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# New sensitive determination method of benzidine–hemoglobin adducts by gas chromatography–electron impact mass spectrometry

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**Abstract**

A gas chromatographic–mass spectrometric assay was developed for the determination of benzidine (BZ)–hemoglobin adducts. Adducts were released from hemoglobin by alkaline hydrolysis and extraction at pH 8 with ethyl ether. The dried extract was completely derivatized with *N*-methyl-*N*-(*tert*.-butyldimethylsilyl) trifluoroacetamide (MTBDMSTFA)–$NH_4I$ (1000:3) under catalysis of dithioerythritol. The recovery of BZ, acetylbenzidine (ABZ) and diacetylbenzidine (DABZ) in the extraction procedure was 76–98%. The detection limits of the assay were 0.1 ng/g for both BZ and ABZ, and 0.5 ng/g for DABZ based upon assayed hemoglobin of 0.1 g. The method was applied to the determination of BZ–hemoglobin adducts formed in young female Sprague–Dawley rats after treatment for 1, 2 and 3 weeks with 0.008% BZ via the drinking water. Two adducts were detected by proposed procedure. The structure of these adducts could be assigned to BZ and ABZ. After 1 week, the total mean amount of adducts determined was 2.8 ng/g hemoglobin. The adduct levels increased up to about 7.5 ng/g after a week and, thereafter, remained essentially constant. The relative contribution of BZ and ABZ to the total hemoglobin adduct level was strongly treatment time-dependent. After 1 week, the BZ and ABZ adducts were formed at similar levels, whereas after 3 weeks the ABZ adducts was predominant. Treatment of rats for 3 weeks in the dose range 12.2–36.8 mg of BZ in drinking water resulted in a dose-proportional increase in the total amount of hemoglobin adducts formed.


*Keywords:* Benzidine–hemoglobin adducts

## 1. 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 man. It is believed that exposure must last for at least 6 months, and that tumors may appear after a latency period to 42 years [1]. Workers exhibiting a high incidence of bladder tumors have urine BZ concentrations of less than 0.160 mg/l [2].

Of an oral dose of BZ, it has been estimated that urinary excretion accounts for 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 [3,4]. The chemical structures of BZ, ABZ and DABZ are shown in Fig. 1.

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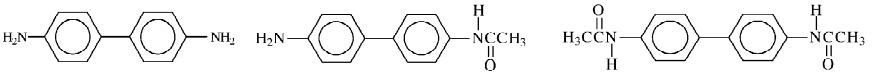

Benzidine Acetylbenzidine Diacetylbenzidine

Fig. 1. Chemical structures of BZ, ABZ and DABZ.

BZ, ABZ and DABZ could interact with hemoglobin and form a new covalent bond with it, leading to adducts. A sensitive quantification method for BZ, ABZ and DABZ needs to be developed in order to provide an insight into the ability of BZ to adduct with hemoglobin in vivo and to monitor the adducts.

Chromatographic methods have been published for the analysis of BZ and its metabolites, involving HPLC methods utilizing electrochemical detection [5–8]. Mass spectrometry has very often been used for detecting BZ and its metabolites. Several mass spectral techniques have been used in this area, however, the most sensitive analysis of BZ and its metabolites is accomplished by gas chromatography–mass spectrometry (GC–MS) [9–12]. A GC–NCI-MS method [10–12] was described to determine the three compounds by converting aromatic amines to their pentafluoropropionyl derivatives. The detection limits were as low as 0.5–1.5 pg/ml in urine sample [10], 0.5 ng/ml in plasma [11] and 25–150 pg/g in hemoglobin [12]. Trimethyl silylation was also attempted to improve the GC–MS behavior of these analytes, but complete derivatization was not achieved due to the lack of stability of the product [10].

This paper describes a complete silylation method for BZ, ABZ and DABZ with *N*-methyl-*N*-(*tert.*-butyldimethylsilyl) trifluoroacetamide (MTBDMSTFA)–$NH_4I$ (1000:3) under the catalysis of dithioerythritol. The method was applied to the determination of BZ–hemoglobin adduct formation in rats.

