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Journal of Chromatography B, 751 (2001) 331–339

JOURNAL OF CHROMATOGRAPHY B

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# Improved coupled column liquid chromatographic method for high-speed direct analysis of urinary *trans*,*trans*-muconic acid, as a biomarker of exposure to benzene

Giorgio Marrubini[a,*], Elbert A. Hogendoorn[b], Teresa Coccini[c], Luigi Manzo[a,c]

[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]*Laboratory of Organic-Analytical Chemistry, National Institute of Public Health and the Environment (RIVM), Bilthoven, The Netherlands*

[c]*Toxicology Division, Salvatore Maugeri Foundation, Medical Centre of Pavia, Pavia, Italy*

Received 6 April 2000; received in revised form 29 August 2000; accepted 1 September 2000

**Abstract**

A coupled column liquid chromatographic (LC–LC) method for high-speed analysis of the urinary ring-opened benzene metabolite, *trans*,*trans*-muconic acid (*t*,*t*-MA) is described. Efficient on-line clean-up and concentration of *t*,*t*-MA from urine samples was obtained using a 3 μm $C_{18}$ column (50×4.6 mm I.D.) as the first column (C-1) and a 5 μm $C_{18}$ semi-permeable surface (SPS) column (150×4.6 mm I.D.) as the second column (C-2). The mobile phases applied consisted, respectively, of methanol–0.05% trifluoroacetic acid (TFA) in water (7:93, v/v) on C-1, and of methanol–0.05% TFA in water (8:92, v/v) on C-2. A rinsing mobile phase of methanol–0.05% TFA in water (25:75, v/v) was used for cleaning C-1 in between analysis. Under these conditions *t*,*t*-MA eluted 11 min after injection. Using relatively non-specific UV detection at 264 nm, the selectivity of the assay was enhanced remarkably by the use of LC–LC allowing detection of *t*,*t*-MA at urinary levels as low as 50 ng/ml ($S/N>9$). The study indicated that *t*,*t*-MA analysis can be performed by this procedure in less than 20 min requiring only pH adjustment and filtration of the sample as pretreatment. Calibration plots of standard additions of *t*,*t*-MA to blank urine over a wide concentration range (50–4000 ng/ml) showed excellent linearity ($r>0.999$). The method was validated using urine samples collected from rats exposed to low concentrations of benzene vapors (0.1 ppm for 6 h) and by repeating most of the analyses of real samples in the course of measurement sequences. Both the repeatability ($n=6$, levels 64 and 266 ng/ml) and intra-laboratory reproducibility ($n=6$, levels 679 and 1486 ng/ml) were below 5%. 

*Keywords:* Exposure biomarker; Muconic acid; Benzene; Coupled column; Urine analysis; Large volume injection

## 1. Introduction

*trans*,*trans*-Muconic acid (*t*,*t*-MA), or *trans*,*trans*-2,4-hexadienedioic acid, is a ring-opened oxidation metabolite which can be detected in both laboratory animals and humans exposed to benzene. The mech-

*Corresponding author. Tel.: +39-0382-556-600; fax: +39-0382-556-615.

*E-mail address:* gmarrubini@fsm.it (G. Marrubini).

0378-4347/01/$ – see front matter 
PII: S0378-4347(00)00497-7

anism of *t,t*-MA formation from benzene is not completely understood. However, *trans,trans*-muconaldehyde (*t,t*-MCHO) a reactive ring-opened six-carbon doubly conjugated dialdehyde which is formed during microsomal metabolism of benzene has been identified as a precursor of *t,t*-MA [1]. Since several studies have indicated that *t,t*-MCHO may play an important role in benzene myelotoxicity, urinary *t,t*-MA has been proposed in occupational and environmental medicine as a biomarker for benzene exposure which also reflects benzene activation to cytotoxic products [2–5]. Recent investigations have indicated that the analysis of *t,t*-MA in urine may provide a convenient approach to biomonitoring of benzene with several advantages including non-invasive testing and good stability of the biomarker in urine [6–20]. Yet, the applicability of this indicator at low benzene concentrations is still controversial since background levels of *t,t*-MA are invariably found in urine of mammals in the absence of obvious benzene exposure [6–11]. Unmodified benzene itself in urine can be a reliable marker of exposure. However, according to recent research published [12], following low-level benzene exposure the data obtained testing *t,t*-MA in urine are generally more reliable and consistent than those observed testing urinary benzene. Therefore, at present the urinary levels of *t,t*-MA are usually preferred for assessing low-level benzene exposure.

