ELSEVIER

www.elsevier.nl/locate/farmac

Il Farmaco 55 (2000) 624–630

IL FARMACO

Metabolic and chemical studies on *N*-(4-chlorobenzyl)-*N*′-benzoylhydrazine

Ş. Güniz Küçükgüzel, İlkay Küçükgüzel, Mert Ülgen *

Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Marmara University, 81010 Istanbul, Turkey

Received 2 January 2000; accepted 10 August 2000

Abstract

The in vitro hepatic microsomal metabolism of *N*-(4-chlorobenzyl)-*N*′-benzoylhydrazine (CBBAH), a model compound representing *N*-alkyl substituted hydrazides, was studied using hepatic washed rat microsomal preparations fortified with NADPH to identify the possible *N*-oxidative, *N*-dealkylated and hydrolytic metabolites. CBBAH and its potential metabolites were prepared, characterized using spectroscopic techniques and then separated using a reversed phase HPLC system with UV detection at 254 nm. CBBAH was chemically converted to the corresponding hydrazone by *m*-chloroperbenzoic acid (*m*-CPBA) oxidation. CBBAH was incubated with rat microsomal preparations in the presence of NADPH, extracted into dichloromethane and evaporated finally under nitrogen. The TLC and HPLC results from the metabolic experiments showed that CBBAH produced the corresponding hydrolytic and *N*-dealkylated metabolites together with the corresponding hydrazone. © 2000 Published by Elsevier Science S.A. All rights reserved.

Keywords: *N*-Alkyl hydrazide; Hydrazone formation; Secondary amines; In vitro metabolism

1. Introduction

Although *N*-(substituted benzyl)-*N*′-substituted benzoylhydrazines have been reported previously to be anticonvulsant [1], MAO inhibitor [2] and anti-HIV agents [3], there is no report regarding their in vitro hepatic microsomal metabolism in the literature. However, there are a few studies on their corresponding hydrazones. For example, the in vitro hepatic microsomal metabolism of benzoic acid 4-amino-(4-fluorophenyl)methylenehydrazide was studied and it was reported that this compound produced the corresponding hydrolytic metabolites [4]. Ülgen et al. [5] also studied the in vitro metabolism of benzoic acid benzylidenehydrazide and found that the aromatic hydroxylation was detected only in the arylidene moiety, but not in the benzoyl ring. This fact prompted us to study the in vitro hepatic microsomal metabolism of *N*-(4-chlorobenzyl)-*N*′-benzoylhydrazine (CBBAH), a previously synthesized and patented insecticide bearing a nonsubstituted benzoyl and a substituted benzyl moieties [6]. Our present aim was not only to investigate the potential *N*-dealkylation and hydrolytic products of CBBAH, but also to detect whether any corresponding hydrazone and/or *N*-oxidative metabolites were formed or not. It was also intended to block the possible aromatic hydroxylation pathway. In the present work, the corresponding hydrazone, benzoic acid(4-chlorophenyl)methylenehydrazide (CBDBAH), which was screened previously for antiepileptic properties [7], was also prepared as a potential metabolic product.

2. Experimental

4-Chlorobenzaldehyde (CB) and 4-chlorobenzylchloride (CBC) and *m*-chloroperbenzoic acid (*m*-CPBA) were purchased from Aldrich. All other chemicals were purchased from Merck. All melting points were recorded on a Büchi 530 melting point apparatus and are uncorrected. UV spectra were recorded on a Shi-

Abbreviations: CBBAH, *N*-(4-chlorobenzyl)-*N*′-benzoylhydrazine; CB, 4-chlorobenzaldehyde; CBC, 4-chlorobenzylchloride; *m*-CPBA, *m*-chloroperbenzoic acid; BAH, benzoic acid hydrazide; CBDBAH, benzoic acid(4-chlorophenyl)methylenehydrazide; BA, benzoic acid.

* Corresponding author. Tel.: +90-216-348 4194.

E-mail address: mulge@turk.net (M. Ülgen).

