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Enhancement of Urinary Elimination of 3-Bromobenzanthrone Metabolites by Oral Supplementation of Ascorbic Acid in Guinea Pigs

RAVINDRA P. SINGH[*], RAJ KHANNA[*], SUBHASH K. KHANNA[**], AND MUKUL DAS[**,#]

[*]*Department of Biochemistry, Lucknow University Lucknow-226001;* [**]*Food Toxicology Laboratory, Industrial Toxicology Research Centre, Postal Box No. 80, M. G. Marg, Lucknow-226 001, India*

Objective 3-Bromobenzanthrone (3-BBA), an anthraquinone intermediate dye, is extensively used in textile industry. Since, our prior studies have shown that 3-BBA caused significant depletion of ascorbic acid (AsA) levels, the effect of exogenous supplementation of AsA on the urinary elimination of 3-BBA metabolites was investigated. **Method** Guinea pigs were treated with single oral dose of 3-BBA (50 mg/kg b. wt.) in groundnut oil while another group was treated with single oral dose of 3-BBA (50 mg/kg b. wt.) along with 3 day prior and post oral supplementation of AsA. Control groups were either treated with groundnut oil or AsA alone. Urine from individual animals was collected, extracted and analysed on HPTLC. **Results** The highest elimination of 3-BBA (75 μg) was found to be in 0-24 h urine fraction which decreased to 18 μg and 5 μg in the two subsequent 24 hourly fractions of urine. Exogenous supplementation of AsA increased the total urinary elimination of 3-BBA by almost 77%. A total of 10 fluorescent metabolites excluding the parent compound were eliminated in the urine of guinea pigs treated with 3-BBA. Densitometric scanning of chromatogram showed different peaks at Rf 0.18, 0.22, 0.27, 0.34, 0.40, 0.48, 0.56, 0.66, 0.72, 0.80, and 0.95 which were eliminated and marked as urinary metabolite 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, and 11 respectively. AsA not only significantly enhanced the elimination of 3-BBA metabolites but also modified the pattern of metabolites drastically in 0-6 h, 6-24 h and 24-48 h urine fractions. **Conclusion** These results indicate that AsA may be useful in protecting the toxicity of 3-BBA by fascilitating the urinary metabolite(s) excretion of 3-BBA.

Key words: Ascorbic acid; 3-Bromobenzanthrone; Metabolites; High performance thin layer chromatography

INTRODUCTION

3-Bromobenzanthrone (3-BBA), an anthraquinone intermediate dye, is frequently being used in textile and allied industries[1,2]. Due to wide application, the demand and utilization of this dye intermediate has been continuously increasing[3]. Industrial workers exposed to 3-BBA during manufacturing, storage, transportation and pulverization processes, have

[#]Correspondence should be addressed to Mukul DAS, E-mail: mditrc@hotmail.com or mditrc@rediffmail.com

Biographical note of the corresponding author: Dr. Mukul DAS is deputy director & head, Food Toxicology Laboratory, has more than 24 years of research experience. He is a member of various scientific socities and regulatory food committees.

Abbreviations: AsA, Ascorbic acid; BA, Benzanthrone; 3-BBA, 3-Bromobenzanthrone; HPTLC, High performance thin layer chromatography.

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complained of dermal manifestations[4]. Our recent studies suggest that topical application of 3-BBA causes skin irritancy which may be related to membrane damage in albino rats[5,6]. Further studies regarding the toxicity of 3-BBA have shown that it may lead to the destruction of cytochrome P-450 dependent monooxygenases along with hepatic damage[7]. These studies have also shown impairment of antioxidant enzymes with a concomitant decrease in ascorbic acid levels following 3-BBA treatment to guinea pigs[7].