Several methods including high-performance liquid chromatography (HPLC) and UV detection [4,10,11,16–19] or mass spectrometry (MS) [20], and gas chromatography (GC) combined with mass spectrometric detection [8,11,13,14] have been used for the analysis of *t,t*-MA in urine. Both HPLC and GC when applied to the determination of *t,t*-MA, as reviewed [11], still require enrichment and clean-up steps, performed either by solid-phase extraction (SPE) on strong anion-exchange (SAX) sorbents [4,8–10,16,18–20] or by liquid–liquid extraction (LLE) using diethyl ether [11] or ethyl acetate [13,14]. With GC, a time-consuming laborious sample pretreatment is required. Usually, after extraction, solvent evaporation and derivatization cannot be avoided owing to the very polar and rather non-volatile nature of *t,t*-MA.

Internal standardization has been used in GC–MS in order to control the quality of the analytical data [8,13,14]. In contrast, internal standardization was not used in most studies determining *t,t*-MA by HPLC–UV-based methods with only one exception [4], despite the inherent variability of the methods related to (e.g.) batch-to-batch reproducibility of SAX cartridges. Recent investigations [21], however, have clearly demonstrated the improved performance of an existing HPLC method [16] when a suitable internal standard for analysis of *t,t*-MA in human urine was used.

Because of the good compatibility of urine with reversed-phase liquid chromatography (RPLC), on-line analysis involving large volume injection and clean-up by means of column switching seems an attractive approach to diminish sample handling and pretreatment. Large-volume injection (200 μl of extract) has been performed with pre-column switching [19], however, off-line SPE on SAX material was applied before instrumental analysis.

As indicated by previous investigations examining highly polar pesticides in aqueous samples [22–27], of β-agonists and corticosteroids in urine [28,29] and serum [30], coupled column RPLC (LC–LC) can be a suitable approach. Compared to a one-column separation or pre-column switching, LC–LC was shown to be efficient in enhancing both selectivity and sensitivity [22–29]. In this study, the screening of urinary *t,t*-MA by LC–LC–UV was investigated in rats to determine the feasibility of this method to process samples originating from experimental exposure studies in a cost-effective way.

## 2. Experimental

### 2.1. Chemicals

*trans,trans*-Muconic acid (*t,t*-MA, *trans,trans*-2,4-hexadienedioic acid, CAS 3588-17-8, 98% purity) was from Sigma–Aldrich (Milan, Italy). Water and methanol both HPLC-grade were from BDH (Milan, Italy). Analytical grade (99% purity) trifluoroacetic acid (TFA) and benzene (99.8% purity) were from Carlo Erba (Milan, Italy).

A stock solution (500 μg/ml) of *t,t*-MA was prepared in methanol and stored at −20°C. For spiking or LC analysis the stock solution was diluted in a solution of 1% TFA in water.

The mobile phase (M-1) applied on the first column (C-1) consisted of methanol–0.05% TFA in water (7:93, v/v). On the second column (C-2), a mobile phase (M-2) of methanol–0.05% TFA in water (8:92, v/v) was used. A rinsing mobile phase (M-R) consisting of methanol–0.05% TFA in water (75:25, v/v) was used for the cleaning of C-1 in between analysis. Sample filtration was done using Titan 0.2-μm nylon 66 filters (13 mm diameter) purchased from SRI (Scientific Resources, Eatontown, NJ, USA).

