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Separation and trace estimation of benzidine and its macromolecular adducts using supercritical fluid chromatography

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Abstract

A sensitive, rapid, selective and reproducible method has been developed to measure blood plasma levels of benzidine (BZ) and its acetylated metabolite, N-OH-*N*,*N*′-diacetylbenzidine (N-OH-DABZ), using supercritical fluid chromatography (SFC) for the first time. Benzidine and N-OH-*N*,*N*′-diacetylbenzidine were extracted from the plasma using ether. Separation was done on a Nucleosil (250 mm × 4.6 mm) 10 μm, Nucleosil-RP-C_{18} column with 7.4% (v/v) methanol-modified supercritical fluid carbon dioxide (2.5 ml min^{-1}) as mobile phase. The column temperature was 45 °C and the outlet pressure was set at 8.83 MPa. The detection was done using a UV-Vis detector set at 280 nm. The limit of quantification was 0.10 ng ml^{-1} (BZ) and 0.14 ng ml^{-1} (N-OH-diacetylbenzidine) using 1 ml plasma specimen. The mean extraction recovery of BZ was found to be 98.6%. The SFC method was directly compared to a published HPLC–UV method. With respect to speed, organic solvent usage, sensitivity, specificity and accuracy, SFC was found to be superior. The method has been successfully used to estimate the BZ, N-OH-diacetylbenzidine levels in blood plasma of the animals who were administered 15 μg kg^{-1} body weight of benzidine.

Further, this method has been also applied for the detection and quantification of benzidine DNA and hemoglobin adducts from the blood and tissue samples of the benzidine dosed animals.

Keywords: Benzidine; Diacetylbenzidine

1. Introduction

Bladder cancer represents about 7% of human malignancies and is the third most prevalent cancer type in men, of the 60 years and older age groups [1,2]. Bladder cancer has long been associated with occupational exposure to specific organic chemicals. The high incidence of bladder cancer among smokers and workers in dyes, rubber and chemical industries is associated with the exposure to aromatic amines [3–8].

Benzidine (BZ) is an aromatic amine bladder carcinogen in humans [4–6]. For the development of the preventive strategies it is important to understanding the mechanism by which benzidine initiates bladder cancer. The carcinogenic potency due to most of the toxicological chemicals can be attributed to their

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ability to interact with DNA and alter its structure [9]. It is known that covalent modification of the genomic DNA is caused by the metabolic active form of the potent carcinogens, including benzidine [10]. Traditionally, the reversed phase HPLC with UV detector has been reported for the detection of benzidine and its acetylated metabolites in biological samples [11–13].

Supercritical fluid chromatography (SFC) is relatively a better technique compared to the conventional liquid chromatography [14–18]. In this technique, a pressurized gas under certain conditions of temperature and pressure acts as a fluid, is used which acts as a mobile phase, as it is, or by changing its polarities with a suitable modifier like methanol or acetonitrile. The low viscosities and high diffusivities of supercritical fluids enhance the chromatographic efficiencies. Further, SFC generates less solvent waste and employs a cheap non-toxic gas, carbon dioxide.

SFC has not been so far utilized to analyze BZ or its metabolites and adducts and hence, in the present investigation, a rapid, sensitive, simple, reproducible and specific SFC technique is described for the first time, for the estimation of benzidine and its acetylated metabolite in blood plasma.

2. Experimental

2.1. Chemicals and reagents

Benzidine (AR grade) was obtained from Sigma. N-OH-*N*,*N*′-diacetylbenzidine (N-OH-DABZ) was prepared as previously described [19]. The identity and purity (>98%) of this compound was established by thin layer chromatography, NMR and mass spectrometry. Methanol (HPLC grade), diethyl ether, ammonium chloride, perchloric acid (all AR grade) were purchased from E. Merck (India). Carbon dioxide was 99% pure, obtained from Bombay Carbon dioxide Co., India. A 2.5 mol l^{-1} ammonium chloride buffer (pH 9) was prepared [13].