## 2. Experimental

### 2.1. Chemicals and reagents

Benzidine, diphenylamine [DA, internal standard (I.S.)], trimethylsilyl imidazole (TMS-I), ammonium iodide ($NH_4I$), *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) and MTBDMSTFA were purchased from Sigma (St. Louis, MO, USA). Acetyl benzidine and diacetyl benzidine (purity of >99.9%) were kindly presented by Professor No (Yonsei University, Seoul, Korea). Their purities were confirmed as above 99.8% by GC–FID. Analytical grade of potassium carbonate, potassium hydroxide, potassium bishydrogen phosphate, sodium sulfate, hydrochloric acid and sodium chloride (Sigma) were used as reagents and ethyl ether, methanol, ethanol, acetone and ethyl acetate (Merck, Darmstadt, Germany) were used as solvents.

### 2.2. Animals and treatment

Twenty young female Sprague–Dawley rats with a body mass of about 220 g were obtained from Haehanbiolink (Chongju, Korea). They were acclimatized for 1 week in Macrolone cages (temperature 18 °C, humidity of 30–70%, illumination time from 6 a.m. to 6 p.m.) and they had free access to tap water and food. The animals (three per group) were treated with BZ in the drinking water for 4 weeks at a concentration of 0.008%. 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 preparations was confirmed by GC–MS. After 1, 2 and 3 weeks of treatment, the animals were killed by open heart puncture. Control animals were left untreated and killed after 3 weeks.

### 2.3. Isolation of hemoglobin

Erythrocytes were isolated from the blood by centrifugation and washed three times in phosphate-buffered saline. Cells were lysed by adding four volumes of 0.1 m*M* EDTA, pH 7.5, at 35–41 °C in ultrasonic bath. Cell debris were removed by centrifugation (10 000 *g*). Hemoglobin was isolated by precipitation with acetonitrile and washed twice with

acetonitrile–water, and finally with 80% ethanol, 96% ethanol, ethanol–diethyl ether and diethyl ether. Hemoglobin samples were dried and stored at 4 °C.

### 2.4. Hydrolysis of hemoglobin

A 100-mg amount of dried hemoglobin was hydrolyzed for 3 h at room temperature with 3 ml of 0.1 *M* NaOH. To each sample, 20 μl of DA 0.1 μg/ml in methanol was added as I.S. BZ, ABZ and DABZ released from hemoglobin was extracted with 7 ml of ethyl ether by mechanical shaking for 10 min. The organic phase was transferred into a 20-ml glass-stoppered test tube and dried in an evaporator and finally in a desiccator over $P_2O_5$–KOH for at least 30 min, before derivatization.

### 2.5. Derivatization

A dry residue of the extract from hydrolysis of hemoglobin is dissolved with 50 μl of MTBDMSTFA–$NH_4I$ (1000:3) containing 0.1 mg of dithioerythritol, and the tubes are heated at 80 °C. At reaction times of 5, 20, 40, 60 and 80 min, a 2-μl sample of the solution was injected in the GC system.

### 2.6. Gas chromatography–mass spectrometry

All mass spectra were obtained with a Agilent 6890/5973 N 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. Separation was achieved with an HP fused-silica capillary column with crosslinked methylsilicone (HP 1), ~30 m length, 0.2 mm I.D., 0.33 μm film thickness. Samples were injected in the split mode with a splitting ratio of 1:8. The flow-rate of the helium was 1.0 ml/min. The operating parameters were as follows: injector temperature, 300 °C; transfer line temperature, 310 °C; oven temperature, programmed from 100 °C at 20 °C/min to 310 °C (held for 2 min). The ions selected in this study were *m/z* 168 and 169 for BZ, *m/z* 397 and 454 for ABZ, *m/z* 439 and 496 for DABZ, and *m/z* 168 and 169 for DA (I.S.).