### 2.2. Apparatus

The LC–LC–UV apparatus is schematically presented in Fig. 1. It consisted of a series 1100 Hewlett-Packard (Waldbronn, Germany) system including a Model G1311A quaternary gradient pump (GP), a Model 1310A isocratic pump (IP), a Model G131A ALS autosampler (AS), a Model G1322A vacuum degasser, a Model G1315A diode-array UV–visible detector (UVD) and a Model G1316A thermostated column oven. In all experiments the oven temperature was set at 22°C. A six-way remote controlled programmable high-pressure valve type ViciAG Valco (Schenkon, Switzerland) was 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.

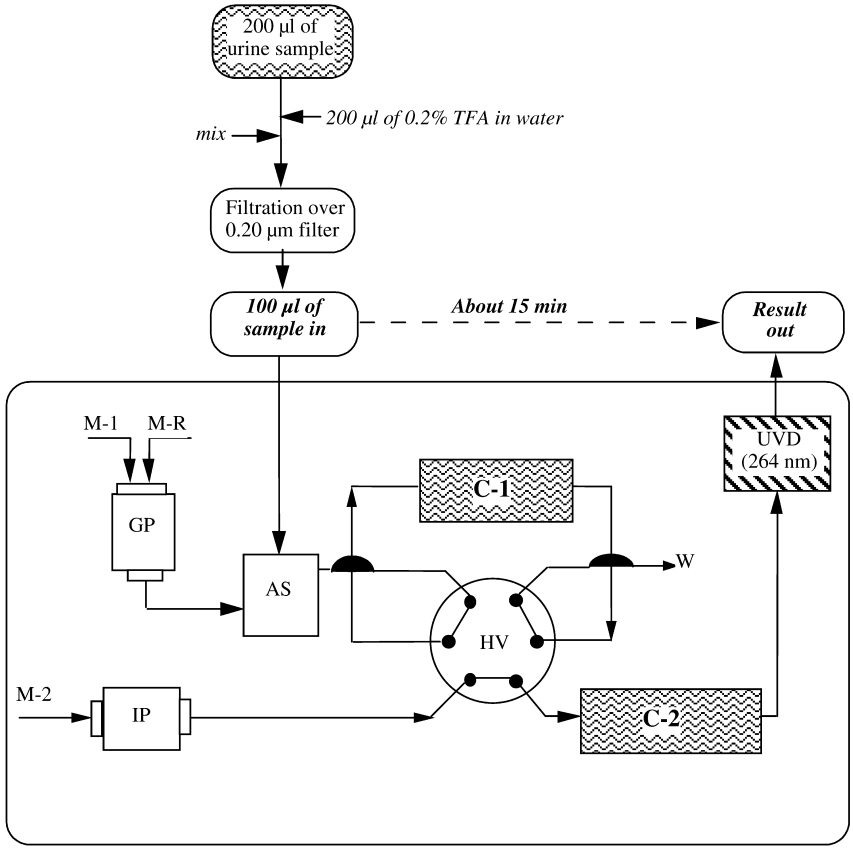


Fig. 1. Scheme of the entire procedure: sample pretreatment and LC–LC–UV analyzer. GP=Quaternary gradient pump; M-1=methanol–0.05% TFA in water (7:93, v/v); M-R=methanol–0.05% TFA in water (25:75, v/v); IP=isocratic pump; M-2=methanol–0.05% TFA in water (8:92, v/v); AS=autosampler; C-1=3 μm particle size, 50×4.6 mm I.D. Microspher $C_{18}$ column; C-2=5 μm particle size, 150×4.6 mm I.D. SPS-5PM-55-100-ODS Regis column; HV=high pressure six-way Valco valve; W=waste outlet; UVD=diode array detector set at 264 nm fixed wavelength.

In LC–LC analysis, a 50×4.6 mm I.D. column packed with 3 μm $C_{18}$ Microspher (Chrompack, Bergen op Zoom, The Netherlands) and a 150×4.6 mm I.D. column packed with 5 μm SPS-5PM-S5-100-ODS (Regis, Morton Grove, IL, USA) were used as the first column (C-1) and second column (C-2), respectively. A pre-column (10×3 mm I.D.) packed with the same packing material of C-1 was installed before the first analytical column (C-1).

