# HPLC/MS analysis of the metabolites of 2,4-toluenediamine (2,4-TDA) and 2,4-toluenediisocyanate (2,4-TDI) in rat urine

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**Abstract**

Profiles of the urinary metabolites of 2,4-toluenediisocyanate (2,4-TDI) and 2,4-toluenediamine (2,4-TDA) have been studied in rats with the aim to find suitable biomarkers of 2,4-TDI exposure. The animals were dosed intraperitoneally with 0.10 mmol/kg of each compound. Urine was cleaned-up by the extraction with acetonitrile and by preparative HPLC to remove polar matrix components. The residuum containing a wide range of metabolites was analyzed by HPLC/MS in the APCI ionization mode. In the rats dosed with 2,4-TDA, most of the previously identified metabolites, namely, 4-acetylamino-2-aminotoluene, 2,4-diacetylaminotoluene, their phenolic derivatives, 4-acetylamino-2-aminobenzoic acid, and 2,4-diacetylaminobenzoic acid, were confirmed. Over ten other compounds were detected and their molecular weight was determined tentatively. The level of urinary metabolites in rats dosed with 2,4-TDI was ca. two orders of magnitude lower compared with the equimolar dose of 2,4-TDA but still a number of products was detected. These were 4-acetylamino-2-aminotoluene, 2,4-diacetylaminotoluene, 2,4-diacetylaminobenzoic acid, three unidentified compounds that were found also as metabolites of 2,4-TDA, and six unknown compounds formed via pathways not including 2,4-TDA („2,4-TDI-specific products“). Identification of the new metabolites will be a subject of future studies.

*Key words:* 2,4-Toluenediisocyanate; 2,4-Toluenediamine; Urinary metabolites

**Abbreviations:** 2,4-TDI, 2,4-toluenediisocyanate; 2,4-TDA, 2,4-toluenediamine; 2,4-TDA-OH(5), 5-hydroxy-2,4-toluenediamine; 2,4-TDA-Ac(2), 2-acetylamino-4-aminotoluene; 2,4-TDA-Ac(4), 4-acetylamino-2-aminotoluene; 2,4-TDA-$Ac_2$, 2,4-diacetylaminotoluene; 2,4-TDA-Ac(4)-OH, hydroxy-4-acetylamino-2-aminotoluene; 2,4-TDA-$Ac_2$-OH, hydroxy-2,4-diacetylaminotoluene; 2,4-DABA, 2,4-diaminobenzoic acid; 2,4-DABA-Ac(2), 2-acetylamino-4-aminobenzoic acid; 2,4-DABA-Ac(4), 4-acetylamino-2-aminobenzoic acid; 2,4-DABA-$Ac_2$, 2,4-diacetylaminobenzoic acid; DMSO, dimethylsulfoxide;

## 1. Introduction

Toluenediisocyanates (TDI) are reactive chemicals used especially in the production of polyurethanes. The most commonly used industrial material is a mixture (4:1) of the 2,4- and 2,6-TDI isomers. Due to their severe immunogenic effects, TDI are the most prevalent chemical cause of occupational asthma in the Western world [1-3]. Therefore, monitoring of exposure to TDI is an important task of occupational health services. Numerous methods for measurement of airborne TDI have been developed [4].

Biological monitoring of the exposure to TDI has been based mainly on the determination of total toluenediamine (TDA) in urine [5-8]. The procedure consists of heating the urine with acid to hydrolyse TDA conjugates, alkalization of the sample, extraction of TDA with toluene, derivatization with a perfluoroacylating reagent, removal of the unreacted reagent, and finally GC/MS analysis. („Total“ TDA is mainly represented by the hydrolysable conjugates, namely, N-acetyl derivatives, glucuronides, and sulphates, which greatly predominate over free TDA.) Biomonitoring of TDI is also possible through analysis of the covalent adducts with plasmatic proteins or hemoglobin. While determination of the labile adducts at the sulfhydryl groups of proteins includes mild hydrolysis to release the corresponding TDA [8,9], the stable adducts at the N-terminal valine of globin are determined following conversion to specific hydantoins [10-11].

The HPLC/MS technique allows highly sensitive and less tedious analytical procedures to be developed. Currently, TDA released by hydrolysis of the urine from TDI-exposed workers was extracted and measured with HPLC/MS, while the derivatization step was omitted [12]. We believe that biomonitoring of 2,4-TDI using HPLC/MS analysis can be further simplified by avoiding the hydrolysis, provided that a suitable biomarker is selected. However, the

complete pattern of urinary metabolites of TDI in humans or other species has not been investigated yet.