PII: S0014-827X(00)00077-X

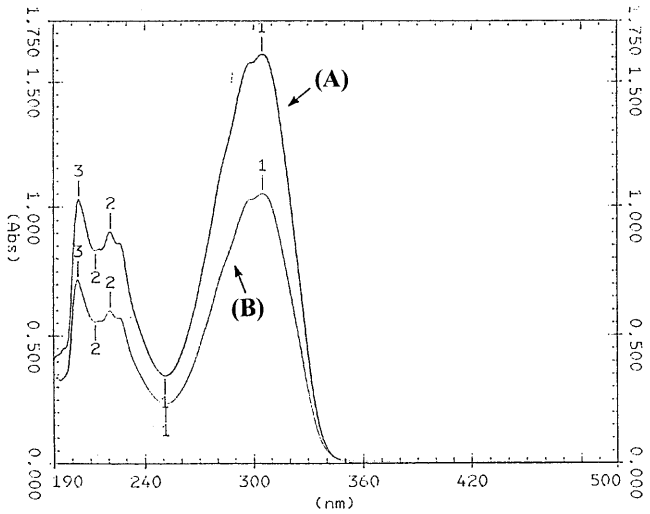

Fig. 1. Curve overlay of UV spectra obtained (A) from synthetic CBDBAH and (B) CBDBAH isolated from the oxidation of CBBAH by preparative TLC.

madzu UV-2100S spectrometer (1 mg/100 ml in methanol). IR spectra were run on a Shimadzu FTIR-8300 spectrophotometer (1 mg/200 mg in KBr). Elemental analyses were performed on a Carlo Erba 1106 instrument. ^{1}H NMR spectrum was run on Bruker AVANC-DPX 400 spectrometer.

This metabolic study required the synthesis of CBBAH together with two of its potential metabolites, namely benzoic acid hydrazide (BAH) and benzoic acid(4-chlorophenyl)methylenehydrazide (CBDBAH). BAH was prepared by the reaction of methyl benzoate and hydrazine hydrate at 110–130°C. This compound had an identical melting point of 115°C to its previously reported value [8]. The attempted oxidation of CBBAH with *m*-chloroperbenzoic acid (*m*-CPBA) was also carried out.

2.1. Preparation of N-(4-chlorobenzyl)-N′-benzoylhydrazine (CBBAH)

BAH (0.004 mol) was dissolved in absolute ethanol. To this solution, an absolute ethanolic solution of CBC (0.002 mol) was added. This mixture was refluxed for 4 h at 110°C. At the end of the reaction, water was added and the solid product obtained was filtered off, dried and recrystallized from a mixture of ethanol–petroleum ether (40–60°C). M.p. 125–127°C. The purity of CBBAH was determined by the use of elemental analysis, TLC and reverse phase HPLC. Analysis for $C_{14}H_{13}ClN_2O$ Calc./Found: 64.49/64.80 (C); 5.02/5.42 (H); 10.74/9.92 (N). The structure of CBBAH was confirmed by the UV, IR, ^{1}H NMR spectral data. The UV and IR spectrum of CBBAH showed the characteristic absorption bands of *N*-alkyl hydrazide. UV (ethanol) $\lambda_{max.}$ (nm): 268, 221 and 205. IR (KBr) cm^{-1} ν: 3269 (CON*H*); 1641 (*CO*NH); 1600, 1508, 1404 (C=C); 1089 (C–Cl). ^{1}H NMR ($CDCl_3$) δ ppm: 4.09 (s, 2H, –NH–CH_2–), 4.30 (s, 1H, –N*H*–CH$_2$), 7.35–7.69 (m, 9H, Ar–H), 7.71 (b, 1H, –CON*H*–). The ^{1}H NMR spectrum of CBBAH in $CDCl_3$ displayed –CON*H*–, –N*H*–CH$_2$–, –NH–CH_2–, protons at 7.71, 4.30, 4.09 ppm, respectively. The –CON*H*–, –N*H*–CH$_2$–N–H protons of iproniazid were observed at 7.58 and 4.31 ppm, respectively [9].