### 2.3. Inhalation exposure and sample collection

Adult male Sprague–Dawley rats (200–250 g) purchased from Charles River Italy (Calco, Lecco, Italy) 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%. They had free access to rodent feed (Mucedola, Settimo Milanese, Milan, Italy) and filtered deionized water. 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. The animals were deprived from food during exposure but water was available ad libitum. The whole-body exposures were performed in a dynamic exposure chamber as described elsewhere [31]. The air supply was passed through activated carbon and high efficiency particulate air (HEPA) filters. 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). 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. The urine collection was accomplished directly from the metabolic cages through a polycarbonate funnel at the bottom of the cage leading into a polycarbonate 20-ml tube. Collection funnel and tube were removed and cleaned after each exposure experiment. All samples were stored at −80°C, and were analyzed within 60 days after collection.

### 2.4. Sample pretreatment

A 200-μl volume of urine sample and 200 μl of 2% TFA in water were brought together in a plastic vial, mixed and filtered over a 0.2-μm filter into an autosampler vial.

### 2.5. LC–LC analysis

The mobile phases were adjusted to a flow-rate of 1 ml/min. A volume of 100 μl of urine sample, after dilution and filtration, was injected on C-1. Following the clean-up with 4.9 ml of M-1 (injection volume included), C-1 was switched on-line with C-2 for 0.45 min for the transfer of the analyte containing fraction to C-2. After transfer, C-1 was rinsed with mobile phase M-R for 8 min at a flow-rate of 1.5 ml/min. Next, C-1 was reconditioned for 5 min with M-1 at a flow of 1.0 ml/min.

### 2.6. Calibration plot, external standardization, and instrumental performance control

Quantitation of *t,t*-MA was performed by means of external calibration using calibration plots of standard additions to blank urine taken from untreated rats: fifteen separate samples were prepared spiking urine with *t,t*-MA at the concentrations of 4000 ng/ml, 1000 ng/ml, 500 ng/ml, 100 ng/ml and 50 ng/ml, and every sample was analyzed once (five points, three replications each point, Table 1).

Standard aqueous solutions were also prepared at

Table 1
Results of the calibration plot[a]

| Spiked level (ng/ml) | Mean concentration±SD (ng/ml) | Mean recovery (%) | RSD (%) |
|---|---|---|---|
| 50 | 46±1 | 92 | 2 |
| 100 | 97±5 | 97 | 5 |
| 500 | 499±6 | 100 | 1.3 |
| 1000 | 1010±6 | 101 | 0.6 |
| 4000 | 3998±19 | 100 | 0.5 |

[a] Samples were prepared in triplicate by spiking urine of untreated animals at different *t,t*-MA concentrations. Each sample was analyzed once. SD, Standard deviation ($n=3$); RSD, relative standard deviation. Linear regression data: equation: $y=511.933x+1.674$. Correlation coefficient: $r=0.999980$ ($r^2=0.99996$).

*t,t*-MA concentrations of about 5000 ng/ml, 500 ng/ml, 100 ng/ml and 50 ng/ml. The solutions were freshly prepared for each analytical session. Control samples were prepared in 1% aqueous TFA at the concentration of 100 ng/ml of *t,t*-MA so as to verify every two urine samples whether the system was being contaminated by the biological matrix or any ''memory effect'' occurred.