Biotransformation is often a pre-requisite for urinary excretion of a toxicant[8,9]. The metabolites excreted via urinary, biliary or faecal routes or those retained in various target organs have a direct bearing on the biotransformation of the parent compound[10]. A number of xenobiotics may not be toxic per se but at times get metabolically activated to highly toxic metabolites[11,12]. Thus, it has been suggested that metabolic bio-elimination of a compound may help to elucidate the mechanism of toxicity. Since 3-BBA causes depletion of ascorbic acid levels[7], attempts have been made to study the metabolic urinary profile of 3-BBA in normal and ascorbic acid supplemented guinea pigs.

MATERIALS AND METHODS

Chemicals

3-Bromobenzanthrone was obtained from ATIC, Valsad, Gujarat, India. HPTLC compatible Silica gel 60 F 254 pre-coated aluminum sheets (20×20 cm size) were procured from E. Merck, Darmstadt, Germany. Ethyl acetate, toluene and methanol were obtained from Qualigens Fine Chemicals, Ltd., Mumbai, India. Ascorbic acid was a product from Sarabhai Chemical Co., Ahemdabad, India. All the other chemicals used were of highest purity available commercially.

Treatment of Animals

Adult male albino guinea pigs (300 g±10 g) were obtained from the animal breeding colony of Industrial Toxicology Research Centre, Lucknow. Animals were raised on commercial pellet diet (Ashirwad, Chandigarh, India) and water ad libitum under laboratory conditions. The animals were maintained in controlled atmosphere of 12 h dark/light cycle, 22℃±2℃ temperature and 50%-60% humidity and treated according to rules laid down by Animal Welfare and Ethics Committee of ITRC. The animals were treated as follows. Twenty guinea pigs were divided into four groups and kept individually in metabolic cages. Group 1: Animals orally treated with groundnut oil and serving as controls. Group 2: Animals treated with oral supplementation of AsA (40 mg/kg b.wt, daily) for 6 consecutive days. Group 3: Animals treated with a single oral dose of 3-BBA (50 mg/kg b.wt) in groundnut oil. Group 4: Animals treated with a single oral dose of 3-BBA (50 mg/kg b.wt) along with 3 day prior and post oral supplementation of AsA. Urine samples of individual animals at different time intervals including 0-24 h, 24-48 h, 48-72 h, and 72-96 h were collected under ice cold conditions.

Estimation of 3-BBA in Urine

Extraction of urine samples and analysis of total 3-BBA was carried out according to Singh *et al.*[13]. Briefly, the urine samples were extracted three times with 5 volumes of chloroform. The extracted fractions were pooled and 1.5 mL aliquot was withdrawn. The volume was expanded to 3.0 mL by the addition of 1.5 mL of methanol. The mixture was

read in Perkin spectrofluorimeter λ 20 using an excitation and emission wavelength of 400 nm and 530 nm, respectively.

Separation of Urinary Metabolites by HPTLC

A similar set of experimental animals with treatment schedule was performed as indicated above. Urine samples at 0-6 h, 6-24 h and 24-48 h were collected in ice cold conditions. The ethyl acetate extract of urine was concentrated under vacuum in a Buchi rotavap and the residue was dissolved in methanol (HPLC grade). The spots of urinary extract were applied by TLC Applicator, (model AS 30, 230 V, Desaga) on pre-coated HPTLC plates (HPTLC Aluminium sheets Silica 60 F 254, 20×20 cm, Merck). The plates were developed under TLC mat 230 (Desaga) using toluene: methanol, 4:1 (v/v) as mobile phase. Fluorescent spots on the developed chromatogram were viewed under 365 nm UV dark room chamber (CN-6, Vilber Lourmat, France). The HPTLC plates were scanned on CD 60- Densitometer (Desaga) equipped with a mercury lamp having a fluorescent excitation wavelength of broad peak at 366 nm and the emission radiations from 366 nm to 550 nm, which is attached with computerized DOS software version.

Statistical Analysis

The analysis of variance (ANOVA) with rank ordering[14] was employed to calculate significance of difference between 3-BBA alone and 3-BBA+AsA treated groups. *P* value less than 0.05 was considered to be significant.