2.2. Standard solutions

Individual solutions of benzidine and N-OH-*N*,*N*′-diacetylbenzidine were prepared by weighing 100 μg of each and dissolving it in 100 ml of methanol to give concentration of 1 μg ml^{-1}. Stock solutions of BZ and N-OH-diacetylbenzidine were further diluted with methanol to 1.0–100 ng ml^{-1}. All these solutions were stored in the refrigerator at the temperature of −4 °C.

For control plasma, fresh frozen human plasma received from a blood bank (Green Cross, Ahmedabad, India) was thawed at room temperature and centrifuged at 1000 × *g* for 5 min. After testing for the absence of interfering endogenous components, using HPLC [13], it was stored at −20 °C.

On every analysis day, calibration samples for BZ and N-OH-diacetylbenzidine were prepared by adding 100 μl of freshly prepared stock solution of both compounds to 1 ml control plasma to obtain plasma BZ and N-OH-diacetylbenzidine concentrations in the range of 0.1–10 ng ml^{-1}. The anticoagulant used was 100 μl of a 5% solution of EDTA added to every 5 ml of blood.

2.3. SFC Instrumentation

2.3.1. For benzidine and N-OH-diacetylbenzidine analysis

A JASCO-900 series (Japan Spectroscopic Co. Ltd., Hachioji, Tokyo, Japan) SF chromatograph was employed for the study. The apparatus was equipped with two pumps (PU-980) which could be adjusted for the flow of both CO_2 and modifier from 0.001 to 10 ml min^{-1}. A Rheodyne model 7125 injection valve was used with an 20 μl external loop for introducing the sample into the analytical column. The temperature of the column was maintained using a JASCO-CO-965 series column oven, while inbuilt cooling circulator thermostatically controlled that of mobile phase. The outlet pressure was adjusted using a JASCO-880-81 back pressure regulator. A programmable JASCO-UV-975 detector equipped with a 4 μl high-pressure cell with a pathlength of 5 mm was used as detector. A study of the wavelength–absorbance relationship of BZ, N-OH-diacetylbenzidine in the range of 200–350 nm obtained by injecting 20 μl of the extracted analytes and measuring absorbances at different wavelengths, showed that BZ exhibited a peak maximum at 280 nm and N-OH-diacetylbenzidine at 293 nm in supercritical fluid CO_2. Hence these wavelengths were chosen as detection wavelengths, respectively. These were chromatographed on a Machey–Nagel,

Nucleosil-RP-C_{18} (250 mm × 4.6 mm) 10 μm column. PC-based Borwin-1.21 (JMBS Developments, Grenoble, France) chromatographic software was used for data integration. The optimum concentration of the mobile phase was found to 7.4% of methanol in supercritical fluid CO_2. The CO_2 flow rate was 2.5 ml min^{-1} with that of modifier being 0.2 ml min^{-1}. The outlet pressure was kept at 8.83 MPa and the temperature of the column and the mobile phase was 45 °C. The injection volume was 20 μl. Benzidine eluted at 3.550 min and N-OH-DABZ at 2.942 min. The total run time was about 10 min.

2.3.2. BZ–DNA adduct analysis

The BZ–DNA adduct analysis could not be carried out with Nucleosil-RP-C_{18} column due to poor retention on the column. Therefore, a C_{18}-μBondapak analytical column (3.9 mm × 300 mm) was employed. The adducts were detected at 280 nm. The conditions like temperature of the column, mobile phase, concentration of the modifier, flow rate of the mobile phase and the outlet pressure were same as described for the benzidine analysis. The *N*-(deoxyguanosin-8-yl)-*N′*-ABZ eluted at 2.733 min.

2.4. Extraction of benzidine and N-OH-diacetylbenzidine from blood plasma

Plasma samples were thawed at room temperature, vortex-mixed for 10 s and centrifuged at 1000 × *g* for 5 min. A 1 ml aliquot of plasma (calibration standard, control or unknown) was mixed with 100 ml methanol, 500 μl water and 100 μl buffer in 15 ml glass centrifuge tubes followed by addition of 5 ml of diethyl ether. Extraction was performed on a rotating mixer for 20 min, followed by centrifugation at 3000 × *g* for 20 min. The aqueous layer was discarded and 4 ml of the organic layer was transferred to another set of glass centrifuge tubes that had been previously rinsed with methanol. The samples were evaporated to dryness in a water-bath at 45 °C under a gentle stream of nitrogen. The residue was reconstituted with 0.2 ml of methanol and 20 μl of the solution were injected into the SFC system under the above specified conditions.