2.2. Preparation of benzoic acid(4-chlorophenyl)methylenehydrazide (CBDBAH)

BAH (0.01 mol) was dissolved in ethanol. To this solution, an ethanolic solution of CB (0.01 mol) was added. This mixture was refluxed for 2 h. The solid product obtained was filtered off, washed with water, dried and recrystallized from ethanol. The purity of CBDBAH was confirmed by elemental analysis, TLC and a reverse phase HPLC. The structure of CBDBAH was established by its UV and IR spectral data. M.p.

Table 1
Chromatographic properties of the substrate and their potential metabolites

Compound	Abbreviations	HPLC system (S)	TLC system (A)
		Retention time (min)	(R_f)
		S [a]	A [b]
Benzoic acid	BA	2.30	
Benzoic acid hydrazide	BAH	3.26	0.07
4-Chlorobenzoic acid	CBA	5.01	
4-Chlorobenzaldehyde	CB	17.60	0.91
N-(4-Chlorobenzyl)-*N′*-benzoylhydrazine	CBBAH	21.25	0.61
Benzoic acid(4-chlorophenyl)methylenehydrazide	CBDBAH	24.95	0.49

[a] S, acetonitrile: 0.02 M phosphate buffer (pH 7.4) (30:70, v/v) final pH 5.00. Flow rate 0.5 ml/min HPLC column (Novapak C_{18} 5 μm, 15 cm length × 3.9 mm).

[b] A, diethylether.

