Simultaneous Determination of Benzidine, Acetylbenzidine and *di*-Acetylbenzidine in Rat Urine

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A gas chromatography/mass spectrometric assay method has been developed for the simultaneous determination of benzidine (BZ), *N*-acetyl benzidine (ABZ) and *N,N*-diacetyl benzidine (DABZ) in rat urine. BZ, ABZ and DABZ were extracted from urine at pH 8 with ethyl ether. Conjugated urinary metabolites were extracted at pH 8 after hydrolysis with 1 M HCl for 30 min at 100 °C. The dried extract was dissolved in 100 μL of ethylacetate and then injected in gas chromatography-mass spectrometric (GC-MS) system without further purification or modification. BZ, ABZ and DABZ have good chromatographic properties and offer very sensitive response for the EI-MS (SIM) without any derivatization. The recoveries for BZ, ABZ and DABZ were about 98.0, 81.8 and 71.4%, respectively, at pH 8.0 and the concentration of 5.0 ng/mL. The coefficients of variation of BZ and ABZ were less than 9.5% from 0.1 to 100 ng/mL and that of DABZ was less than 13% in the same concentration range. The detection limits of the assay were 0.01 ng/mL for both BZ and ABZ, and 0.05 ng/mL for DABZ in urine or plasma 1.0 mL.

Keywords : Benzidine, *N*-Acetyl benzidine, *N,N*-Diacetyl benzidine, GC-MS (SIM).

Introduction

Benzidine(4,4-diaminobiphenyl, BZ) is widely employed in the manufacture of dyes. Chronic exposure to BZ is known to produce urinary bladder cancer in human, so studies show that exposure must last for at least six months and that tumors may appear after a latency period of 2-42 years.[1,2] Workers exhibiting a high incidence of bladder tumors had urine BZ concentrations of less than 0.160 mg/L.[3] The measurement of unchanged BZ in urine has been used as an index of exposure to the compound. Urinary BZ concentrations were about 0.009 mg/L in workers exposed to air levels of 0.007-0.011 mg/m^3. The range was 0.100-0.200 mg/L for workers exposed to air containing 0.150-0.400 mg/m^3 of the chemical.[4]

Of an absorbed dose of BZ, urinary excretion accounts for an estimated 4-10% as the parent compound, 7-16% as acetyl benzidine (ABZ) and diacetyl benzidine (DABZ) and much of the remainder as the sulfate conjugate of 3-hydroxybenzidine.[4,5]

ABZ can interact with biological macromolecules, such as hemoglobin, RNA and DNA and lead to a new covalent bond between BZ or ABZ and macromolecules, leading to adducts.

Developing a sensitive quantification method of BZ, ABZ and DABZ in urine or plasma is important to monitoring the compounds and providing insight into the ability of BZ to interact with biological macromolecules *in vivo*.

Chromatographic methods have been published for the analysis of BZ and its metabolites in urine or plasma, involving HPLC methods utilizing electrochemical detection.[6-9] Mass spectrometry has the potential to become a standard analytical tool for detecting BZ and its metabolites. Several mass spectral techniques have been used in this area, however, sensitive analysis of BZ and its metabolites *in vivo* sources was usually accomplished by gas chromatography/mass spectrometry (GC/MS).[10-12] Hsu and colleagues[11] describe a GC-NCI-MS method to determine the three compounds by converting aromatic amines to their pentafluoropropionyl derivatives. Detection limits were as low as 0.5, 0.8, and 1.5 pg/mL for BZ, ABZ and DABZ. The method is, however, too complex and time consuming. Jedrzejczak and colleagues[12] also attempted to determine benzidine in urine using capillary gas chromatography and negative-ion chemical-ionization mass spectrometry after pentafluoropropionyl derivatization. The lower limit of detection for benzidine in this case was 0.5 mg/L.

In this paper we discuss a rapid and simple method for the simultaneous determination of BZ, ABZ and DABZ in rat urine without derivatization.