2.5. Calibration and calculation

Eight calibration plasma samples covering the expected concentration range viz. 0.1, 0.5, 1.0, 5.0, 8.0 and 10.0 ng ml^{-1} of benzidine and N-OH-diacetylbenzidine were processed daily with control and the unknown samples. The calibration graph was obtained by plotting the peak area ratio against the benzidine or diacetylbenzidine concentrations. A linear least squares analysis gave the best result as

$$Y = aX \pm b$$

$$Y = 0.00167X - 0.008 \quad \text{(benzidine)}$$

$$Y = 0.0033X + 0.0003 \quad \text{(N-OH-diacetybenzidine)}$$

where X is the peak area, Y the BZ or N-OH-diacetylbenzidine concentration (ng ml^{-1}) in plasma, a the slope of the calibration graph, b the intercept on Y-axis.

Everyday the above calibration standards were studied at the beginning of each analytical batch. The regression lines established were used to calculate test analyte compositions by interpolation.

2.6. Quality control

To estimate the accuracy and precision of the method, quality control samples of BZ and N-OH-diacetylbenzidine free plasma (from control animals), supplemented with 0.1, 1.5, 5.0, 10.0 ng ml^{-1} of BZ or N-OH-diacetylbenzidine were prepared and stored at −20 °C. These samples were analyzed along with the test samples.

2.7. Toxicological application

2.7.1. Animals

Sprague–Dawley and Wistar rats were obtained from Surekh Educational, Ahmedabad and bred in the laboratory. Animals were housed in the laboratory's animal care facility minimum for a week to allow acclimatization.

Animals were allowed food and water ad libitum. The temperature (20–30 °C), humidity and day–night cycles of the animal room were controlled. Adult male animals (100), of average age (1 year) and weight (100 g) were assigned randomly for treatment and non-treatment groups. Care was taken to perform blood sampling and other treatment at the same time of the day, at the interval of 7 days for all animals to minimize variation due to circadian rhythms. Animals

grouped according to age and weight, were weaned when 2 weeks of age. They were housed in plastic cages in units of two.

2.7.2. Dose administration

The assay method was successfully employed to measure the plasma concentrations of BZ and N-OH-diacetylbenzidine in the above mentioned animals. The BZ was administered orally in two different concentration as described below:

1. *Acute study*: A 15 $\mu g\,kg^{-1}$ bodyweight dose of benzidine was given to animals at a single time point.
2. *Chronic study*: A total dose of 15 $\mu g\,kg^{-1}$ bodyweight benzidine was given to animals at a rate of 5 $\mu g\,kg^{-1}$ bodyweight per week, on days 0, 6 and 12.

Blood samples were collected daily and the concentration of BZ, N-OH-diacetylbenzidine were monitored for a period of 21 days. The samples were frozen immediately after collection, stored at −20 °C and analyzed by SFC within 7 days. Animals from each study group were sacrificed at the end of specified period. Ten milliliters blood sample from the sacrificed animal was used for hemoglobin analysis. The livers were removed, DNA was isolated and purified [20]. The DNA was dried, quantified [21] and stored for further analysis.

2.8. DNA adduct analysis

For DNA adduct analysis 1 $mg\,ml^{-1}$ DNA (extracted from the treated animals) solution was prepared in double distilled water. It was enzymatically hydrolyzed by dissolving 0.1 $mg\,ml^{-1}$ DNAse I in NaCl solution (0.9%) and adding to it the DNA solution. The mixture was incubated for 3 h at 37 °C under argon. Then 20 $\mu g\,ml^{-1}$ nuclease P1 (dissolved at 0.5 $mg\,ml^{-1}$ in 1 mM $ZnCl_2$), 0.5 $U\,ml^{-1}$ alkaline phosphatase and 0.3 $U\,ml^{-1}$ acid phosphatase were added. The mixture was incubated for 12–18 h under argon and the *N*-(deoxyguanosin-8-yl)-*N*′-ABZ adducts were extracted in butanol. The butanol was removed by rotary evaporation and the residue was redissolved in 0.2 ml methanol for analysis by SFC as described in Section 2.3.2.