## 3. Results and discussion

### 3.1. General LC aspects

Given the highly polar and acidic properties of *t,t*-MA, RPLC using a large chain alkyl-bonded stationary phase, e.g., $C_{18}$, and a mobile phase at low pH appears to be an attractive technique to obtain efficient separation of the analyte. When UV detection is used, the analysis of *t,t*-MA in urine is severely hampered by the presence of a large excess of interfering substances having RPLC–UV properties very similar to those of the analyte. Hence, RPLC–UV involving direct urine injection in the absence of efficient clean-up cannot provide much selectivity and thus sensitivity. In the authors experience, reversed-phase LC–LC provided a valid solution to the problem of separating analytes at trace levels in the presence of a large excess of matrix interfering substances [22–30]. The approach of simply dividing the separation power over two analytical columns of equal selectivity by means of column switching can significantly improve selectivity in comparison to either a one-column or a two-column chromatography without column switching. Studies measuring trace levels of analytes in environmental [22–27] or biological [28,29] samples have shown that the most powerful feature of LC–LC is to elucidate peaks in the first part of the chromatogram which, in a single column operation, are frequently obscured by the excess of early eluting interfering substances. This advantage is provided by the separation power of the first column, C-1, which eliminates co-elution of the major part of interferences to the second column, C-2. Clearly, the attainable sensitivity is dependent on the size of the transfer volume, making it most effective in case of single residue methods. On this basis, the potential of LC–LC was investigated for the on-line direct analysis of *t,t*-MA in urine as described below.

### 3.2. LC–LC–UV method development

According to published guidelines [26], the first step in the development of a suitable LC–LC–UV method is to select proper mobile and stationary phases and the dimensions of the columns. Highly polar analytes show poor hydrophobic retention on reversed-phase stationary phases; in order to obtain sufficient separation between a polar analyte such as *t,t*-MA and the matrix interfering substances, a first column with high separation power is desirable. However, an increased separation power usually implies the use of longer columns, and the greater dimension of the column will inversely affect sensitivity.

Based on our previous experience [26,28,30], we firstly selected 3 μm particle size $C_{18}$ columns (50× 4.6 mm I.D.) for both C-1 and C-2. Preliminary experiments performed to select optimal organic modifier showed better selectivity when using methanol instead of acetonitrile. Regarding the mobile phase pH, a study was undertaken for defining the exact p$K_a$ values for *t,t*-MA [32] which apparently have not been reported in previous investigations [11]. As expected, the acidity of *t,t*-MA is very similar to that of fumaric acid. However, and less obviously because of theoretical and practical aspects so far under examination, the p$K_a$ values of *t,t*-MA measured turned out to be slightly higher than those measured for fumaric acid. Hence, a mobile phase consisting of methanol–0.05% TFA in water, pH 2.4 (7:93, v/v) was firstly selected as eluent on both columns, and all samples were acidified at least to a pH value as low as 2 with TFA. Under these conditions *t,t*-MA showed a *k*-value allowing a relatively large clean-up volume of 4.9 ml and a transfer volume of 0.45 ml.

After selecting the appropriate mobile phase, diode array detection and spectral investigation of *t,t*-MA revealed a maximum of UV absorbance at 264 nm which was thus selected for UV detection in the following experiments.

The next step was selecting a proper injection

volume. Early experiments indicated that large-volume injection of *t,t*-MA in 0.05% TFA in water was possible up to a 400 μl with no significant additional band broadening of the analyte compared to an injection volume of 20 μl. Thus, an injection volume of 100 μl was selected, as an acceptable balance between sensitivity and unnecessary high sample load. Urine samples were buffered to pH 2.0 by 1 to 1 dilution with a solution of 2% of TFA in water (see Fig. 1) in order to avoid changes in retention and in peak shape after injection. During the LC–LC–UV processing of standards, the retention of *t,t*-MA was shown to strongly depend on the column temperature: a temperature change of 2°C (22 to 24°C) resulted in a 3% decrease in *t,t*-MA retention, indicating the need to thermostat the columns.

When the selected LC–LC conditions were applied on blank and spiked urine samples, the determination of *t,t*-MA was severely hampered by the co-elution of an interference.