### 2.7. Calibration and quantification

Calibration curves for BZ, ABZ and DABZ were established by extraction and derivatization after adding 0.1, 0.5, 1.0, 5.0 and 10 ng of standards and 2 ng of I.S. in 0.1 g hemoglobin. The ratio of the peak area of standard to that of the I.S. was used in the quantification of the compound.

## 3. Results and discussion

### 3.1. Derivatization

For the enhancement of GC performance (e.g. peak symmetry, resolution, peak height) and sensitivity of BZ, ABZ and DAB, derivatization of the amino group is necessary. In our previous work [12], the aniline nitrogen of BZ and ABZ, and the amide nitrogen of ABZ and DABZ were completely converted to a silylated derivative from each. The reaction rate of BZ, ABZ and DABZ with MTBDMSTFA–$NH_4I$ (1000:3) containing 0.1 mg of dithioerythritol was determined by the detection of the products (Fig. 2). The derivatives were analyzed at reaction times of 5, 20, 40, 60 and 80 min. As a result, only bis-TBDMS–BZ, bis-TBDMS–ABZ and bis-TBDMS–DABZ were detected as products of the silylation reaction of the aromatic amines in about 30 min at 80 °C. These experiments show that silylation of the aromatic amines with MTBDMSTFA produced a sterically crowded branching derivative, which may hinder mono-TBDMS–nitrogen from more silylation. The derivative was stable in the chromatographic system and minimum for 1 week in reagents at room temperature.

### 3.2. Mass spectrometry

The mass spectra of the three silylated derivatives are shown in Fig. 3. The molecular ion at *m/z* 412 and the diagnostic ions at *m/z* 397, 355, 297 and 149 of spectrum A indicated that BZ was silylated to the corresponding bis-TBDMS–BZ with MTBDMSTFA. The molecular ion was the base peak. The ions at *m/z* 397 and 355 were from the losses of one methyl or butyl group from the molecular ion. The molecular ion at *m/z* 397 and the

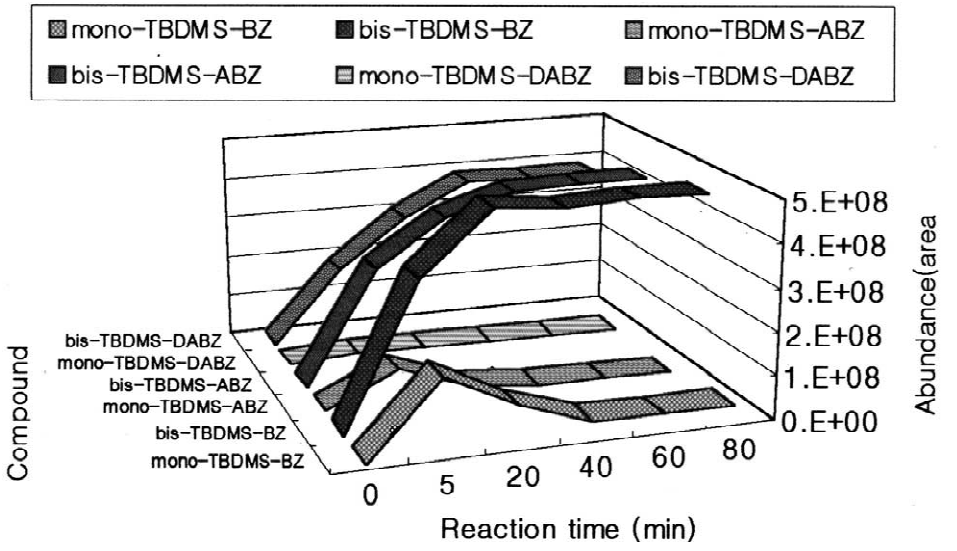


Fig. 2. Time course of the reaction of BZ, ABZ and DABZ with MTBDMSTFA–$NH_4I$ (1000:3) containing 0.1 mg of dithioerythritol.

diagnostic ions at $m/z$ 439, 397 and 341 of spectrum B indicated that ABZ was silylated to the corresponding bis-TBDMS–ABZ with MTBDMSTFA. The base peak $m/z$ 397 was due to the loss of the butyl group from molecular ion. Also, the molecular ion at $m/z$ 496 and the diagnostic ions at $m/z$ 481, 439 and 365 of spectrum C indicated that DABZ was silylated to the corresponding bis-TBDMS–DABZ with MTBDMSTFA. The base peak $m/z$ 439 was due to the loss of the butyl group from the molecular ion.