2.9. Hb adduct analysis

Blood samples collected from treated animals were centrifuged at 3000 rpm to separate serum and red blood cells. The isolated RBC were washed three times with 0.9% saline, lysed with 15 ml of deionized water and 2 ml of toluene and the mixture was shaken vigorously for 30 min. It was then centrifuged at 10,000 rpm for 10–20 min to separate the hemoglobin. Dialysing with distilled deionized water at 4 °C for 2 days, purified Hb. The concentration of hemoglobin thus obtained was determined by Drabkin's method.

The concentration of BZ, in the adducted Hb, was determined by reacting the Hb with 0.1 M NaOH and incubating it for 3 h at 35 °C. Then BZ was extracted with ether and analyzed by SFC. The analysis was as described in Section 2.3.1.

3. Results and discussion

Selectivity of this method was confirmed by analyzing 1.0 ml of plasma of control animals by the procedure described above. Fig. 1 shows the chromatogram of plasma from benzidine administered animals.

3.1. Limit of quantification and limit of detection

The limit of quantification of the assay, defined as the minimum concentration that could be measured with a precision [coefficient of variation (CV)] about 10% was found to be 0.10 $ng\,ml^{-1}$ for BZ and 0.14 $ng\,ml^{-1}$ for N-OH-diacetylbenzidine. The limit of detection, defined as concentration giving a signal-to-noise ratio 3:1 was 0.014 $ng\,ml^{-1}$ of BZ and 0.019 $ng\,ml^{-1}$ for N-OH-DABZ using chromatographic and instrumental parameters defined above.

3.2. Recovery

Extraction recovery of the carcinogen was determined by assaying spiked concentrations of BZ and N-OH-diacetylbenzidine in plasma and methanolic solutions ($n = 5$ each case) at four levels. Results are given in Table 1.

3.3. Linearity

A linear correlation between peak area ratios and the concentration was found in the range 0.1–10 $ng\,ml^{-1}$.

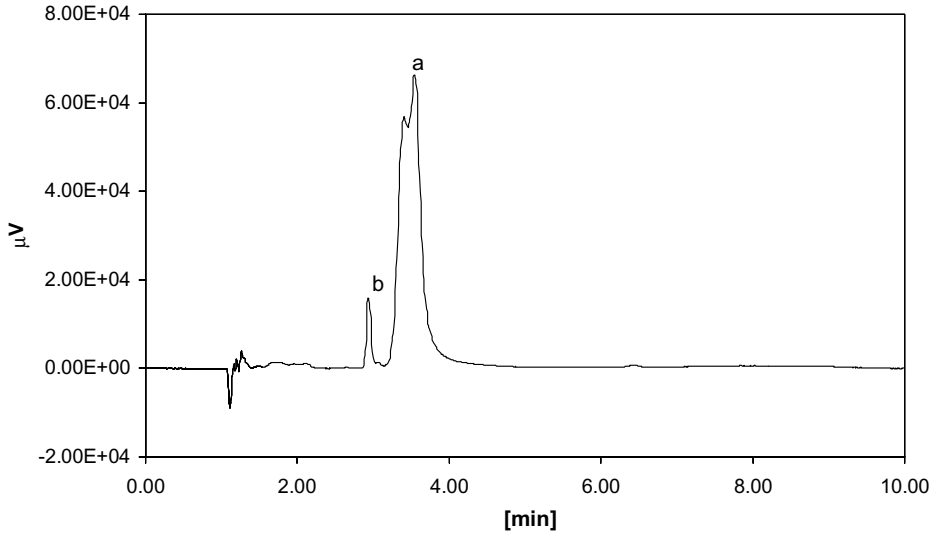

Fig. 1. Separation of (a) benzidine and (b) N-OH-diacetylbenzidine from blood plasma using SFC. The benzidine and N-OH-diacetylbenzidine eluted at 3.550–2.942 min, respectively.