The major portion of inhaled TDI is covalently bound to tissues. Only a small portion undergoes direct hydrolysis to TDA [13] but additional TDA is probably produced by catabolic breakdown of the covalent adducts. The metabolic route of TDI that involves TDA is assumed to produce identical end-products as the metabolism of TDA itself, which is known to include oxidation on the aromatic ring and methyl group, and formation of N-acetyl-, O- or N-glucuronyl-, and O-sulphate conjugates (Fig. 1). These reactions occur also in combination. The major metabolite of 2,4-TDA in rabbit, rat, and guinea-pig was tentatively identified as 5-hydroxy-2,4-toluenediamine [2,4-TDA-OH(5)], other metabolites were 4-acetylamino-2-aminotoluene [2,4-TDA-Ac(4)], 2-acetylamino-4-aminotoluene [2,4-TDA-Ac(2)], 2,4-diacetylaminotoluene [2,4-TDA-$Ac_2$], hydroxy-4-acetylamino-2-aminotoluenes [2,4-TDA-Ac(4)-OH], several other phenols, and possibly an N-glucuronide [14]. Other authors detected in the urine of rats and mice 2,4-TDA-Ac(4), 2,4-TDA-$Ac_2$, 4-acetylamino-2-aminobenzoic acid [2,4-DABA-Ac(4)], and 2,4-diacetylaminobenzoic acid [2,4-DABA-$Ac_2$] (Fig. 1) [15]. Similar metabolite pattern was found in rats dosed with 2,4-TDA, 0.5 mmol/kg b.w., whereas no urinary metabolites were detected after intraperitoneal administration of 2,4-TDI, 0.15 mmol/kg [16]. Trace amounts of 2,4-TDA-Ac(4) and 2,4-TDA-$Ac_2$ were identified in rats exposed to 2,4-TDI intraperitoneally or by inhalation, using a highly sensitive GC/MS/MS method [13,17,18].

Detection of 2,4-TDI metabolites in humans is hampered by their very low concentration in exposed workers´ urine. Therefore, we first investigated the urinary metabolic profiles in rats injected intraperitoneally with high doses of 2,4-TDI and 2,4-TDA. Comparison of both profiles enabled us to distinguish which of the metabolites found in the urine of 2,4-TDI-dosed rats were 2,4-TDI-specific and which were formed via 2,4-TDA. The study revealed

several new metabolic products of both types, detailed identification of which will be a subject of forthcoming investigations.

## 2. Experimental

### *2.1. Chemicals*

2,4-TDI (purum, >98%) and 2,4-TDA (pract., >98%) were bought from Fluka. 2,4-TDA-Ac(2), 2,4-TDA-Ac(4) and 2-TDA-$Ac_2$ were prepared in our laboratory according to Glinsukon et al. [18]. 2,4-diaminobenzoic acid [2,4-DABA] (free acid) was liberated from its dihydrochloride using 0.1 M sodium hydroxide. 2,4-DABA dihydrochloride was prepared by heating of 2,4-DABA-$Ac_2$ with concentrated hydrochloric acid. The 2,4-DABA-$Ac_2$ was prepared by the oxidation of 2,4-TDA-$Ac_2$ using potassium permanganate. 2,4-DABA-Ac(4) and 2-acetylamino-4-aminobenzoic acid [2,4-DABA-Ac(2)] were prepared by the acetylation of 2,4-DABA (free acid) with an equimolar amount of acetic anhydride and subsequent separation by preparative TLC. All other reagents and solvents were of analytical grade, from various sources.

### *2.2. Animal experiment*

Male Wistar rats (350-430 g) (Charles River, Czech Republic), 3 groups each consisting of 2 animals, were individually placed in glass metabolic cages to collect control urine for 24 h. Then, solution of 2,4-TDI in olive oil (8.7 mg/ml × 2 ml/kg, i.e., 0.10 mmol/kg), or solution of 2,4-TDA in DMSO (6.1 mg/ml × 2 ml/kg, i.e., 0.10 mmol/kg), or DMSO alone (2 ml/kg), was administered intraperitoneally, and the animals were again placed in metabolic cages. Water was provided ad libitum. After 24 h, the animals were removed, the cages were rinsed

with distilled water (5-10 ml) to wash down the urine completely, and the diluted urine was frozen until analysis.

*2.3. Sample clean-up procedure*

To the rat urine sample (2 ml) was added cold acetonitrile (8 ml). The mixture was briefly shaken and centrifuged at 1000 g for 3 min to separate two layers. The clear upper layer was transferred and evaporated in a rotary vacuum evaporator to dryness. The residue was dissolved in distilled water (1 ml).

The acetonitrile-precleaned urine from 2,4-TDI-dosed rats was further treated as follows. An aliquot (400 μl) was fractionated using a preparative HPLC system which consisted of a LC-10AT pump, SCL-10A system controler, CTO-10A column oven, SPD-M10A diode-array detector, and FRC-10A fraction collector (Shimadzu, Japan). A stainless steel column 150×10 mm with Pegasil ODS, 5μm (Senshu Pak, Japan) was used. Mobile phase: water-MeOH with MeOH contents (v/v) as follows (time - % of MeOH): 0-5, 10-5; 40-50, 45-80, 48-5; 60-5 (linear gradients). Flow-rate: 2 ml/min. Column temperature: 40 $^{o}$C. The eluate fractions were collected in 2-min intervals during the first 10 min, then in 5-min intervals until 55 min. An aliquot of each fraction (20 μl) was used for GC/MS determination of total 2,4-TDA. The remaining eluate of all fractions collected over 10-55 min was then pooled, evaporated in Speedvac to dryness, and the residue was reconstituted in distilled water (500 μl) for the HPLC/MS analysis.

