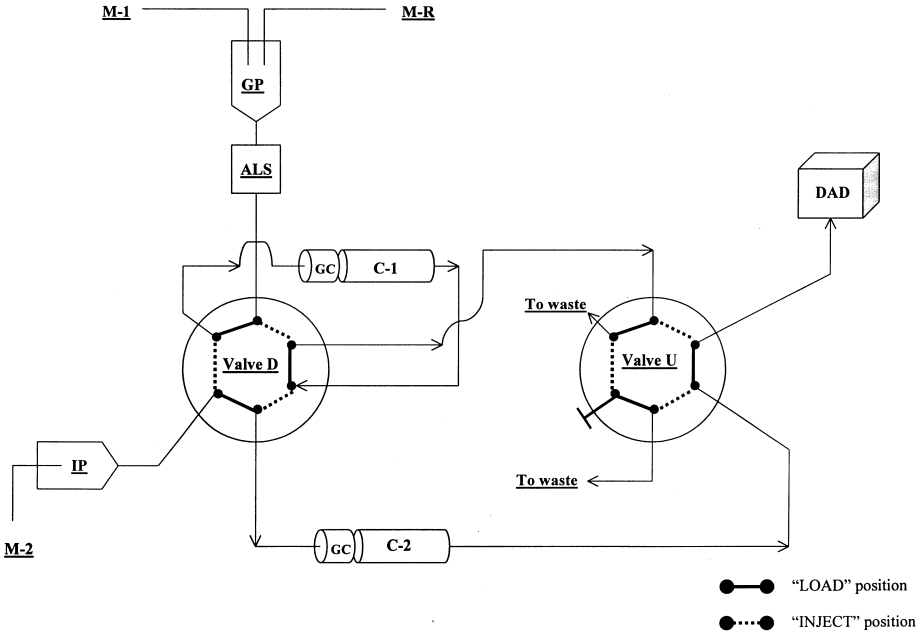

Fig. 2. Scheme of the instrumental set-up. GP=Quaternary gradient pump, M-1=mobile phase 1, M-R=rinsing mobile phase, ALS=autosampler, IP=isocratic pump, M-2=mobile phase 2, GC=guard column, C-1=first analytical column, C-2=second analytical column, DAD=diode array detector. Valves D and U=high pressure six-way remote-controlled switching valves. Full lines indicate the flow direction when the valve is switched in the ''LOAD'' position. Broken lines indicate the flow direction when the valve is switched in the ''INJECT'' position.

autosampler (ALS), a Model G1322A vacuum degasser, a Model G1315A diode-array UV–visible detection (DAD) system and a Model G1316A thermostated column oven. In all experiments the oven temperature was set at 22.0±0.5°C. Quantitative analysis was performed at single wavelength setting the detector at 264 nm. Two six-way remote controlled programmable high-pressure valves type ViciAG Valco (Schenkon, Switzerland) were used for column switching. Fully automated operation of the LC–LC–UV system was performed by a Hewlett-Packard Vectra VE 6/350 personal computer equipped with ChemStation software version A.06.03.

The columns used for the LC–LC separations were (C-1) a 50×4.6 mm I.D. column packed with 3 μm C_{18} Microspher material, (C-2) a 100×4.6 mm I.D. packed with 3 μm Hypersil ODS stationary phase (Chrompack, Bergen op Zoom, The Netherlands). Both columns were installed with 10×2 mm I.D. Chromsep guard columns packed with reversed-phase material. For rat urine processing, and during the very early phase of method development on human urine a 150×4.6 mm I.D. semi permeable surface column packed with 5 μm SPS-5PM-S5-100-ODS stationary phase (Regis, Morton Grove, IL, USA) was used as the second column.

2.5. Sample pre-treatment

Aliquots of 0.25 ml of urine samples were diluted (1:1, v/v) with 0.1 *M* aqueous HCl in screw-capped 1.8-ml plastic vials. The acidic solutions were mixed on a Vortex stirrer for a few seconds and centrifuged at 4500 rpm for 15 min. Of the resulting clear solutions at pH between 1 and 2, 100 μl were directly injected into the LC–LC–UV analyser.