This wide range of concentrations necessitated the use of a weighing factor ($1/X^2$) in order to avoid division into sub-ranges. The correlation coefficient of determination was generally better than 0.9990 and the accuracy (added − found/added) for the daily studies was better than 5%.

3.4. Precision

The precision of the method was evaluated by replicate analysis of spiked quality control plasma samples. The intra-assay data were obtained by replicate analysis of plasma samples ($n = 5$). The inter-assay data were obtained by analyzing the same plasma over a period of 7 weeks for 40 times. The data shown in Table 2 (BZ and N-OH-diacetylbenzidine), demonstrate the good precision of the method over the concentration range investigated. The results presented have been determined from the means of replicates per assay per day.

Table 1
Extraction recoveries of BZ, N-OH-diacetylbenzidine from plasma

Concentration (ng/ml)	Recovery (±S.D.) (%)[a]	
	BZ	N-OH-diacetylbenzidine
0.5	96.9 ± 1.5	99.3 ± 0.8
1.0	99.5 ± 0.8	96.5 ± 1.0
5.0	98.5 ± 1.4	98.2 ± 1.2
10.0	99.6 ± 0.5	98.8 ± 0.8

[a] Average of the five determinations.

3.5. Stability

For stabiity of BZ and N-OH-diacetylbenzidine in plasma vials containing known concentrations of BZ/N-OH-diacetylbenzidine, at three levels, low, medium and high were stored at −20 °C for a period of 120 days. The concentrations were chosen from the calibration range. Every day of analysis, one vial was taken and assayed together with the animal plasma samples. In these experiments BZ was stable for 100 days whereas N-OH-diacetylbenzidine was stable for 80 days.

3.6. Toxicological results

The mean maximum peak plasma concentration (C_{max}) of BZ and N-OH-diacetylbenzidine in animals obtained by SFC, following administration of 15 μg kg^{-1} body weight orally are as shown in Fig. 2. The mean maximum plasma concentration (C_{max})

Table 2
Accuracy and precision of the SFC method for the determination of benzidine and N-OH-diacetylbenzidine in spiked plasma samples

Concentration added ($ng\,ml^{-1}$)	Concentration found[a] ($ng\,ml^{-1}$)	Error (%)	Accuracy (%)	R.S.D. (%)
Benzidine				
Inter-assay precision				
0.10	0.098	2.00	98.00	2.25
1.50	1.47	2.00	98.00	1.54
5.00	4.88	2.40	97.60	0.94
8.00	7.88	1.50	98.50	0.62
10.00	9.90	1.00	99.00	0.44
Intra-assay precision				
0.10	0.097	3.00	97.00	2.98
1.50	1.46	2.70	97.30	1.87
5.00	4.89	2.20	97.80	1.09
8.00	7.84	2.00	98.00	0.72
10.00	9.88	1.18	98.81	0.52
N-OH-diacetylbenzidine				
Inter-assay precision				
0.10	0.097	3.00	97.00	3.00
1.50	1.46	2.66	97.34	1.90
5.00	4.85	3.00	97.00	1.00
8.00	7.88	1.50	98.50	0.78
10.00	9.89	1.06	98.94	0.67
Intra-assay precision				
0.10	0.097	3.00	97.00	3.00
1.50	1.45	3.33	96.66	2.10
5.00	4.84	3.20	96.80	1.15
8.00	7.85	1.80	98.20	0.86
10.00	9.88	1.12	98.87	0.55

[a] Average of the five determinations ($n = 5$).

of BZ was $10\,ng\,ml^{-1}$ at 5.0 h (T_{max}) after oral administration and the elimination half-life ($T_{1/2}$) was 60 h. The results for N-OH-diacetylbenzidine were $C_{max} = 7\,ng\,ml^{-1}$, $T_{max} = 7.0$ h and $T_{1/2} = 20$ h.

3.7. Covalent binding of BZ to rat liver DNA and blood Hb (in vivo)

Two types of studies were performed. In acute study, where $15\,\mu g\,kg^{-1}$ bodyweight BZ was given at a time, a constant dose response relationship between the DNA and Hb adduct concentration was observed (Fig. 3). Out of 21 days of analysis, the adducts were detectable only till the sixth day (144 h).