The acetonitrile-precleaned urine from rats dosed with 2,4-TDA in DMSO or with DMSO alone was also purified by preparative HPLC as described above. However, the fractionation was omitted and all eluate collected over 10-55 min was pooled and evaporated to dryness. The residue was analyzed as described for 2,4-TDI.

*2.4. Determination of total 2,4-TDA*

Total 2,4-TDA was determined by the method of Williams et al. [19], modified by Morita et al. [20]. The procedure included hydrolysis of urine with sulfuric acid, alkalization, extraction of 2,4-TDA with dichloromethane, derivatization with heptafluorobutyric anhydride (HFBA), and GC/MS-NCI analysis.

*2.5. HPLC/MS*

The measurements were carried out on an HPLC/MS system consisting of a liquid chromatograph model 1100 (Hewlett-Packard, USA) or Rheos 2000 pump (LC Packings, The Netherlands) coupled to a LCQ ion trap mass spectrometer (Finnigan, USA) set in positive APCI mode. Capillary temperature 150 $^{o}$C, APCI vaporizer temperature 450 $^{o}$C. Detector range: *m/z* 50-700. Helium was used as a collision gas. Collision-induced dissociation mass spectra were obtained at a relative collision energy 35%. Daughter ions were monitored at *m/z* 50-500.

The metabolic profiles of 2,4-TDI and 2,4-TDA were measured in the pooled (10-55 min) eluate from the preparative HPLC under the experimental conditions I and II, respectively.

*Experimental conditions I* The measurements were carried out in Occupational Poisoning Centre, Tokyo. Stainless steel column Synergi 4μm Polar-RP 80Å, 150×4.6 mm (Phenomenex, USA). Sample volume: 20 μl. Mobile phase: (A) 0.1% acetic acid in water, (B) 0.1% acetic acid in MeOH, (time - % of B): 0-0, 18-80, 20-80, 21-0, 30-0 (linear gradients). Flow-rate: 1 ml/min. Column temperature: 25 $^{o}$C.

*Experimental conditions II* The measurements were carried out in the Institute of Entomology, České Budějovice. Stainless steel column Synergi 4μm Polar-RP 80Å, 150×2 mm (Phenomenex, USA). Sample volume: 5 μl. Mobile phase: (A) 0.04% TFA in water, (B)

0.04% TFA in acetonitrile, (time - % of B): 0-10, 18-80, 20-80, 21-10, 30-10 (linear gradients). Flow rate: 0.25 ml/min. Column temperature: 30 $^{o}$C.

*2.6. HPLC/MS data analysis*

The full scan MS data collected over 0-12 min of HPLC analysis of the cleaned-up urines were divided into 0.5-min intervals. Average full scan mass spectra measured over matching intervals in the pre-exposure (control) and postexposure urine from the same animal, were visually inspected for characteristic differences. Presence of ions in spectra of the postexposure urine, when they were absent or significantly less abundant in spectra of the control urine, was an indication of the possible presence of a metabolite. Mass chromatograms at the given *m/z* were compared. We considered as confirmed presence of a metabolite when a peak was observed in chromatogram of the postexposure urine but there was no peak at that retention time in chromatogram of the control urine.

## 3. Results

*3.1. HPLC profile of the 2,4-TDI metabolites hydrolysable to 2,4-TDA*

Urine of rats dosed with 2,4-TDI was precleaned by the extraction with acetonitrile and fractionated on a preparative HPLC column. Aliquots of the collected fractions were heated with sulfuric acid to liberate 2,4-TDA from its conjugates. This was derivatized with HFBA and determined by GC/MS-NCI. Fig. 2 indicates that the major part of the metabolites hydrolysable to 2,4-TDA was eluted between 25 and 55 min. On the other hand, almost the entire amount of polar endogenous components of urine was eluted between 2-6 min (visible

as a residue obtained after evaporation of the mobile phase). Thus, the fractions eluting after 10 min were pooled to efficiently separate the 2,4-TDI metabolites from the polar matrix. The same procedure was used to isolate the metabolites of 2,4-TDA.

### *3.2. Metabolic products of 2,4-TDA*

Rat urine samples collected before and after intraperitoneal administration of 2,4-TDA, 0.1 mmol/kg, were precleaned by the extraction with acetonitrile and preparative HPLC, and investigated using HPLC/MS in the APCI mode (*Experimental conditions I1*). About ten metabolites of 2,4-TDA could be detected in the total ion current (TIC) chromatogram of the postexposure urine (Fig. 3). The following compounds were identified by comparison with the synthetic standards: 2,4-TDA-Ac(4), 2,4-TDA-$Ac_2$, 2,4-DABA-Ac(4), and 2,4-DABA-$Ac_2$ (Fig. 1, Table 1). Another two major metabolites, both providing ions at *m/z* 205, 223, and 240 ($t_R$: 3.66 and 4.18 min), are most likely the isomeric phenols 2,4-TDA-$Ac_2$-OH. The compound at *m/z* 181 ($t_R$: 2.79 min) is probably phenol 2,4-TDA-Ac(4)-OH. However, since aromatic amines are known to undergo also oxidation to N-hydroxylamines, presence of N-hydroxy-2,4-TDA derivatives with $M_r$ identical to phenols cannot be excluded. A complete list of detected metabolic products of 2,4-TDA is presented in Table 2. Parent 2,4-TDA as well as the potential metabolites 2,4-TDA-Ac(2), 2,4-DABA, and 2,4-DABA-Ac(2) were not found.