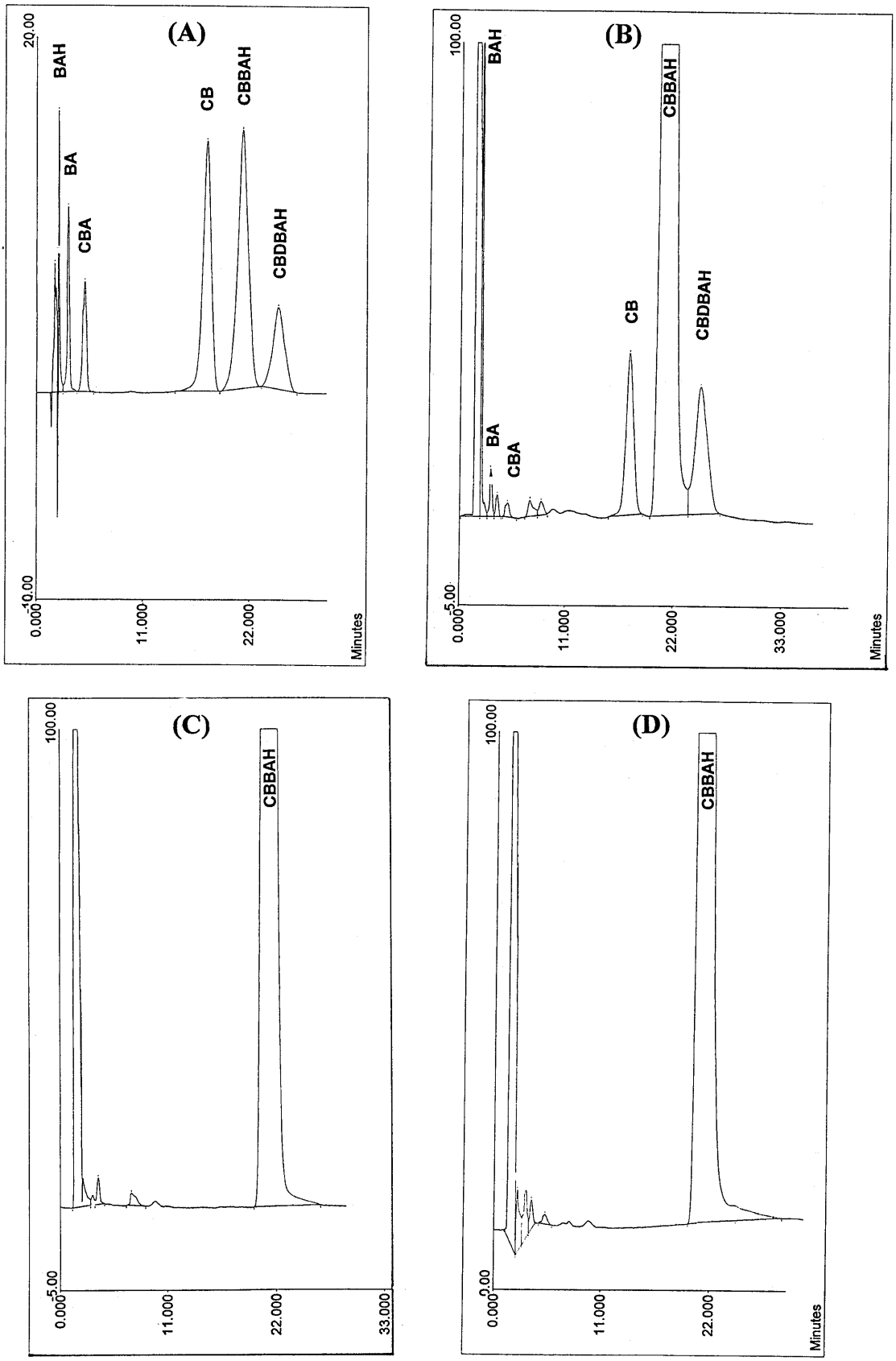

Fig. 2. RP-HPLC chromatogram obtained (A) from authentic CBBAH and its potential metabolites; (B) following extraction of male rat microsomal incubation mixture with CBBAH as substrate; (C) from incubation with denatured microsomes; (D) from incubation with microsomes in which co-factors were omitted; (E) from incubation of microsomes in which substrate and co-factors were omitted; (F) from co-incubation of BAH and CB with denatured microsomes.