In chronic study, where a total $15\,\mu g\,kg^{-1}$ bodyweight BZ was given at a rate of $5\,\mu g\,kg^{-1}$ bodyweight per week, the variation in concentration of BZ–DNA and BZ–Hb adduct was non-linear (Fig. 4). In this case the peak concentration of the DNA adducts was obtained on days 1, 7 and 13, which was 500, 645 and $755\,pg\,mg^{-1}$ of DNA, respectively. The maximum concentration of Hb adducts ($450\,pg\,mg^{-1}$ Hb) was obtained after the third dose deliverance on the 12th day and remained almost constant after the 16th day.

In both the studies, the concentration of Hb adducts was less than the DNA adducts. However, the ratio between the adduct concentrations of DNA and Hb was constant during the period of analysis in both acute and chronic studies.

The advantages of SFC became particularly striking when the solvent usage, sample throughput, waste disposal, sensitivity, specificity, accuracy and precision for SFC and HPLC were compared. Table 3 gives a comparison for these factors in the case of benzidine plasma analysis. The conditions

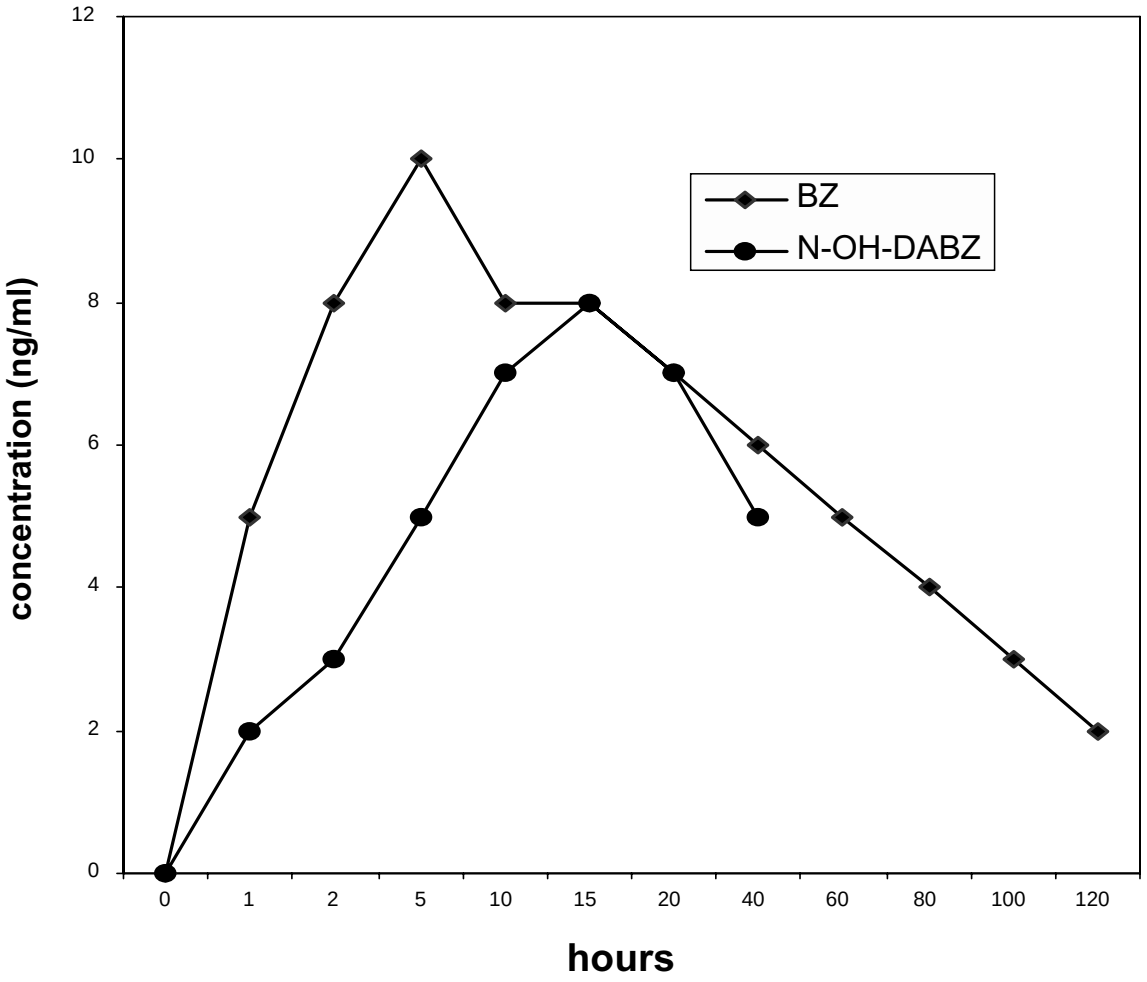

Fig. 2. Mean plasma concentration of BZ and N-OH-DABZ after administration of 15 μg kg^{-1} body weight BZ in animals obtained by SFC.

for HPLC were the same as described elsewhere [22,23].