### *3.3. Metabolic products of 2,4-TDI*

Rat urines collected before and after intraperitoneal administration of 2,4-TDI, 0.1 mmol/kg, were analysed similarly as described for 2,4-TDA, with the exception that the conditions of

the HPLC/MS analysis were slightly different (*Experimental conditions 1*). Such change resulted in prolonged retention times but the elution order and APCI spectra of the compounds of interest remained almost unaffected.

The level of urinary metabolites after dosing rats with 2,4-TDI was about two orders of magnitude lower when compared with the equimolar dose of 2,4-TDA. In the total ion chromatograms (*m/z* 50-500), no significant differences between pre- and post-exposure urines were observed. However, a systematic search (see 2.6.) resulted in the detection of numerous compounds (Table 3), though at very low levels, with the ratio of signal to noise usually being less than 10. Of these compounds, 2,4-TDA-Ac(4), 2,4-TDA-$Ac_2$, and 2,4-DABA-$Ac_2$ were confirmed using synthetic standards, and 2,4-TDA-$Ac_2$-OH was identified tentatively. Other 2,4-TDA-related but unidentified components are those characterised as follows: $t_R$ 7:08 min (m/z 297), $t_R$ 7.56 min (*m/z* 326), and $t_R$ 8.16 min (m/z 321). The remaining compounds listed in Table 3, namely, those at $t_R$ 7:69 min (*m/z* 252), $t_R$ 8.83 min (*m/z* 361+379+396), $t_R$ 9:41 and 10:39 min (m/z 308+325), $t_R$ 9:55 min (m/z 317+318+334+335), and $t_R$ 9.87 min (m/z 304+322) were not detected after administration of 2,4-TDA. Therefore, they appear to be the 2,4-TDI-specific metabolites.

## 4. Discussion

HPLC/$MS^n$ is an effective method for detection and identification of unknown components in complex mixtures, because a wide array of compounds of various properties can be analyzed simultaneously and, for each compound, highly characteristic mass spectra can be obtained. Analysis of biological material for xenobiotics usually includes a clean-up procedure to isolate the analytes from the matrix, which may overload or contaminate the HPLC column

and MS detector. In this study, i.e., profiling of TDI/TDA metabolites in rat urine, a nonselective work-up procedure had to be developed to cover a wide range of compounds, which may be alkaline, acidic, amfoteric or nonionic molecules of various polarity. Since neither liquid-liquid extraction with water-immiscible solvents nor solid phase extraction appeared suitable for this purpose, an alternative two-step clean-up procedure was employed.

First, the urine was mixed with acetonitrile. This resulted in the formation of two separated liquid phases in most samples. A large amount of inorganic salts and highly polar matrix components such as urea (up to 60% by weight) remained in the aqueous layer, while the TDI/TDA metabolites were recovered in the upper acetonitrile layer. (Acetonitrile is a water-miscible liquid. The phase separation occurs only with highly concentrated samples such as rat urine, which contains ca. 50-200 mg of solid per ml, but would not occur with human urine.) It cannot be excluded that some polar metabolites may remain in the aqueous phase. Nevertheless, the recovery of the total 2,4-TDA in the acetonitrile phase, assessed by the comparison of total amounts of 2,4-TDA in both phases, was >90%. Despite the usefulness of the extraction with acetonitrile, semipreparative HPLC proved to be a superior clean-up procedure in that it permitted separation of the TDI/TDA metabolites from polar matrix with a great efficiency. When the clean-up by HPLC was omitted, the final HPLC/MS analyses provided distorted chromatograms.

The HPLC/MS screening of rat urine for the TDI/TDA metabolites revealed numerous compounds. Some of them were unambiguously identified using the synthetic standards. Since positive APCI detection mode produces characteristic ions $[M\text{-}OH]^+$, $[M+H]^+$, $[M+H_2O]^+$, and $[2M+H]^+$, such ionization pattern permitted assessment of the molecular weights of unknown compounds more reliably than a single ion. If only a single ion was detected, it was assumed to be $[M+H]^+$ (Table 2,3), although in some known compounds the most abundant ions were $[M\text{-}OH]^+$ or $[M+H_2O]^+$ (Table 1). The assessed molecular weights

were tested as to whether they correspond to hypothetical products generated by known metabolic reactions of 2,4-TDA. Mass increments produced by these reactions are shown in Table 4. For example, the values of 180 and 222 amu correspond to the previously identified metabolites 2,4-TDA-Ac(4)-OH and 2,4-TDA-$Ac_2$-OH, respectively [14]. Similarly, the values of 251 and 325 amu correspond to acetyl-2,4-diaminohippuric acid and dihydroxy-2,4-diacetylhippuric acid, respectively. However, not all compounds characteristic for post-exposure urines have to be necessarily exogenous metabolic products. Administration of relatively high doses of irritant diisocyanate or diamine may seriously affect endogenous metabolism, thus inducing highly enhanced levels of physiological components in the urine.