Therefore, selectivity was changed by choosing another type of $C_{18}$ material as the second column. Based on previous favorable applications [27], a 150×4.6 mm I.D. column packed 5 μm particle size $C_{18}$ semi-permeable surface (SPS) material was tested. In this column, a ''restricted access material'' is designed to improve the separation of small analytes and to exclude large molecules from retention in order to process directly biological fluids. In addition to the $C_{18}$-bonded groups, the SPS material comprises a covalently bonded polyoxyethylene polymer network which provides an additional and different retention mechanism [33]. In our study, the application of the SPS column resolved the selectivity problem.

Calibration plots resulting from aqueous standards and standards additions in urine were very similar, demonstrating clearly the selectivity of the method. During the processing of both standards in water and urine no significant changes were observed in *t,t*-MA peak, which indicates the robustness of the approach. However, in order to prevent system contamination caused by interferences present in the matrix, e.g., deposition of salts, sample processing was carried out by alternating injections of samples and urinary standards with injections of aqueous standard solutions and/or aqueous blanks as controls. In such approach the occurrence of any (unexpected) matrix effect can be controlled by comparing the response of equally concentrated aqueous and urinary standard solutions.

### 3.3. Results

The selectivity and sensitivity features of the LC–LC–UV analyzer are illustrated by Fig. 2 comparing four superimposed chromatograms. Chromatogram A shows the peak of *t,t*-MA corresponding to 100 ng/ml of analyte in an aqueous acidic reference solution. Chromatograms C and D compared with the blank (chromatogram B) demonstrate that urinary *t,t*-MA is well distinguishable from the matrix background noise when concentrations of benzene as low as 0.1 ppm are administered to the animals. Quantitative data are presented in Table 1, showing a calibration plot obtained by the measurement of blank urine spiked with *t,t*-MA at levels from 50 to 4000 ng/ml. For each concentration level, three samples were examined (five points, three replications each point). The results showed high correlation coefficients ($r=0.99998$).

Repeatability and intra-laboratory reproducibility of the method were tested daily by multiple analyses of real samples. The results of four sets of these experiments are summarized in Table 2. Both repeatability and intra-laboratory reproducibility were less than 5% for direct determination of urinary *t,t*-MA at concentration levels ranging from 64 to 1486 ng/ml.

### 3.4. Discussion

The advantage of LC–LC using the $C_{18}$/SPS column combination in comparison to the use of the same columns without column switching (i.e., direct coupling) is illustrated by Fig. 3. In comparative experiments measuring a rat urine sample containing 75 ng/ml of *t,t*-MA, the column switching technique provided much better selectivity than a two-column approach.

The LC–LC–UV method described in this report is rapid and simple in application, especially in the sample preparation procedure which consists only in dilution and filtration of the biological fluid over disposable filters. This simplicity is associated with the consistent precision of measurements, the re-