To support the results obtained using an animal model based on differential effect of age and dosage on kinetics of BZ, a screening study on occupationally exposed industry workers has been carried out. The blood and urine samples have been collected from the workers of the various dyes industries of Vatva, Ahmedabad and the BZ level was measured spectrophotometrically, as well as by SFC. The donors were medically examined and subjected to questionnaire to develop their case history. It was observed that out of 500 workers examined, 60 were detected with average 20 ± 5 ppm benzidine concentration in blood and urine samples. The same workers were also diagnosed for bladder cancer, which was further confirmed by histological examination of needle and surgical biopsies. Though benzidine has been banned in India since 1994, the industries under survey in our study used many BZ-based dyes. It is an established fact that BZ-based dyes get metabolized to benzidine in vivo resulting into similar toxicity effect. The information obtained by monitoring exposed individuals for benzidine concentration has helped to define the role of benzidine-based dye exposure in bladder cancer.

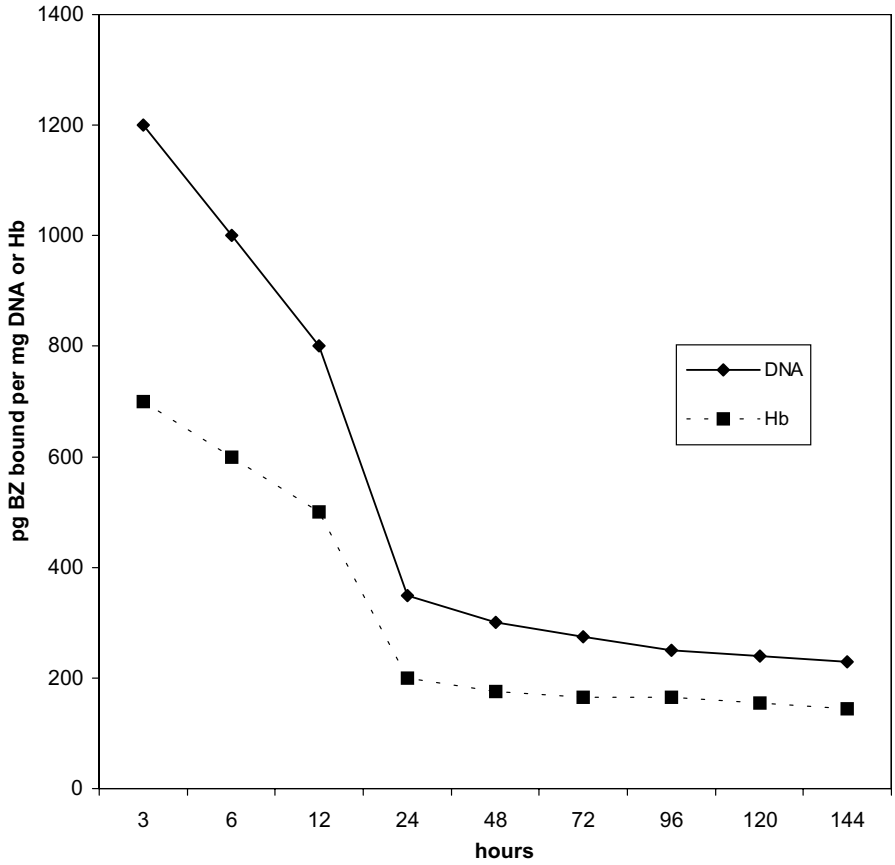

Fig. 3. Concentration of DNA and Hb adducts on exposure of single dose of 15 μg kg^{-1} body weight BZ.