The HPLC/MS analysis of urine from 2,4-TDA-exposed rats enabled to detect within a single analysis most of the metabolites reported previously [14-16]. The slight differences in results of this and previous studies may be due to differences in rat strain and administered dose. Although the current analytical procedure is highly nonselective, it cannot be excluded that some extremely polar metabolites, such as glucuronides or sulphates, were removed by the sample clean-up. Failure to detect them in this study may also be due to low response in the APCI ionization technique.

Nevertheless, the present investigation pointed out a number of unknown metabolic products of 2,4-TDA and 2,4-TDI. Forthcoming studies are planned to elucidate their structure, explain their metabolic origin, and evaluate their usefulness for biomonitoring of exposure.

**Acknowledgements**

The study was supported by grant 310/00/0934 from the Grant Agency of the Czech Republic, and by Health and Labour Sciences Research Grants (Research on Occupational Safety and

Health) from the Ministry of Health, Labour and Welfare, Japan, which is gratefully acknowledged. The authors also thank to Dr. I. Linhart for his valuable comments on the manuscript.

**References**

1. M.H. Karol, R. Jin, Chem. Res. Toxicol. 4 (1991) 503-509.
2. X. Baur, W. Marek, J. Ammon, A.B. Czuppon, B. Marczynski, M. Raulf-Heimsoth, H. Roemmelt, G. Fruhmann, Int. Arch. Occup. Environ. Health 66 (1994) 141-152.
3. J.A. Bernstein, Toxicology 111 (1996) 181-189.
4. R.P. Streicher, C.M. Reh, R.J. Key-Schwartz, P.C. Schlecht, M.E. Cassinelli, P.F. O´Connor, Am. Ind. Hyg. Assoc. J. 61 (2000) 544-556.
5. C. Rosenberg, H. Savolainen, J. Chromatogr. 367 (1986) 385-392.
6. T. Brorson, G. Skarping, C. Sango, Int. Arch. Occup. Environ. Health 63 (1991) 253-259.
7. A. Maître, M. Berode, A. Perdrix, S. Romazini, H. Savolainen, Int. Arch. Occup. Environ. Health 65 (1993) 97-100.
8. G. Sabbioni, Environ. Health Perspect. 102, Supplement 2 (1994) 61-67.
9. O. Sepai, D. Schütze, U. Heinrich, H.G. Hoymann, D. Henschler, G. Sabbioni, Chem.-Biol. Interact. 97 (1995) 185-198.
10. J. Mráz, E. Gálová, M. Hornychová, Abstracts of the International Symposium on Biological Monitoring in Occupational and Environmental Health, Espoo, September 1996, 103-104.
11. G. Sabbioni, R. Hartley, S. Schneider, Chem. Res. Toxicol. 14 (2001) 1573-1583.
12. T. Sakai, Y. Morita, Y. Kim, Y.X. Tao, Toxicol. Letters 134 (2002) 259-264.
13. C. Timchalk, F.A. Smith, M.J. Bartels, Toxicol. Appl. Pharmacol. 124 (1994) 181-190.

14. R.H. Waring, A.E. Pheasant, Xenobiotica 6 (1976) 257-262.

15. P.H. Grantham, L. Mohan, T. Benjamin, P.P. Roller, J.R. Miller, E.K. Weisburger, J. Environ. Pathol. Toxicol. 3 (1980) 149-166.

16. J. Mráz, Š. Bouškova, H. Nohová, I. Linhart, Abstracts of the International Symposium on Biological Monitoring in Occupational and Environmental Health, Espoo, September 1996, 101-102.

17. M.J. Bartels, C. Timchalk, F.A. Smith, Biol. Mass. Spectrometry 22 (1993) 194-200.

18. T. Glinsukon, E.K. Weisburger, T. Benjamin, P.P. Roller, J. Chem. Eng. Data 20 (1975) 207-209.

19. N.R. Williams, K. Jones, J. Cocker, Occup. Environ. Med. 56 (1999) 598-601.

20. Y. Morita, T. Sakai, Y. Kim, Jap. J. Occup. Med. Traumatol. 51 (2003) 154-157.

**Table 1.** HPLC/MS-APCI analysis of the synthetic standards. The measurements were conducted as described in *Experimental conditions II*.

| Compound | $M_r$ | MS (m/z) [a] | $MS^2$ [b] (m/z) [a] | $t_R$ (min) |
|---|---|---|---|---|
| 2,4-TDA | 122 | 123, 164 | 123: 106, 108 | 2.45 |
| 2,4-TDA-Ac(2) | 164 | 165, 206, 329 | 165: 106, 124, 147 | 2.97 |
| 2,4-TDA-Ac(4) | 164 | 165, 206, 329 | 165: 106, 123, 124, 147 | 3.52 |
| 2,4-TDA-$Ac_2$ | 206 | 165, 207, 224, 329, 371 | 207: 165, 177 | 5.15 |
| 2,4-DABA | 152 | 109, 153 | 153: 135 | 3.57 |
| 2,4-DABA-Ac(2) | 194 | 151, 177, 195, 234 | 195: 151, 177 | 6.05 |
| 2,4-DABA-Ac(4) | 194 | 151, 177, 195 | 195: 151, 177 | 5.21 |
| 2,4-DABA-$Ac_2$ | 236 | 219, 237, 254 | 237: 177, 195, 219 | 6.23 |