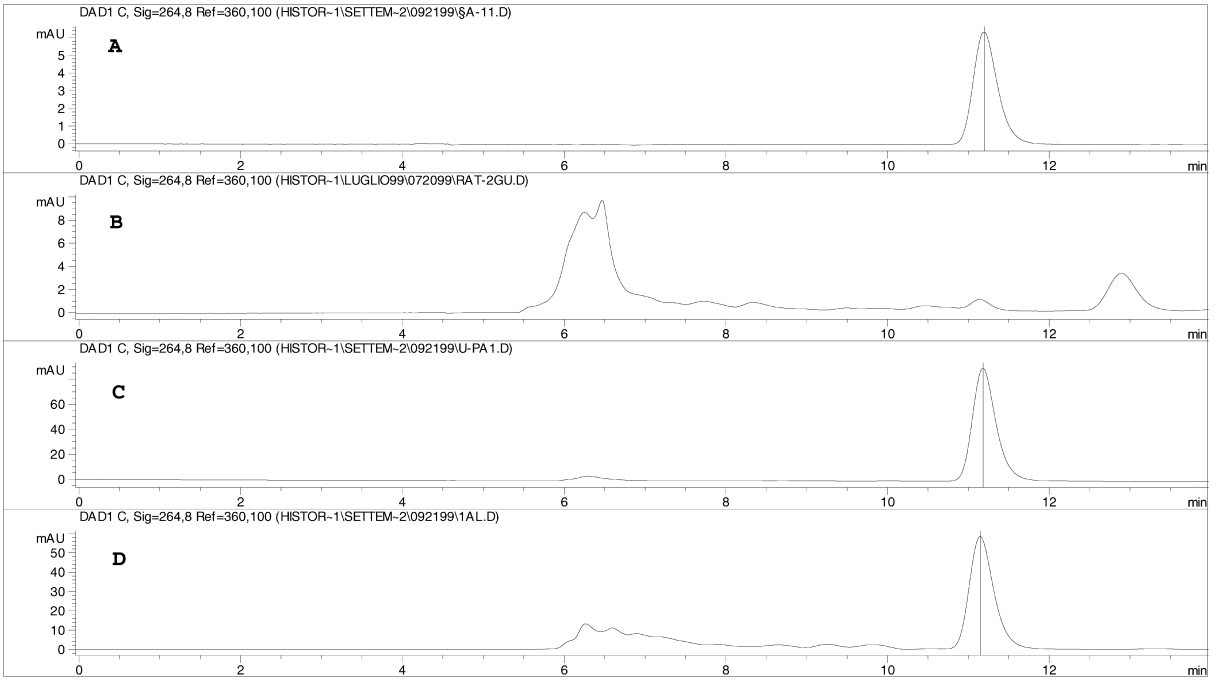


Fig. 2. LC–LC–UV (264 nm) analysis of *t,t*-MA. Injection volume of 100 μl. (A) 100 ng/ml of *t,t*-MA acidic (1%, v/v, TFA) aqueous standard solution; (B) blank urine; (C) blank urine spiked with 2000 ng/ml of *t,t*-MA; (D) urine taken from a rat exposed for 6 h to 0.1 ppm of benzene in air: *t,t*-MA concentration found was 1800 ng/ml.

peatability, the intra-laboratory reproducibility, and the linearity of the calibration plot. As a demonstration of the robustness of the instrumental apparatus, more than 550 samples of urine were processed with the same couple of columns, and no particular care was dedicated to instrument maintenance if we except the daily washing of both HPLC columns with M-R.

The speed of the entire procedure was estimated by a sample throughput of 30–40 samples per day; the upper limit was readily reached when less control samples and replicates were inserted in the analytical sequences.

## 4. Conclusions

Coupled-column reversed-phase chromatography and UV detection (LC–LC–UV) at 264 nm has proven to be a selective and sensitive approach for the direct high speed analysis of *t,t*-MA in urine samples. The results indicated that *t,t*-MA can be

Table 2
Repeatability (*r*) and intra-laboratory reproducibility (*R*) of measurements performed on urine samples taken from rats exposed to 0.1 ppm benzene in air[a]

| | Number of experiments | Mean concentration of *t,t*-MA (ng/ml) | Standard deviation (ng/ml) | *r* (%) | *R* (%) |
|---|---|---|---|---|---|
| A | 3 | 64 | 9 | 3.0 | |
| B | 3 | 266 | 2 | 3.0 | |
| C | 3 | 1486 | 28 | | 2.0 |
| B | 3 | 679 | 8 | | 1.0 |

[a] Samples were taken A, 42-h after exposure; B, 18-h after exposure; C, 6-h during exposure.