Experimental Section

Materials. Benzidine and diphenylamine (internal standard) were obtained from Sigma (St. Louis, MO, USA). Acetylbenzidine and *di*-acetylbenzidine were providede after synthesis by Prof. No (Yonsei University, Seoul, Korea). Analytical grade of potassium carbonate, potassium hydroxide, potassium bishydrogen phosphate, sodium sulfate, hydrochloric acid and sodium chloride (Sigma, St. Louis, MO, USA) were used as reagents and ethyl ether, methanol, ethanol, acetone and ethylacetate (E. Merck, Darmstadt, Germany) were used as solvents.

Animals and treatment. Seven female Sprague-Dawley

rats, each with a body weight of about 220 g, were obtained from Haehanbiolink (Chongju, Korea). They were acclimatized for one week in Macrolone cages (temp. of 18 °C, humidity of 30-70%, illumination time from 6 a.m. to 6

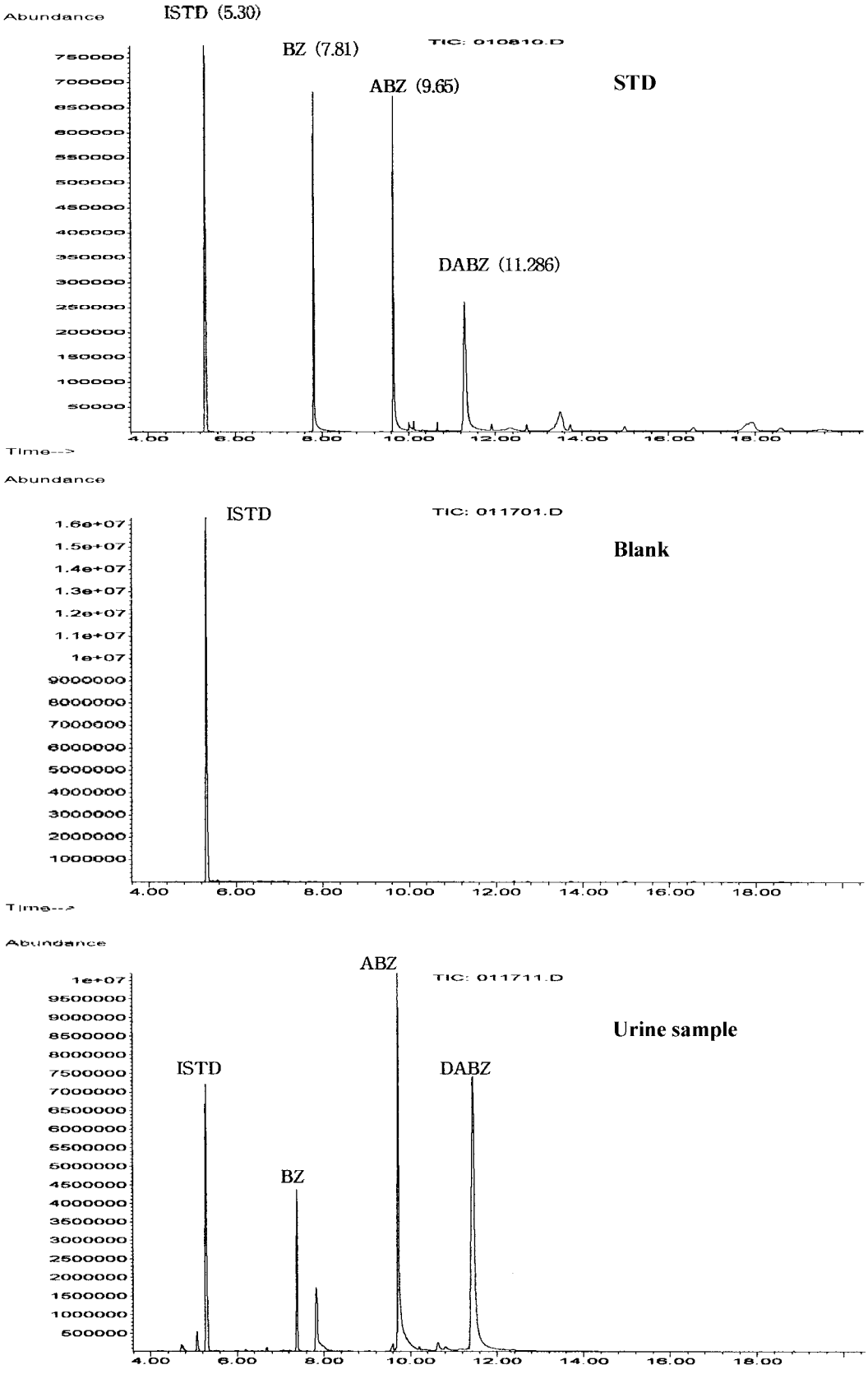

Figure 1. Chromatograms of standard (0.5 mg/L), blank and the extract from rat urine.

p.m.), and they had free access to tap water and food. Benzidine was given orally to one group (3 per group) at a dose of 2.0 mg/kg as a 2.0 mg/mL aqueous solution. Urine samples were collected during 24 h. Another group were treated with BZ in the drinking water for 3 weeks at a concentration of 0.008 %, and urine samples were collected daily. BZ in the drinking water was prepared by dissolving 0.8 g of BZ in 0.5 mL of ethanol and dilution with 10 L of mineral water. The content of BZ in the drinking water was confirmed by GC-MS. One control rat was left untreated and destroyed after 3 weeks.

Extraction from biological samples. In a 20 mL test tube, was placed 1 mL of urine. About 2 g of KH_2PO_4 and 100 μL of diphenylamine (1 μg/mL in acetone) as an internal standard were added to the solution, and the sample was extracted with 7 mL of ethyl ether by mechanical shaking for 10 min. The organic phase was transferred into a 20 mL glass test tube, which was stoppered and dried in an evaporator and finally with nitrogen. The dried residue was dissolved with 100 μL ethyl acetate, and a 2 μL sample of the solution was injected in the GC system.