[a] the most abundant ion is underlined

[b] parent ion: doughter ions

**Table 2.** Characteristic compounds detected by HPLC/MS-APCI analysis of rat urine after administration of 2,4-TDA. The measurements were conducted as described in *Experimental conditions II*.

| $t_R$ (min) | m/z [a] | $M_r$ (assessed) | Structure [b] |
|---|---|---|---|
| 2.22 | 137 | 136 | |
| 2.58 | 182 | 181 | |
| 2.79 * | 151, 163, <u>181</u> | 180 | 2,4-TDA-Ac(4)-OH |
| 2.96 | <u>155</u>, 196, 309 | 154 | |
| 3.48 | 403 | 402 | |
| 3.52 * | <u>165</u>, 182, 206, 329 | **164** | **2,4-TDA-Ac(4)** |
| 3.66 * | <u>205</u>, 223, 240 | 222 | 2,4-TDA-$Ac_2$-OH |
| 3.70 | 281 | 280 | |
| 4.05 | 297 | 296 | |
| 4.18 * | 205, 223, <u>240</u> | 222 | 2,4-TDA-$Ac_2$-OH |
| 4.40 * | 151, 163, 206, <u>326</u>, 651 | 325 | |
| 4.46 | 321 | 320 | |
| 5.15 * | 165, 207, <u>224</u>, 371, 430 | **206** | **2,4-TDA-$Ac_2$** |
| 5.21 * | 151, 177, <u>195</u> | **194** | **2,4-DABA-Ac(4)** |
| 6.23 * | 219, 237, <u>254</u> | **236** | **2,4-DABA-$Ac_2$** |
| 6.67 * | 431, <u>448</u> | 430 | |
| 7.88 * | 447, <u>464</u> | 446 | |
| 8.13 | 220 | 219 | |
| 8.20 * | <u>421</u>, 438 | 420 | |
| 8.33 | <u>321</u>, 338 | 320 | |
| 8.79 | 300 | 299 | |

[a] the most abundant ion is underlined

[b] structure of compounds printed in bold was confirmed using the synthetic standards

* peak visible in the total ion current chromatogram (Fig. 3)

**Table 3.** Characteristic compounds detected by HPLC/MS-APCI analysis of rat urine after administration of 2,4-TDI. The measurements were conducted as described in *Experimental conditions I.*

| $t_R$ (min) | m/z [a] | $M_r$ (assessed) | Structure [b] |
|---|---|---|---|
| 6.47 | 165 | **164** | **2,4-TDA-Ac(4)** |
| 6.83 | 240 | 222 | 2,4-TDA-$Ac_2$-OH |
| 7.08 | 297 | 296 | |
| 7.55 | 326 | 325 | |
| 7.69 | 252 | 251 | |
| 8.09 | 207, <u>224</u> | **206** | **2,4-TDA-$Ac_2$** |
| 8.16 | 321 | 320 | |
| 8.83 | 361, <u>379</u>, 396 | 378 | |
| 9.07 | 237 | **236** | **2,4-DABA-$Ac_2$** |
| 9.41 | <u>308</u>, 325 | 307 | |
| 9.55 | <u>317</u>, 318, 334, 335 | 316 | |
| 9.87 | <u>304</u>, 322 | 321 | |
| 10.39 | 165, <u>308</u>, 325 | 307 | |

[a] the most abundant ion is underlined

[b] structure of compounds printed in bold was confirmed using the synthetic standards

**Table 4.** Known or possible metabolic reactions of 2,4-TDA

| Metabolic reaction | Product | Mass increment (amu) | Number of groups |
|---|---|---|---|
| N-acetylation | M-H+$COCH_3$ | 42 | 1, 2 |
| oxidation of methyl to carboxyl | M-$CH_3$+COOH | 30 | 1 |
| oxidation of methyl to carboxyl followed by conjugation with glycine | M-$CH_3$+CONHCHCOOH | 87 | 1 |
| C- or N-hydroxylation | M-H+OH | 16 | 1, 2, 3... |
| O- or N-glucuronidation | M+$C_6H_{10}O_7$-$H_2O$ | 176 | 1, 2, 3... |
| O-sulfatation | M+$H_2SO_4$-$H_2O$ | 80 | 1, 2, 3... |

**Figure captions**

**Fig. 1** Proposed metabolic scheme of 2,4-TDI and 2,4-TDA, and formulae of the known or possible 2,4-TDA-related metabolites

**Fig. 2.** HPLC fractionation of the urine of rats dosed intraperitoneally with 2,4-TDI, 0.10 mmol/kg: elution profile of 2,4-TDA in the hydrolysed fractions (a), in non-hydrolyzed fractions (b)

**Fig. 3.** HPLC/MS chromatograms (*m/z* 50-700) of rat urine before (a) and after (b) intraperitoneal administration of 2,4-TDA, 0.1 mmol/kg. The peaks indicated by an arrow denote the exposure-related compounds visible in the TIC chromatogram. The analysis was conducted as described in *Experimental conditions II*.