### 3.3. Chromatography

Fig. 3 also shows the chromatogram of the bis-TBDMS–BZ, bis-TBDMS–ABZ and bis-TBDMS–DABZ derivatives. For the GC separation of the derivatives, the use of a nonpolar stationary phase was found to be efficient. The peaks are symmetrical and no tailing can be seen. The derivatives do not show any adsorption effects in the GC system. The retention times of DA, bis-TBDMS–BZ, bis-TBDMS–ABZ and bis-TBDMS–DABZ were 9.15, 12.31, 12.75 and 13.18 min, respectively. The separation of the derivatives and the I.S. from the background compounds of hemoglobin was good. There were no extraneous peaks observed in a chromatogram of a blank biological sample at the retention times of the analytes.

### 3.4. Linearity

Examination of typical standard curve by computing a regression line of peak area ratios of bis-TBDMS–BZ, bis-TBDMS–ABZ and bis-TBDMS–DABZ to I.S. on concentration using least-squares demonstrated a good fit with correlation coefficients being consistently greater than 0.995. The lines of best fit for BZ, ABZ and DABZ are $y = 0.4662x - 0.0184$ ($r^2 = 0.9951$), $y = 0.6624x - 0.2213$ ($r^2 = 0.9967$) and $y = 0.9524x - 0.0006$ ($r^2 = 0.9992$) over a range of 1.0–100 ng/g, where $x$ is the analyte concentration (ng/g) and $y$ is the peak area ratio of the analyte to I.S.

### 3.5. Extraction yield

Several hemoglobin samples of varying composition were prepared and the extraction yield was calculated by the percentage of the derivatives recovered. The recoveries of BZ, ABZ and DABZ were about 98.0, 82.5 and 76.4 at a concentration of