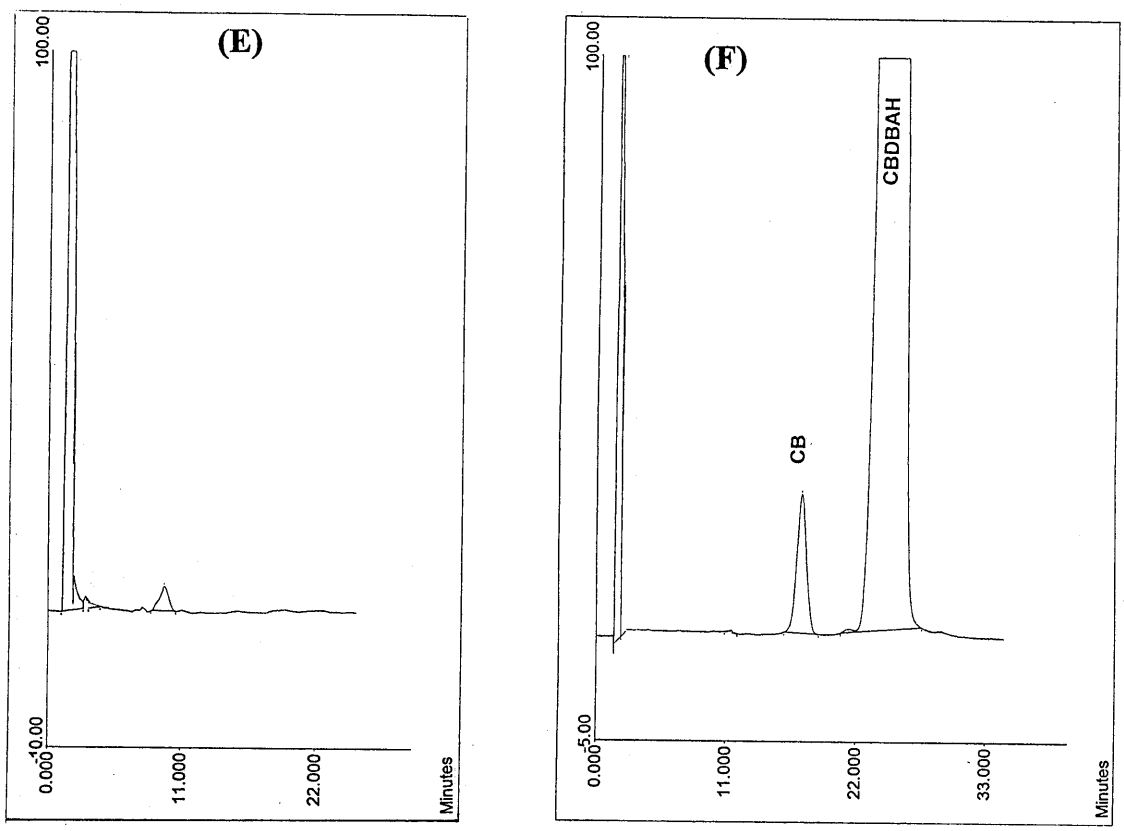

Fig. 2. (*Continued*)

164–165°C (lit. 170–175°C) [10]. Analysis for $C_{14}H_{11}ClN_2O$ Calc./Found: 64.99/64.89 (C); 4.28/4.56 (H); 10.83/10.85 (N). The UV spectrum exhibited a characteristic E band for aromatic group at 202 nm and a characteristic K band for chromophoric –CH=N– group at 306 nm [4]. UV (ethanol) $\lambda_{max.}$ (nm): 306, 221 and 204. IR spectrum of compound CBDBAH showed the characteristic absorption bands of hydrazone (N–H 3288; C=O 1664; –CH=N– 1620 cm^{-1}). IR (KBr) $cm^{-1}\nu$: 3288 (CON*H*); 1664 (*CO*NH); 1620 (CH=N); 1602, 1543, 1487 (C=C); 1086 (C–Cl).

2.3. Attempted oxidation of N-(4-chlorobenzyl)-N′-benzoylhydrazine

The oxidation products of CBBAH were synthesized by the oxidation reaction with *m*-CPBA [11]. CBBAH (0.001 mol, 0.261 g) was dissolved in dry acetone and the flask kept in the dark. To this stirred and cooled solution, *m*-CPBA (0.002 mol, 0.43 g 80%, in dry acetone) was added dropwise over 30 min. The reaction was continued for further 5 h and terminated by removal of the solvent under reduced pressure. The residual solid was dissolved in dichloromethane (25 ml) and this solution was subsequently washed with aqueous K_2CO_3 (0.5 M, 3×25 ml); dried with anhydrous Na_2SO_4 and concentrated under reduced pressure to leave a mixture of a pale yellow and white solid. This mixture was dissolved in acetone and chromatographed on preparative silica gel plates (0.5 mm thickness) in conjunction with diethylether. The corresponding hydrazone (CBDBAH) and 4-chlorobenzaldehyde (CB) were obtained from the bottom and top bands, respectively. The silica-layers corresponded to these bands were scraped off and extracted into dichloromethane and the solvent was evaporated. Two solid products were obtained. The solid corresponding to the bottom band gave a m.p. at 160–165°C, UV (ethanol) $\lambda_{max.}$ (nm): 305, 221 and 204 (Fig. 1). HPLC $R_t = 24.90$, TLC $R_f = 0.48$ whereas the solid corresponding to the top band gave a HPLC $R_t = 17.50$, and a TLC $R_f = 0.90$.

2.4. Analytical separation

The separation techniques were based on TLC and an isocratic HPLC system. TLC was carried out using prepared silica-gel GF254 0.25 mm on glass plates (E. Merck) with diethylether as solvent system A. Compounds were identified by their absorbances at 254 nm (Table 1). A mixture of 4-dimethylaminocinnamaldehyde (0.2% w/v) in hydrochloric acid (1 M)–ethanol (1:1, v/v) reagent was sprayed on to the plates after

development. A dark red color was obtained. The HPLC chromatograph consisted of an isocratic system, a Rheodyne syringe loading sample injector valve (model 7725) fitted with a 20 μl sample loop, a model 481 Waters wavelength UV detector and a Unicam 4880 Chromatography Data Handling System integrator. The HPLC column (Spherisorb, μ-Bondapak C_{18} 5 μm, 25 cm length × 4.6 mm and Novapak C_{18} 5 μm, 15 cm length × 3.9 mm) was purchased from Phase Separations Limited, UK. The guard column packing material (Whatman Pellicular ODS) was purchased from Whatman International Ltd., Maidstore, Kent, UK. The composition of the mobile phases, flow rates and the retention times of the CBBAH and its potential metabolites were given in Table 1. All these compounds were detected by their absorbance at 254 nm.