**Fig. 1.**

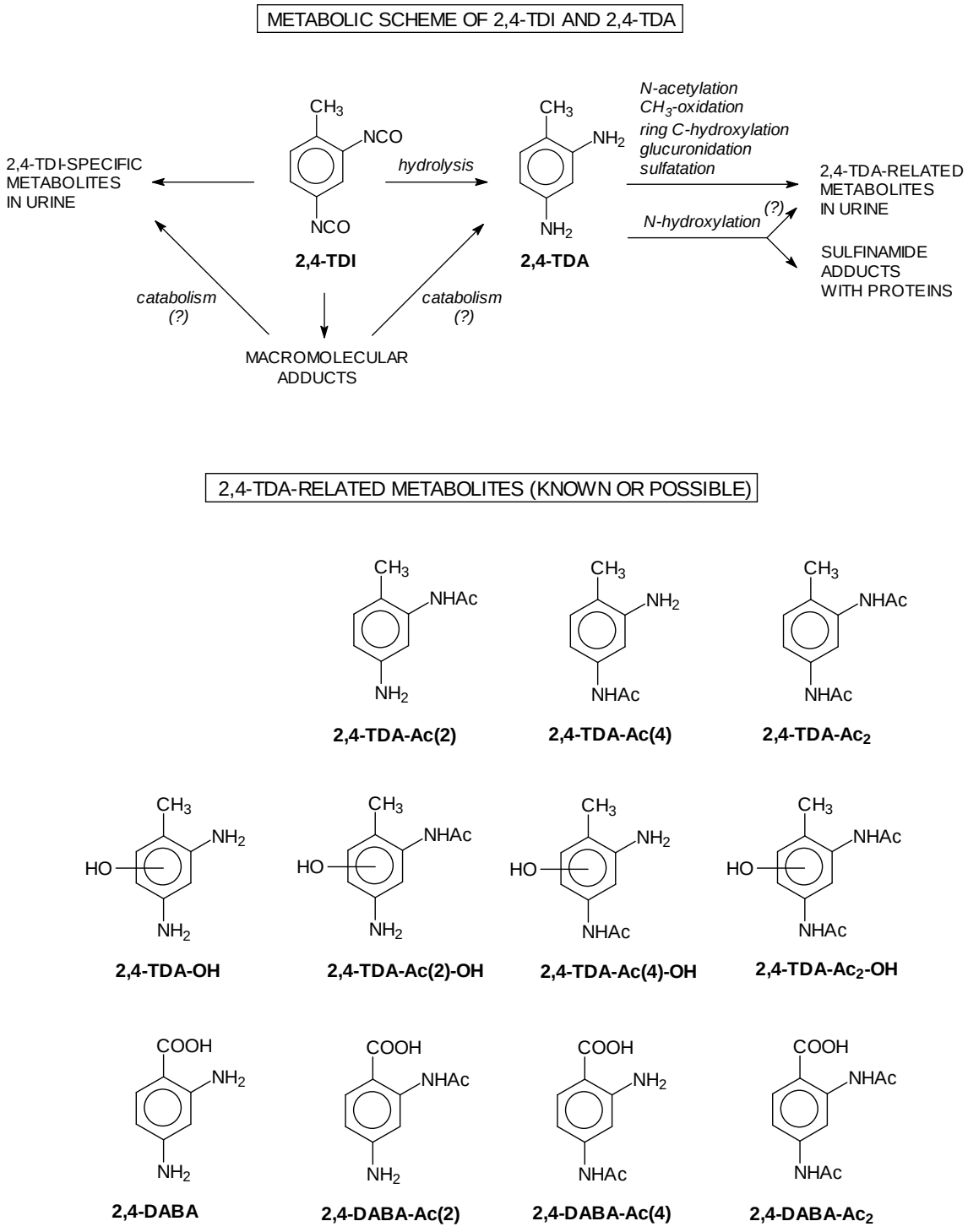

METABOLIC SCHEME OF 2,4-TDI AND 2,4-TDA
2,4-TDI-SPECIFIC METABOLITES IN URINE
hydrolysis
2,4-TDI
2,4-TDA
N-acetylation
CH3-oxidation
ring C-hydroxylation
glucuronidation
sulfatation
2,4-TDA-RELATED METABOLITES IN URINE
N-hydroxylation
(?)
SULFINAMIDE ADDUCTS WITH PROTEINS
catabolism (?)
catabolism (?)
MACROMOLECULAR ADDUCTS
2,4-TDA-RELATED METABOLITES (KNOWN OR POSSIBLE)
2,4-TDA-Ac(2)
2,4-TDA-Ac(4)
2,4-TDA-Ac2
2,4-TDA-OH
2,4-TDA-Ac(2)-OH
2,4-TDA-Ac(4)-OH
2,4-TDA-Ac2-OH
2,4-DABA
2,4-DABA-Ac(2)
2,4-DABA-Ac(4)
2,4-DABA-Ac2