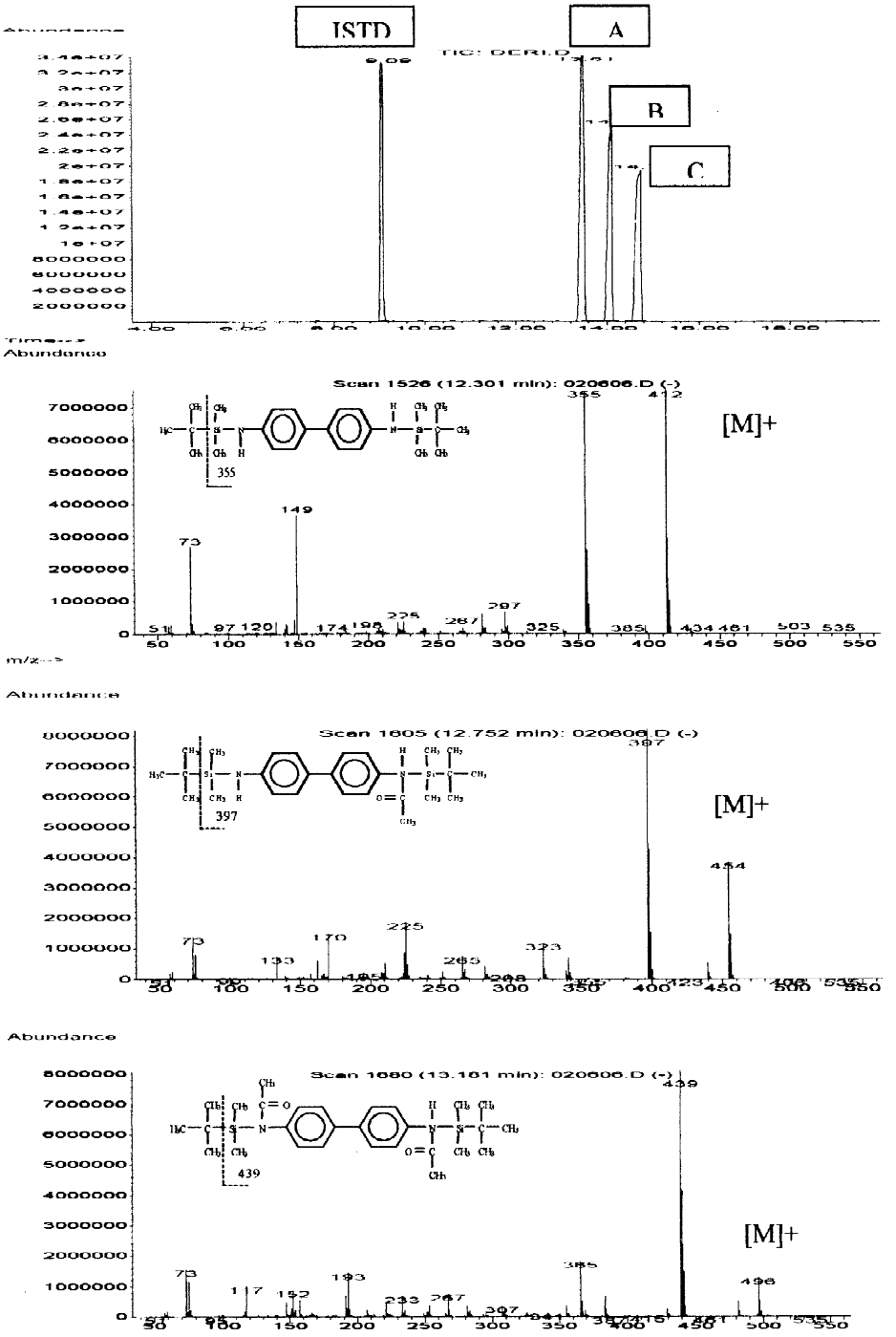


Fig. 3. Chromatogram and EI spectra of (A) bis-TBDMS–BZ derivative, (B) bis-TBDMS–ABZ derivative and (C) bis-TBDMS–DABZ derivative.

5.0 ng/g hemoglobin, respectively, and it was found to be nearly constant at several concentrations.

In order to test whether, with the given procedure for the isolation of hemoglobin, noncovalently bound BZ, ABZ and DABZ were eliminated, hemoglobin was precipitated in the presence of the I.S. and washed with the sequence of organic solvents presented in the isolation method of hemoglobin. No I.S. could be detected in the spiked or control samples.