Acid hydrolysis. In a 20 mL test tube, was placed 1 mL of urine and 0.25 mL of 5 M HCl. The solution was heated at 100 °C for 30 min. The acid-hydrolyzed samples were neutralized with 0.2 mL of 5M KOH and buffered by adding about 2 g of KH_2PO_4 before extraction. The recoveries were compared by the concentrations of BZ, and its metabolites were recovered at 10, 20, 30, 40, 50 and 60 min after acid treatment.

Gas chromatography-mass spectrometry. All mass spectra were obtained with a Agilent (Palo Alto, CA, USA) 6890/5973N GC-MSD instrument. The ion source was operated in the electron ionization mode (EI: 70 eV, 230 °C). Full-scan mass spectra (m/z 40-800) were recorded for analyte identification. Selected ion monitoring (SIM) detection mode was used for the quantification of the analytes. The fragments with m/z 169 and 168 from the internal standard were recorded between 3.50 and 7.00 min, the fragments with m/z 92 and 184 from BZ were recorded between 7.00 and 8.50 min, the fragments with m/z 184 and 226 from ABZ were recorded between 8.50 and 10.00 min and the fragments with m/z 184, 226 and 268 from DABZ were recorded between 10.00 and 20.00 min with a dwell time of 50 ms. An HP 1 capillary column (30 m × 0.2 mm i.d. × 0.33 μm F.T.) was used. Samples were injected in the pulsed splitless mode. The flow rate of the helium was 1.2 mL/min. The GC operating temperatures were: injector temperature, 300 °C; transfer line temperature, 310 °C; oven temperature, programmed from 100 °C at 20 °C/min to 320 °C (held for 2 min).

Calibration and quantification. Calibration curves for BZ, ABZ and DABZ were established by extraction after adding 0.1, 50, 100, 500, 1000, 2500, 5000 and 10000 ng of standards and 100 ng of internal standard in 1 mL of urine. The ratios of the peak areas of standards to that of internal standard was used in the quantification of the analytes.