**Fig. 2.**

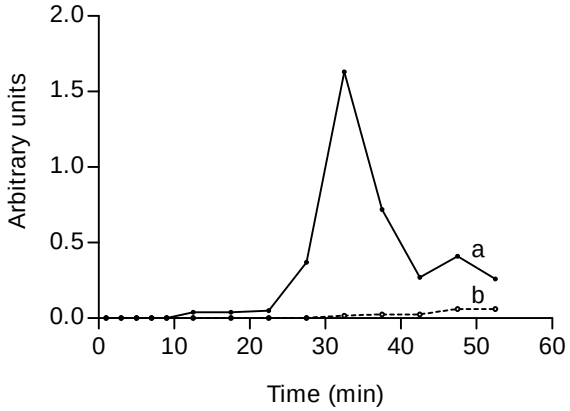
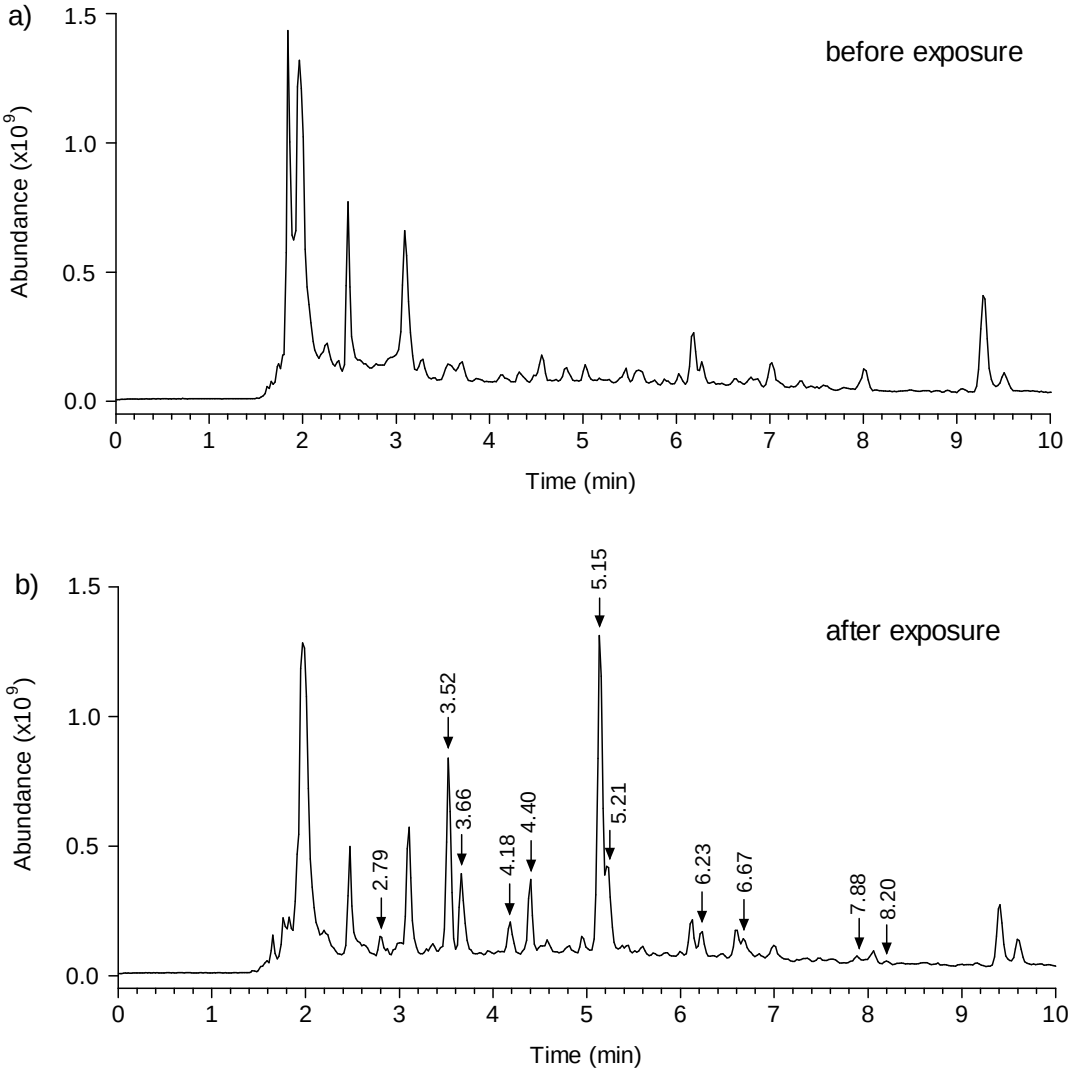


**Fig. 3.**


a)
before exposure
Abundance (x10^9)
Time (min)
b)
after exposure
Abundance (x10^9)
Time (min)
2.79
3.52
3.66
4.18
4.40
5.15
5.21
6.23
6.67
7.88
8.20