The proposed method for the determination of benzidine and N-OH-*N*,*N*′-diacetylbenzidine in blood plasma has an advantage of high reproducibility and speed. As CO_2 is much cheaper than acetonitrile, the cost of analysis is significantly reduced as compared to the HPLC method. The technique offers a viable alternative to HPLC. The present data shows that the SFC method is highly sensitive (0.1–10 ng ml^{-1})

Table 3
Data on the usage and waste of organic solvents for BZ estimation in blood plasma using SFC and HPLC

	SFC/UV	HPLC/UV
Mobile phase	7.4% (v/v) methanol in CO_2 at 2.5 ml min^{-1}	Methanol gradient: 0–100%, 1.0 ml min^{-1}
Run time (min)	10.0	15.0
Sample throughput (h^{-1})	6.0	4.6
Volume of mobile phase (h^{-1})	160 ml	60 ml
Volume of organic solvent (h^{-1})	12 ml	20 ml
Volume of disposable waste (h^{-1})	12 ml	60 ml
Sensitivity (BZ)	0.1 ng ml^{-1}	25 μg l^{-1}
Diacetylbenzidine	0.2 ng ml^{-1}	0.5 mg l^{-1}
Accuracy (BZ)	98.22%	–
Diacetylbenzidine	97.70%	–

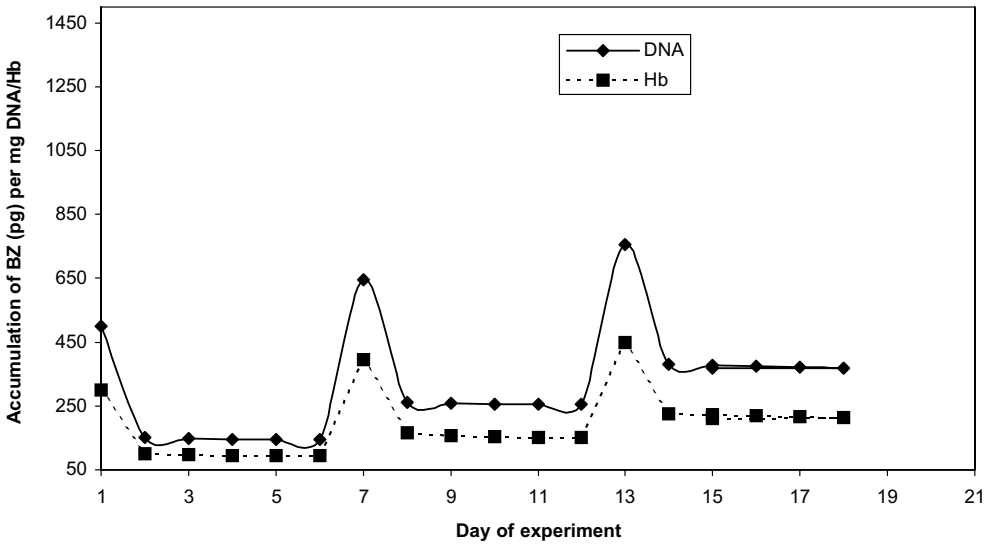

Fig. 4. Concentration of DNA and Hb adducts on exposure of $5\,\mu g\,kg^{-1}$ body weight per week on days 0, 6 and 12.

compared to HPLC. The linearity, precision and specificity was excellent. The present method is also selective for the detection and quantification of BZ–DNA and BZ–Hb adducts from plasma samples of animals administered $15\,\mu g\,kg^{-1}$ bodyweight BZ. It can be therefore inferred that this method can be used for risk assessment of benzidine and benzidine-based dyes at low exposure. Further, the method of SFC can be applied for assessment of other carcinogens under study.

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