2.6. Calibration plot

Quantitation of *t,t*-MA in human samples was performed by means of external calibration using a calibration plot of standard additions to pooled urine obtained from non-exposed non-smoking subjects. Fifteen separate samples were prepared spiking urine with *t,t*-MA at the concentrations of 2.0, 1.0, 0.5, 0.1 and 0.05 mg/l, and every sample was analysed once (Table 1). Standard solutions were prepared in aqueous 0.01 *M* HCl at the same concentrations of urinary standards. Control samples were also prepared in aqueous 0.01 *M* HCl at the concentration of 0.1 mg/l of *t,t*-MA. All the solutions were freshly prepared for each analytical session. Aqueous 0.01 *M* HCl control samples and standard solutions were randomly inserted in the automatic injection sequences.

Quantitation of *t,t*-MA in rat urine was carried out using a calibration plot obtained by standard additions to pooled urine taken from untreated animals. Blank urine was added with *t,t*-MA at the concentrations of 4, 1, 0.5, 0.1 and 0.05 mg/l, and every sample was analysed once (data not shown).

2.7. Sample analysis

A method described previously for rat urine [24], was initially adopted for analysing *t,t*-MA in human urine. However, the results of pivoting experiments demonstrated that the LC–LC conditions used for rat urine did not provide sufficient selectivity for the

Table 1
Calibration plot data obtained from pooled urine of subjects non-smoking, non-exposed to benzene

t,t-MA addition in pooled urine (mg/l)	Average peak area±SD	Corrected area±SD	Concentration found±SD (mg/l)	RSD (%)	*n*
Blank	20.2±1.3	–	–	–	3
0.05	56.6±0.7	36.4±0.7	0.044±0.001	2.2	3
0.10	94.1±0.8	73.0±2.2	0.105±0.004	3.8	3
0.50	326±5	306±5	0.491±0.008	1.6	3
1.0	643±20	622±19	1.01±0.03	3.0	3
2.0	1230±3	1210±3	1.99±0.01	0.5	3

SD: standard deviation, RSD: relative standard deviation. Equation of the calibration curve: $y=601.9x+10.1$, where y corresponds to the corrected peak area, and x to the concentration of *t,t*-MA expressed in mg/l. Correlation coefficient: $r=0.9998$ ($r^2=0.9995$).

separation of *t,t*-MA from interfering substances existing in human urine. In particular, the following changes were introduced in the original method [24], and the modified procedure for routine analysis is described below. A weaker mobile phase was used on C-1 (changing from methanol–water, 7:93, v/v, to methanol–water, 4:96, v/v). The column chosen as C-2 was a 100×4.6, 3 μm particle size (p.s.) column packed with Hypersil ODS material. The pH of the mobile phases M-1, M-2, and M-R was lowered to 2.1 by enhancing the % of TFA from 0.05% (v/v) to 0.074% (v/v). The LC–LC procedure was thus the following. At the beginning of each analytical session the retention time and peak shape of *t,t*-MA on C-1 was controlled by analysis of three consecutive injections of an aqueous solution of *t,t*-MA at the concentration of 0.1 mg/l (Fig. 2, valves configuration D: LOAD, U: INJECT). The injection of 100 μl of spiked urine at the concentration of 2 mg/l followed, and the outcome of this testing was used for transfer-time selection. In the LC–LC experiments, both valves D and U were switched in the position LOAD, while the mobile phases were adjusted to a flow-rate of 1 ml/min. After dilution and centrifugation, a volume of 100 μl of urine sample was injected on C-1. Following the clean-up with 7 ml of M-1, C-1 was switched on-line with C-2 for 0.45 min for the transfer of the analyte-containing fraction to C-2 (Fig. 2, valves configuration D: INJECT, U: LOAD). After transfer, the valves were switched back to the initial configuration (D: LOAD, U: LOAD), and C-1 was rinsed with mobile phase M-R for 18 min at a flow-rate of 1.2 ml/min. Next, C-1 was reconditioned for 5 min with M-1 at a flow-rate of 1.0 ml/min. A shorter procedure was applied to control samples and aqueous standards: 100 μl of aqueous 0.01 *M* HCl containing *t,t*-MA were injected and eluted with M-1 on C-1 and with M-2 on C-2. The same transfer timing was applied as in the case of urine samples, but no rinsing of C-1 was necessary. The analysis of *t,t*-MA aqueous standards every three injections of diluted urine was chosen to monitor the repeatability of the analyte retention time and to prevent column contamination. Every eight runs, rinsing of both columns was carried out; C-1 was eluted for 30 min with M-R at the flow-rate of 1.2 ml/min, and C-2 was eluted with M-2 at 1 ml/min flow-rate. After allowing five min for C-1 equilibration, a new series begun with one aqueous standard injection. At the end of each analytical session, both columns were washed separately with at least 30 ml of methanol.