2.4.1. Biological studies

Glucose-6-phosphate dehydrogenase (G6PH) was purchased from the Boehringer Mannheim Corporation (London) Ltd. Nicotinamide adenine dinucleotide phosphate mono sodium salt (NADP) and glucose-6-phosphate (G6P) disodium salt were obtained from Sigma Ltd. The animals used in this investigation were male rats. Hepatic washed male rat microsomes were prepared at 0°C using the calcium chloride precipitation method [12]. Incubation was carried out in a shaking water-bath at 37°C using a standard co-factor solution consisting of $NADP^+$ (disodium salt, 1 μmol), G6P (disodium salt, 5 μmol), G6PDH (0.5 unit) and $MgCl_2$ (50% w/w, 10 μmol) in 1 ml of phosphate buffer (0.2 M, pH 7.4) for each flask. Co-factor solutions were pre-incubated for 5 min before the addition of substrate (2 μmol) in methanol (50 μl) and microsomes equivalent to 0.25 g original liver. Incubation was continued for 30 min, terminated and the incubates were extracted with dichloromethane (2 × 5 ml). Extracts were evaporated to dryness under a stream of nitrogen at 20°C. Dry organic residues were reconstituted in 200 μl of methanol for HPLC and 50 μl of methanol for TLC analysis.

3. Results and discussion

Following the metabolic experiments using CBBAH as a substrate, the *N*-dealkylation products, i.e. BAH and CB were observed by RP-HPLC (Fig. 2) and TLC (Fig. 3). The formation of the corresponding hydrazone (CBDBAH) as a major in vitro metabolic product was also demonstrated. The control experiments were performed to investigate the mechanism of formation of this metabolite by co-incubation of BAH with CB in the presence of denatured microsomes. The result indicated that the formation of CBDBAH was most probably via condensation of CB and BAH. This was in

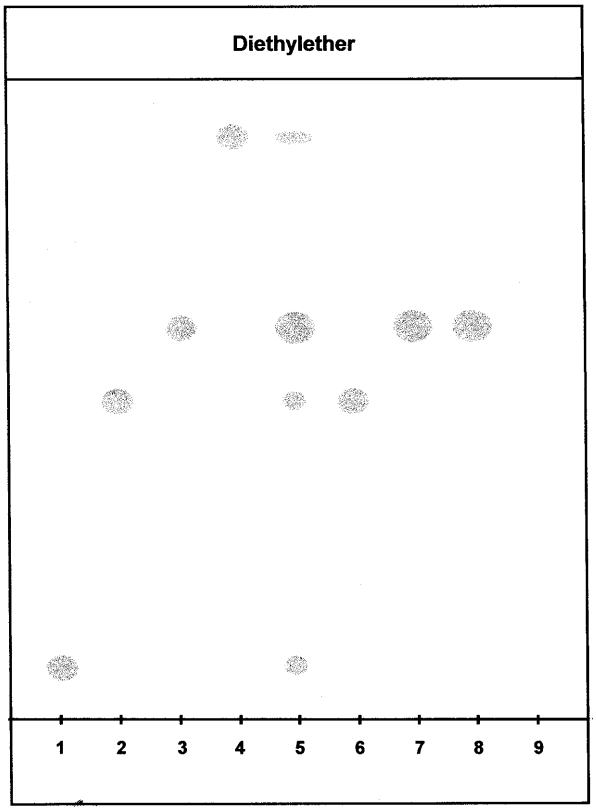

Fig. 3. TLC chromatogram obtained following extraction from male rat microsomal incubation mixture with CBBAH as substrate. 1, BAH; 2, CBDBAH; 3, CBBAH; 4, CB; 5, test; 6, incubation of CB and BAH in the presence of denatured microsomes; 7, control (denatured microsomes); 8, control (omission of co-factors); 9, control (omission of the substrate).