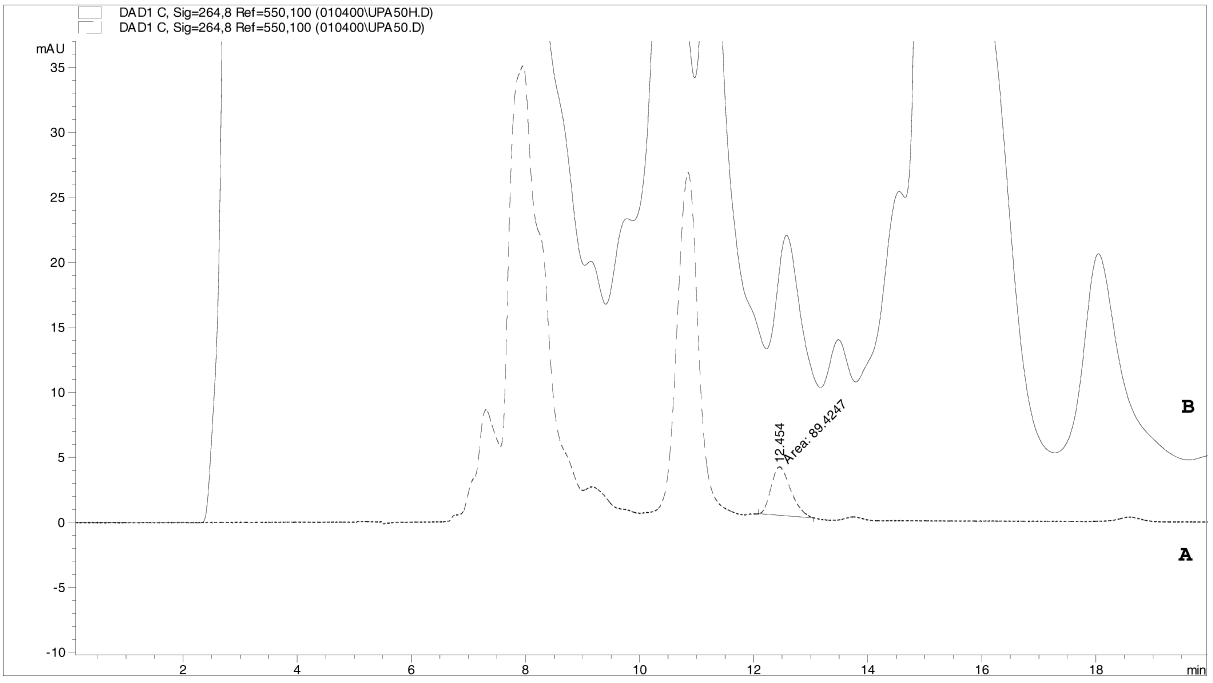


Fig. 3. RPLC–UV (264 nm) of 100 μl of a rat urine sample obtained after dilution and filtration and containing 75 ng/ml *t,t*-MA. (A) LC–LC on 3 μm $C_{18}$ (50×4.6 mm I.D.)/5 μm SPS (150×4.6 mm I.D.) columns; M-1, methanol–0.05% aqueous TFA, pH 2.4 (7:93, v/v) and M-2, methanol–0.05% aqueous TFA, pH 2.4 (8:92, v/v) both at 1 ml/min; clean-up volume, 4.9 ml; transfer volume, 0.45 ml. (B) LC without column switching (C-1 and C-2 directly coupled online); the mobile phase used was methanol–0.05% aqueous TFA, pH 2.4 (8:92, v/v).

measured in rat urine at levels as low as 50 ng/ml in less than 20 min. Consequently, *t,t*-MA can be used as a marker to assess the benzene exposure at ambient air concentrations as low as 0.1 ppm. This may represent a remarkable progress in the biomonitoring of low-level exposure to benzene compared to many published methods measuring urinary *t,t*-MA in laboratory animals or humans [1,11]. Because of a rapid, minimal sample handling and pretreatment, the developed LC–LC procedure affords a sample throughput of 30–40 samples per day which provides a cost-effective way for the routine biomonitoring of benzene exposure. The validation data demonstrate the wide applicability of the LC–LC–UV method, and its suitability for the precise determination of urinary *t,t*-MA over a concentrations range spanning from 50 to 4000 ng/ml.

Further developments will include the validation of the LC–LC–UV method for human urine by inter-laboratory comparison with a different method (i.e., HPLC–MS) and with a procedure involving internal standardization, SPE on SAX cartridges, and RPLC–UV such as those suggested recently by other authors [4,21].

## Acknowledgements

The authors thank Mr. Davide Acerbi for the skilled collaboration offered.

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