RESULTS

The urinary elimination of 3-BBA in controls and AsA supplemented guinea pigs is given in Table 1. Single oral administration of 3-BBA to guinea pigs showed the highest elimination of 3-BBA (75.4 μg) in between 0-24 h urine fraction. Subsequently, two 24 hourly fractions of urine showed a much lesser elimination of 3-BBA (5.4-18.3 μg), while 72-96 h urine fraction virtually exhibited no elimination. Exogenous supplementation of AsA (40 mg/kg b.wt) increased the total urinary elimination of 3-BBA by almost 77%. The animals exogenously supplemented with AsA showed 8231 and 509% higher elimination of 3-BBA in 0-24, 24-48 and 48-72 h urine fractions, respectively when compared to urine fractions of 3-BBA alone treated animals (Table 1). There was no detectable amount of fluorescence in the urine of control and AsA alone treated animals (Data not shown).

TABLE 1

Urinary Elimination Pattern of Orally Administered 3-BBA Alone and Supplemented Guinea Pigs

Time Period (h)	Levels of 3-BBA Eliminated (μg)	
	3-BBA Alone	3-BBA±AsA
00-24	75.42±4.80	81.78±5.11(8)
24-48	18.32±0.09	60.67±4.78* (231)
48-72	5.37±0.04	32.68±2.33* (509)
72-96	Nil	1.47±0.02*
Total	99.61±5.47	176.60±8.43* (77)

Note. Data represent $\bar{x}\pm s$ of 5 animals. Values in parenthesis indicate percent increase over 3-BBA alone treated group. *$P<0.05$, significant when compared to 3-BBA treated group.

Effect of AsA administration on urinary fluorescent metabolites of 3-BBA in guinea pigs is shown in Figure 1. HPTLC resolution of fluorescent spots with Rf 0.18, 0.22, 0.27, 0.34, 0.40, 0.48, 0.56, 0.66, 0.72, 0.80, and 0.95 were marked as metabolites 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, and 11, respectively. In 0-6 h urine samples, metabolites 1, 5, 7, 8, 10, and 11