3. Results

The changes introduced in the method developed for rat urine [24] were directed to achieve a narrower peak and a stronger retention of the analyte on the columns used. Following an investigation on *t,t*-MA acidity, we recognised that the pK_a values were pK_{a1}=2.7–3.5 and pK_{a2}=4.7 [31,32]. Thus, the pH of the mobile phases M-1, M-2, and M-R was lowered to 2.1 by enhancing the % of TFA from 0.05% (v/v) to 0.074% (v/v). Nonetheless, the SPS column used in preliminary experiments had to be substituted because of poor selectivity. The Hypersil ODS column chosen provided stronger retention for *t,t*-MA in the ion suppression operating mode. Furthermore, the column substitution allowed also to select a slightly stronger eluent M-2 (changing from methanol–water, 7:93, v/v [24], to methanol–water, 10:90, v/v). Under these conditions, the LC–LC analyser could process up to 300 μl of urine per injection without loss of column performance. However, given the levels of interest of the target urinary biomarker, namely above 0.05 mg/l, the volume of injection selected was 100 μl. Accordingly, the calibration was studied on urinary concentrations ranging from 0.05 to 2.0 mg/l. As shown in Fig. 1, the mobile phases composition and the columns combination provided a satisfactory separation of *t,t*-MA from the main interfering peaks in the chromatogram. The retention time of *t,t*-MA after column switching is at about 15 min while the interfering peak elutes just before *t,t*-MA. Regarding resolution (R_s), in some samples where *t,t*-MA was at levels close to 0.05 mg/l in urine, the separation achieved was not baseline to baseline. However, since the worst R_s obtained was 1.5, this was usually considered a condition sufficient for a tentative determination of the analyte (Fig. 1C). Table 1 shows the features of linearity of the method. The quantitation limit of 0.05 mg/l was set at the lower concentration point of the calibration plot, while the limit of detection was calculated at 0.02 mg/l. The

data presented in Table 1 evidence that the limit of detection of 0.02 mg/l is sufficient to detect *t,t*-MA in pooled urine taken from non-exposed non-smoking subjects. Therefore, the raw area of the calibration samples had to be corrected by subtracting the peak area of *t,t*-MA in the blank. Linearity was observed in the range from 0.05 to 2.0 mg/l *t,t*-MA in urine and resulted in a correlation coefficient of 0.9998.

Table 1 reports figures of accuracy and precision of the method: replicate measurements of spiked urine samples showed relative standard deviations (RSDs) ranging from 0.5 to 3.8%. In this respect, the control of the columns temperature by means of a thermostated oven is of critical importance, because the *t,t*-MA peak retention time is very sensitive to even small changes of column temperature. An enhancement in room temperature as little as from 22 to 24°C can cause a significant shift in the analyte retention time on C-1 (a 3–5% decrease in retention time) which in turn causes poor recoveries on C-2 after column switching.

Fig. 1D shows one chromatogram obtained analysing the post-shift urine sample collected from a worker (sample code 54) who was exposed to about 0.5 ppm of benzene in air. The concentration of *t,t*-MA found in urine was around 1.0 mg/l. Replicate analyses on five real samples for within-session repeatability testing resulted in an RSD ranging from 3 to 8% (Table 2).

Fully automated analytical sessions as long as 2 consecutive days and 1 night were planned. During the entire course of these sessions the instrumental performance proved consistently to be at the level illustrated by data reported in Tables 1 and 2.