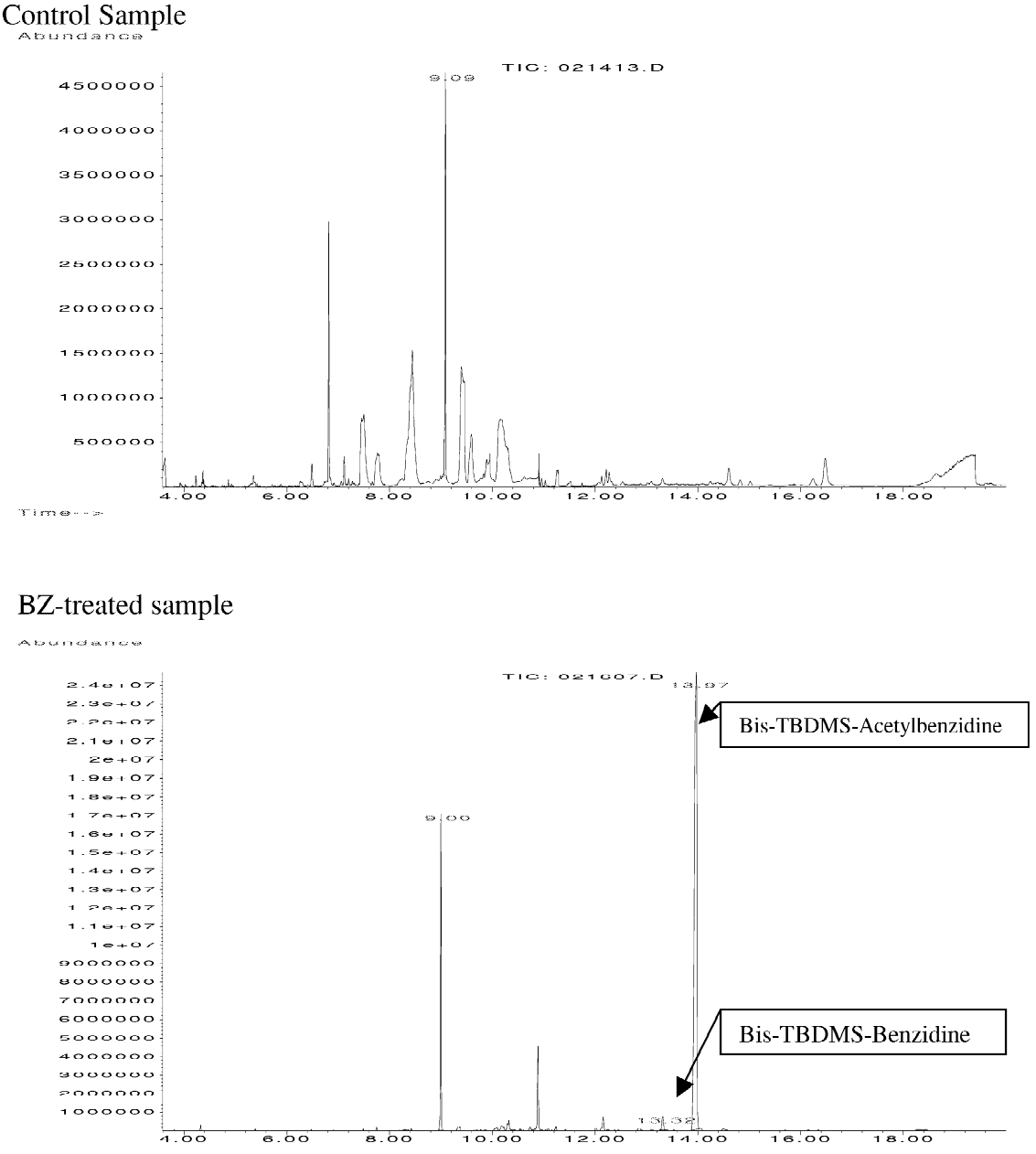


Fig. 4. GC–MS (SIM) chromatograms after hydrolysis of hemoglobin samples isolated from rats, extraction and derivatization; (A) control sample, (B) BZ-treated sample.

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

| Concentration (ng/g) | | $X \pm$SD (RSD, %)[a] |
|---|---|---|
| Added | Found | |
| 1.0 | 1.0, 0.9, 0.9, 0.8, 1.2 | 1.0±0.1 (7.1) |
| 5.0 | 5.1, 5.0, 4.6, 5.2, 4.9 | 5.0±0.2 (4.2) |
| 10.0 | 8.9, 9.5, 10.7, 10.2, 9.8 | 9.8±0.6 (6.2) |

[a] $X$, mean value; SD, standard deviation; RSD, relative standard deviation.

### 3.6. Precision and accuracy

The reproducibility of the assay was very good, as shown in Table 1. For five independent determinations at 1.0, 5.0 and 10.0 ng/g, the RSD was <6%.

### 3.7. Sensitivity

The combination of high derivatization yield and high sensitivity of the derivative by EI-MS (SIM) permits the determination of hemoglobin adducts at concentrations well below those reported previously. The LOD for the derivatized standards is 10 pg for BZ and ABZ, and 50 pg for DABZ. Method detection limits were 0.1 ng/g for BZ and ABZ, based on an assayed hemoglobin of 0.1 g. Limits were defined by a minimum signal-to-noise ratio of 3 and RSD values for replicate determinations ($n=5$) of 15% or less.