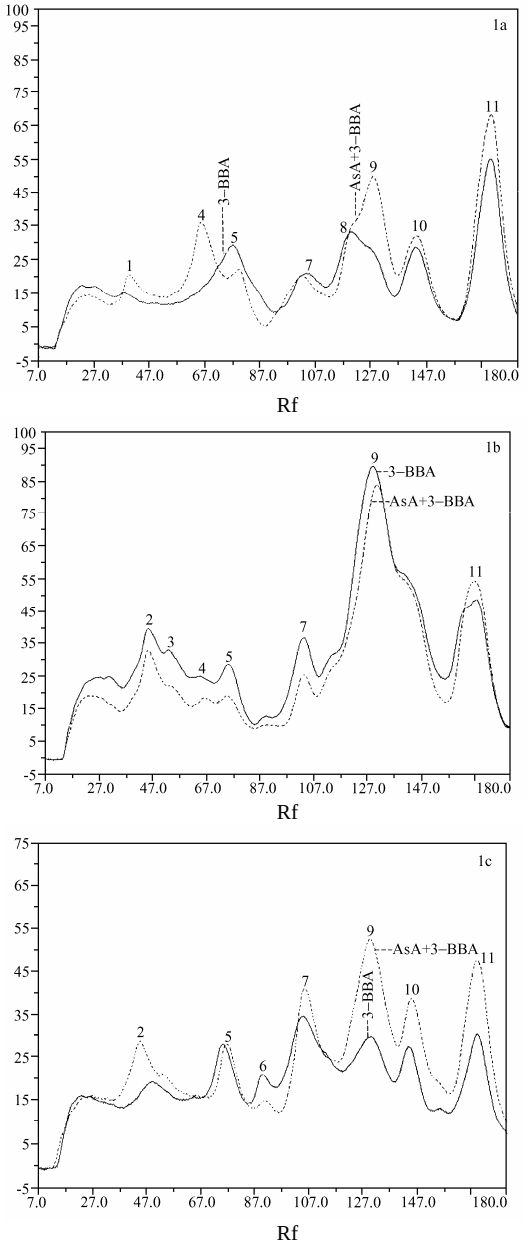

FIG.1. Densitometric scanning of HPTLC resolved urinary metabolites of 3-BBA in normal and ascorbic acid supplemented guinea pigs. (a) 0-6 h; (b) 6-24 h; and (c) 24-48 h. ——3-BBA treated animals. --------- 3-BBA+AsA treated animals. The scanned urine fractions at different time interval was taken for further analysis as mentioned in Table 2.

with respective Rf values of 0.18, 0.40, 0.56, 0.66, 0.80, and 0.95 were eliminated (Fig. 1a). AsA treatment resulted in an enhancement of metabolite 1 while extra peak 4 and 9 emerged as new metabolites. Further, AsA treatment showed decrease in metabolite 5 while metabolite 8 completely disappeared in the urine fraction (Fig. 1a). In 6 h-24 h urine samples, metabolites 2, 3, 5, 7, 9, and 11 were eliminated (Fig. 1b). AsA supplementation to 3-BBA treated animals showed a disappearance of metabolite 3 and an appearance of a new metabolite 4 while metabolite 2 was enhanced when compared to urine of 3-BBA treated animals (Fig. 1 b). Metabolites 2, 5, 6, 7, 9, 10, and 11 were found to be eliminated in the urine fraction of 24-48 h (Fig. 1c). AsA supplementation showed substantial enhancement of metabolites 2, 6, 7, 9, and 10 in the 24-48 h urine fraction (Fig. 1c)

Effect of AsA supplementation on urinary metabolite excretion pattern in 3-BBA exposed guinea pigs is given in Table 2. The metabolites at Rf 0.18, 0.22, 0.27, 0.34, 0.40, 0.48, 0.56, 0.66, 0.72, and 0.80 were numbered as 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10, respectively. The spot at Rf 0.95 matches with the Rf of standard 3-BBA. AsA not only enhanced the elimination of 3-BBA metabolites but also modified the pattern of metabolites drastically. In the 0-6 h urine fraction, formation of metabolite 5 was significantly reduced while metabolite 8 was found to be absent after AsA supplementation to 3-BBA treated animals. Metabolites 4 and 9 emerged as the two new peaks while metabolites 1 and 11 were significantly increased following AsA supplementation to animals. Urine samples of 6-24 h period showed that AsA caused disappearance of metabolite 3 while an extra metabolite 4 appeared as a new peak. Further, formation of metabolites 2 and 11 were significantly enhanced while metabolites 5 and 7 were significantly reduced in AsA treated animals. In the case of the urine fraction of 24-48 h, metabolites 2, 7, 9, 10, and 11 were significantly increased while metabolite 6 was significantly decreased by AsA supplementation. Interestingly, AsA supplementation to 3-BBA treated animals led to 42, 16 and 164% enhancement of 3-BBA urinary metabolites elimination at 0-6 h, 6-24 h and 24-48 h, respectively (Table 2). No fluorescent peaks in the urine of controls and AsA alone treated animals were detected (data not shown).

TABLE 2

Effect of AsA Supplementation on Urinary Metabolite Excretion Pattern in 3-BBA Exposed Guinea Pigs