accordance with the findings reported by Low et al. [13]. Since the chemical oxidation of CBBAH using *m*-CPBA yielded CBDBAH as the major product, it could be proposed that this hydrazone would also arise from a direct oxidation of CBBAH via dehydratation of an unstable *N*-hydroxy intermediate (Fig. 4) [14]. CB was further oxidized to 4-chlorobenzoic acid (CBA). BA was found as a metabolite which may arise either from the hydrolysis of CBBAH or alternatively from the hydrolysis of BAH. All these metabolites were not observed with control incubates with denatured microsomes or incubates lacking co-factors. This indicates that these metabolites formed via enzymatic reactions. Metabolic and chemical reactions possibly involved in the biotransformation of CBBAH are shown in Fig. 4.

N-Dealkylated and hydrolytic metabolites were detected together with a hydrazone function formed. Benzoic acid hydrazide produced by the *N*-dealkylation reaction is possible to form hydrazone with reactive carbonyl groups, i.e. pyruvic acid and α-ketoglutaric acid. If this occurs, all α-keto acid resources needed for the pyruvate oxidation by tricarboxylic acid cycle would be depleted. This may interrupt the carbohydrate

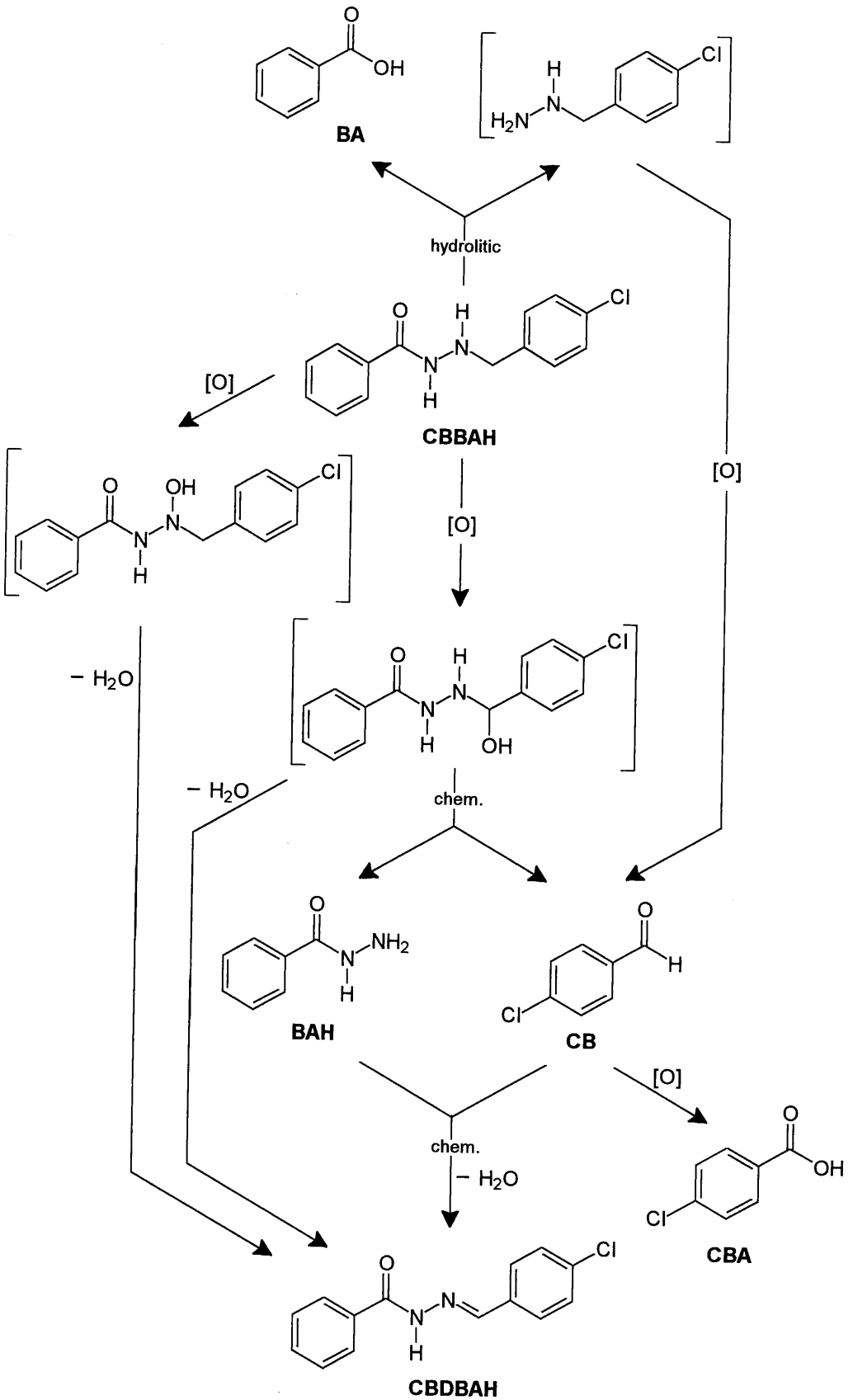

Fig. 4. In vitro microsomal metabolic reactions of CBBAH.

and the amino acid metabolisms. However, in our structure, the hydrazone formation from the benzoic acid hydrazide and 4-chlorobenzaldehyde produced by *N*-dealkylation may prevent, to some extent, these potential toxic responses. In addition, it may be possible that BAH produced by *N*-dealkylation from CBBAH may result in hepatic necrosis by a similar mechanism as in isoniazid and isopropylhydrazine. Isoniazid is known to produce a hydrazone with vitamin B_6 and a deficiency for this vitamine occurs. BAH may similarly have this undesired effect.