### 3.8. Application

After hydrolysis of hemoglobin samples isolated from BZ-treated rats, extraction and derivatization, two adducts were detectable by GC–MS. The structure of these adducts could be assigned to BZ and ABZ by their retention times and fragmentation pattern obtained by GC–EI-MS (Fig. 4).

To assess the formation of the steady state of hemoglobin adducts during treatment with BZ (0.008% BZ in drinking water, 6.2 mg BZ/kg body mass per day), blood samples from rats were taken 1, 2 and 3 weeks after initiation of treatment. After 1 week, the total mean amount of adducts determined was 2.8 ng/g hemoglobin. The adduct levels increased up to about 7.5 ng/g after week 1 and, thereafter, remained essentially constant (Fig. 5). The relative contribution of BZ and ABZ to the total hemoglobin adduct level was strongly treatment time-dependent. After 1 week, the BZ and ABZ adducts were formed at similar levels, whereas after 3 weeks the DABZ adducts was predominant. Treatment of rats for 3 weeks in the dose range 12.2–36.8

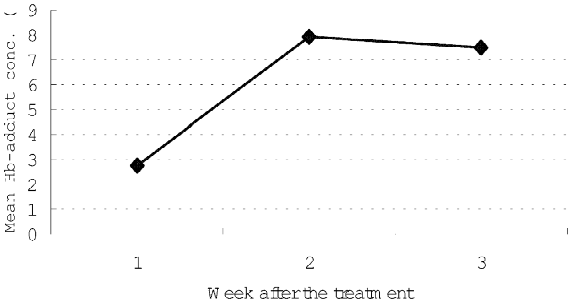


Fig. 5. Accumulation of hemoglobin adducts (mean of three animals) in rats during treatment for 3 weeks with 0.008% BZ in the drinking water.

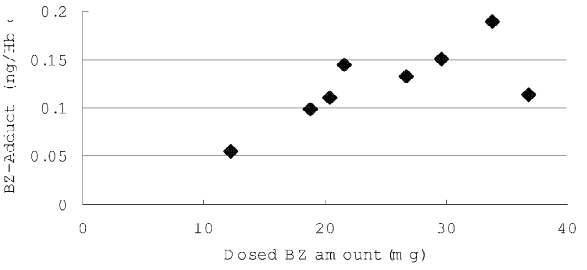


Fig. 6. Dose–response relationship of BZ–hemoglobin adduct levels in rats after treatment with different levels of BZ in the drinking water.

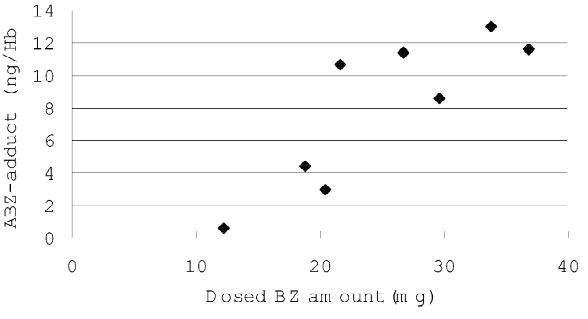


Fig. 7. Dose–response relationship of ABZ–hemoglobin adduct levels in rats after treatment with different levels of BZ in the drinking water.

mg of BZ in drinking water resulted in a dose-proportional increase in the total amount of hemoglobin adducts formed (Figs. 6 and 7).

## 4. Conclusions

The silylation derivatives of BZ, ABZ and DABZ with MTBDMSTFA–$NH_4I$ (1000:3) containing dithioerythritol have good chromatographic properties and offers very sensitive response for the EI-MS (SIM). The extraction of these compounds from hemoglobin with ethyl ether also gave high recovery with small variation. Quantitation of BZ, ABZ and DABZ are excellent, with linear calibration curves over a range of 1.0–100 ng/g and detection limit of 0.1 ng/g. The present method may also be applicable to the analysis of benzidine–DNA adducts.

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