Metabolite	Rf Values	Total Urinary Metabolites (μg)					
		0-6 h		6-24 h		24-48 h	
		3-BBA	3-BBA+AsA	3-BBA	3-BBA+AsA	3-BBA	3-BBA+AsA
1	0.18	0.20±0.01	0.46±0.02*	–	–	–	–
2	0.22	–	–	1.90±0.02	3.49±0.04*	1.55±0.01	6.84±0.03*
3	0.27	–	–	1.82±0.02	–	–	–
4	0.34	–	1.80±0.02	–	0.43±0.01	–	–
5	0.40	2.18±0.28	1.27±0.11*	2.23±0.02	0.92±0.01*	2.95±0.20	3.10±0.26
6	0.48	–	–	–	–	0.67±0.01	0.30±0.01*
7	0.56	0.65±0.03	0.58±0.30	3.63±0.04	2.02±0.01*	4.95±0.51	10.23±0.63*
8	0.66	2.06±0.12	–	–	–	–	–
9	0.72	–	4.10±0.24	39.65±3.67	47.83±2.92	2.68±0.20	17.45±0.81*
10	0.80	1.31±0.08	1.28±0.14	–	–	2.04±0.02	5.63±0.28*
11	0.95	3.62±0.03	4.74±0.39*	11.68±0.73	16.23±1.49*	5.55±0.35	10.26±0.53*
Total		10.02	14.23(42)	60.91	70.92(16)	20.39	53.81(164)

Note. Data represent $\bar{x}+s$ of 5 animals. Values in parenthesis indicate percent increase over respective 3-BBA treated group. Total metabolites in urine of 3-BBA animals upto 48 h were 91.32 μg while 52% enhancement was observed in urine of 3-BBA+AsA treated animals. *$P<0.05$, significant when compared to 3-BBA treated animals.

DISCUSSION

Endogenous biosynthesis of AsA involves an enzyme L-gulano-gamma-lactone oxidase which is present in several rodent species like rats and mice[15]. However, this enzyme is absent in a variety of other species like men, monkeys and guinea pigs. Hence, these animals are not able to synthesize AsA and have to depend on exogenous or dietary source[16]. Earlier reports suggest that benzanthrone (BA) caused a significant depletion of AsA levels in blood, adrenal and liver of rats, mice and guinea pigs[17,18]. Measurement of 3-BBA in the urine of guinea pigs in the presence of exogenously supplemented AsA indicates that it leads to an appreciable enhancement (77%) in the urinary elimination of 3-BBA. Similar enhancement in the urinary elimination kinetics was earlier noted in the case of benzanthrone by AsA supplementation as well[19].

It is generally believed that urinary excretion of lipophilic xenobiotics would be negligible in the absence of metabolism which makes the compound more polar[20]. Thus, it is quite likely that metabolism of 3-BBA might be occurring in liver through cytochrome P-450 mediated system after getting absorbed from GI tract. The hepatic metabolites may enter the systemic circulation and/or may enter the gut again through bile via enterohepatic route and may re-enter systemic circulation and finally pass out in urine through kidney[21]. An understanding of the extent and nature of the metabolites may be derived from serial analysis of the concentrations of the chemical in the plasma and urine which may give an opportunity to rationalize the extrapolation of toxicity observed in animals[10]. Mammals can synthesize xenobiotic conjugates that are more polar, thereby facilitating their elimination from the body[22]. It can therefore be suggested that the metabolism and urinary clearance of 3-BBA may be altered as a consequence of decrease in CYP 1A1/1A2 isozyme with simultaneous induction of phase II enzymes[7].

Results of HPTLC profile of urinary fluorescent metabolites of 3-BBA show that the exogenous supplementation of AsA substantially enhanced the urinary elimination of various metabolites. Also the pattern of elimination of urinary metabolites of 3-BBA are considerably modulated including emergence of new metabolites and disappearance of others in AsA supplemented animals. In this regard AsA has not only been shown to enhance the lowered metabolic activity which is associated with the normal synthesis of haem protein, cytochrome P-450 but also increases the phase I and phase II enzymes in control animals[23,24]. Further experiments are required to characterize the structure of these metabolites and their modulation in experimental animals which will contribute to understanding the mechanism of toxicity of 3-BBA. Overall, the results suggest that AsA may be useful in protecting the toxicity of 3-BBA by significantly facilitating the urinary metabolite excretion.

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