Recovery. Urine samples at various pH values were prepared and the relative recoveries were calculated as percentages of recovered BZ and its metabolites.

Method detection limit. Method detection limits were calculated by a minimum signal-to-noise ratio of 3 and coefficients of variation for replicate determinations (n = 5) of 15% or less after adding 0.01 and 0.02 ng of standards and 100 ng of internal standard in 1 mL of urine.

Results and Discussion

Chromatogram. The present work describes a GC-MS (SIM) method to quantify benzidine and its metabolites in rat urine using diphenylamine as an internal standard. The chromatograms are shown in Figure 1. As can be seen from the Figure, the peaks of BZ, ABZ and DABZ are relatively symmetrical. The retention times of ISTD, BZ, ABZ and DABZ were 5.30, 7.81, 9.65 and 11.29 min, respectively. Separation of the analytes and the internal standard from the background compounds of urine were very good. There were no extraneous peaks observed in a chromatogram of blank biological sample at the retention times of analytes and internal standard.

Acid hydrolysis. Acid hydrolysis was tested in 1 M HCl at 100 °C. The recoveries of BZ, ABZ and DABZ were found to be maximum at 30 min and, thereafter, remained constant (Figure 2). Therefore, the hydrolysis time was decided as 30 min.

Recovery. The recoveries of BZ and its metabolites from urine samples at various pH values were studied (Figure 3). Those of BZ, ABZ and DABZ were about 98.0, 81.8 and 71.4 at pH 8.0 and the concentration of 5.0 ng/mL, respectively, and it was found to be nearly constant at several concentrations. The recoveries decrease according to the increase of the polarity of the compounds.

Linearity. Examination of typical standard curve by computing a regression line of peak area ratios of BZ, ABZ and DABZ to an internal standard on concentration, using a least-squares fit demonstrated a linear relationship with correlation coefficients being consistantly greater than 0.993. The lines of best fit for BZ, ABZ and DABZ are $y = 0.1937x - 0.0181$ ($r^2 = 0.9998$), $y = 0.2557x - 0.0270$ ($r^2 = 0.9994$) and

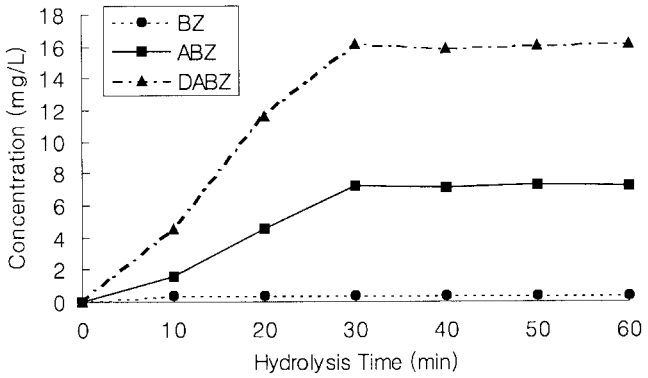

Figure 2. The time course in the acid hydrolysis of conjugated BZ, ABZ and DABZ in urine.

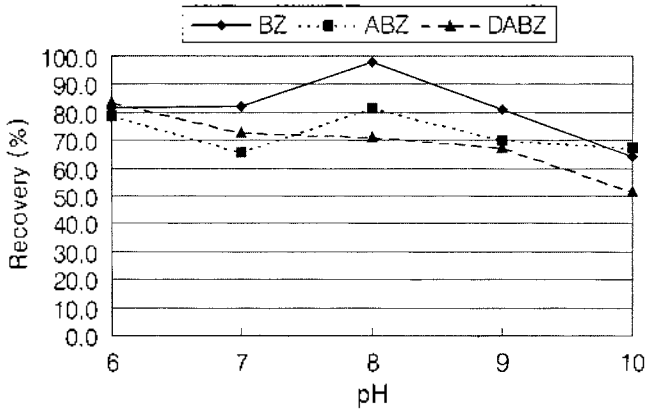

Figure 3. The recoveries of BZ, ABZ and DABZ at various pH.