4. Discussion

The present work reports that the method developed for the determination of *t,t*-MA in rat urine previously described [24] is not appropriate for monitoring *t,t*-MA in human urine. In order to process human samples, substantial changes had to be introduced in the instrumental set-up. The reversed-phase columns coupling, together with mobile phases pH, relative composition, and strength were modified to achieve the selectivity requested.

Human urine, in broad terms, is a complex aqueous mixture of compounds compatible with reversed-phase HPLC. Aqueous samples loaded with organic carbon and inorganic salts can be injected directly onto common C_8 and C_{18} columns, providing that no particulate matter is suspended in the sample, and using a strong eluent as mobile phase. Nonetheless, direct injection of biological samples (e.g., urine, serum), can rapidly damage or clog most HPLC columns if an adequate clean-up is not adopted. Moreover, compounds like *t,t*-MA are not easily determined with sufficient selectivity by direct injection on a single reversed-phase HPLC column as early recognised [27], since they are eluted hardly separated from the solvent front. The column switching technique allows to fractionate the sample injected; it thus permits one to introduce into the analytical reversed-phase column a small volume of the complex aqueous mixture containing polar compounds. Therefore, column switching can help in minimising stationary phase damage and improve the selectivity of separations of polar compounds from matrix interferences.

Our main purpose has been to provide a reliable

Table 2
Determination of *trans,trans*-muconic acid in urine of benzene-exposed workers

Sample code	Benzene in air (ppm)	Mean concentration±SD (mg/l)	RSD (%)	*n*
54	0.5	1.09±0.05	5	3
56	0.2	0.12±0.01	8	3
61	0.4	0.23±0.01	4	3
64	0.4	0.061±0.002	3	3
66	0.2	<0.05	n.a.	3

SD: standard deviation, RSD: relative standard deviation, n.a. not applicable.

and robust way of performing a large number of sensitive, specific, and precise determinations of urinary *t,t*-MA. The samples of human urine provided by refinery workers illustrate the utility of our method. At benzene air concentration of 0.2–0.5 ppm, four of the five subjects studied had urinary levels of *t,t*-MA lower than 0.5 mg/l and clearly determinable. The threshold limit value (TLV) for benzene on workplace according to the Italian present laws is 3 ppm (9.75 mg/m^3; Italian Government Decreto Legislativo No. 66, 24 March 2000 [33]). Therefore, the sensitivity of our method was considered sufficient for biological monitoring purposes on workplace. Levels in urine ranging from 0.05 to 2 mg/l have been indicated to define the most expectable interval of concentrations for *t,t*-MA in exposed workers and non-exposed subjects [4,13,26,34]. The present method allows the direct determination of *t,t*-MA in urine with satisfactory sensitivity and precision in the range 0.05–2 mg/l. It can thus be considered a useful resource also for investigating benzene exposure as it occurs in the general environment.

A short comment is due regarding the biomedical significance of the data presented in Table 2.

Large differences in *t,t*-MA urinary levels have been reported among workers equally exposed to low air concentrations of benzene [5,7,9]. The variation of individual data for urinary *t,t*-MA can be the consequence of several causes in addition to the accuracy of the analytical procedure adopted. For instance, sampling and analysis of environmental benzene, diet and smoking [5,12–14], polymorphism of benzene biotransformation and excretion of *t,t*-MA [15] are all possible confounding factors. Therefore, in spite of the increasing sensitivity and precision of the analytical techniques applied for the determination of *t,t*-MA in human urine, the wide variations in the biomarker levels at present remain an inherent feature of the biomarker itself when exposure to low airborne benzene concentrations (i.e., <0.5 ppm) is investigated.

5. Conclusions

A sensitive and selective method for the analysis of *t,t*-MA in human urine by direct injection of the biological fluid into a coupled column HPLC analyser is described. The method provided an adequate cost–benefits ratio for routine analysis of *t,t*-MA; its valuable features are sufficient sample throughput (2 h^{-1}), robustness, precision, and minimal sample handling. The unattended LC–LC–UV analyser in our laboratory could process samples with a high degree of automation, in the course of 2 consecutive days without the need of particular maintenance.

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