Table 1. Within-run precision and accuracy of BZ, ABZ and DABZ in urine (n=5)

Added concentration (ng/mL)	Found concentration (ng/mL), X ± SD (RSD)		
	BZ	ABZ	DABZ
0.10	0.11±0.01 (9.1)	0.12±0.01 (8.3)	0.92±0.12 (13.0)
5.00	4.92±0.19 (3.9)	4.89±0.23 (4.7)	4.87±0.42 (8.6)
100.0	102.0±2.0 (2.0)	97.8±2.5 (2.6)	98.0±4.2 (4.3)

X = mean value; SD = standard deviation; RSD = relative standard deviation

$y = 0.4199x - 0.1256$ ($r^2 = 0.9938$) over the very wide range of 0.1-10000 ng/mL, where x is the analyte concentration (ng/mL) and y is the peak area ratio of the analyte to internal standard.

Precision and accuracy. The reproducibility of the assay was very good, as shown in Table 1. For five independent determinations at 0.1, 5.0 and 100.0 ng/mL, the coefficient of variation was less than 13%.

Sensitivity. Detection limits were 0.01 ng/mL for BZ and ABZ, and 0.05 ng/mL for DABZ based upon an assayed urine volume of 1 mL. Limits were defined by a minimum signal-to-noise ratio of 3 and coefficients of variation for replicate determinations (n = 5) of 15% or less. This method permits the determination of BZ, ABZ and DABZ in urine of workers exposed to air levels of 0.007-0.011 mg/m^3.

Application to rat urine samples. Rat urine was analyzed by the proposed method. Acid hydrolysis was used for the determination of the conjugated forms of benzidine and its metabolites in urine.

After administration of a single oral dose of 2 mg/kg BZ, 0.6% was excreted in 24 hr as unchanged benzidine, 11.3% as ABZ (60% among that as conjugated form), and 34.3% as DABZ (78% as conjugated form).

Daily rat urine concentrations of BZ, ABZ and DABZ during the treatment of 3 weeks with BZ at concentration of 0.008% are shown in Figure 4. During the initial 1 week after the treatment of BZ, the excretion amounts of two acetylated metabolites were in the concentration range of 1-9 mg/L, but thereafter, those of two metabolites increased to the concentration range of 10-23 mg/L.

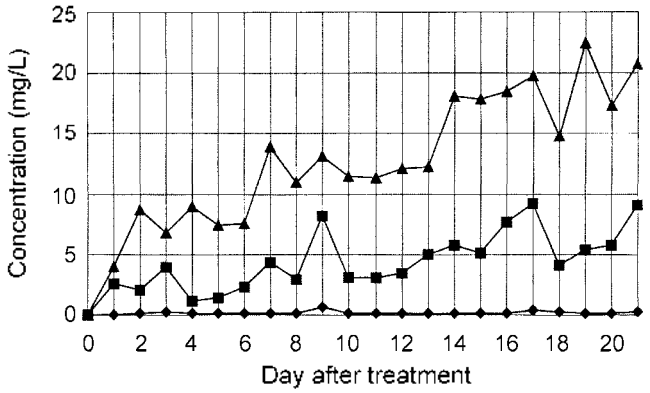

Figure 4. Rat urine concentrations of BZ, ABZ and DABZ during the treatment of 3 weeks with BZ at concentration of 0.008%.

Conclusions

BZ, ABZ and DABZ have good chromatographic properties and offer very sensitive responses for the EI-MS (SIM) without any derivatization. The single-step extraction of these compounds in biological samples with ethyl ether also gave very high recovery with small variation. Quantitation of BZ, ABZ and DABZ are excellent, with linear calibration curves over a range of 0.1-10.000 ng/mL and a detection limit of 0.01-0.05 ng/L. The present method can be used for the rapid monitoring of benzidine and its metabolites in biological samples and may be applicable to the determination of benzidine-hemoglobin adducts.

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