Acknowledgements

This work was supported by the Research Fund of Marmara University Project number SBDYD-102.

References

[1] V.K. Agarwal, T.K. Gupta, S.S. Parmar, Substituted indolebenzylhydrazines as anticonvulsants, J. Med. Chem. 15 (1972) 1000–1001.
[2] O.M. Aboul-Wafa, M.A.E. El-Metwalli, Novel benzo[*b*]tienylhydrazine and 1,3,4-oxadiazole derivatives as potential antidepressant agents, Arch. Pharm. (Weinheim) 325 (1992) 603–608.
[3] H. Zhao, N. Neamati, S. Sunder, H. Hong, S. Wang, G.W.A. Milne, Y. Pommier, T.R. Burke, Hydrazide-containing inhibitors of HIV-1 integrase, J. Med. Chem. 40 (1997) 937–941.
[4] Ş.G. Kömürcü, S. Rollas, M. Ülgen, J.W. Gorrrod, A. Çevikbaş, Evaluation of some arylhydrazones of *p*-aminobenzoic acid hydrazide as antimicrobial agents and their in vitro hepatic microsomal metabolism, Boll. Chim. Farm. 134 (1995) 375–379.
[5] M. Ülgen, B.B. Durgun, S. Rollas, J.W. Gorrod, The in vitro hepatic microsomal metabolism of benzoic acid benzylidenehydrazide, Drug Metab. Drug Interact. 13 (1997) 285–294.
[6] M. Hayashi, Y. Higuchi, (Nissan Chemical Industries, Ltd.) Jpn. Kokai Tokkyo Koho 7959,326 (Cl.AO1 N9/20), 12 May, 1979, Appl. 77/124,913, 18 October, 1977, pp. 5, C.A. 91, 118683g.
[7] J.R. Dimmock, J.M. Brenner, O.A. Philips, Evaluation of some benzenesulphonyl hydrazones of aryl aldehydes and ketones as antiepileptic agents, Pharmazie 42 (1987) 376–378.
[8] K. Sung, A.R. Lee, Synthesis of [(4,5-disubstituted-4*H*-1,2,4-triazol-3-yl)thio] alkanoic acids and their analogues as possible antiinflammatory agents, J. Heterocycl. Chem. 29 (1992) 1101–1109.
[9] M.G. Vigorita, R. Ottana, R. Maccari, F. Monforte, G. Bisignano, F.C. Pizzimenti, Synthesis and in vitro antimicrobial and antitumoral screening of novel lipophilic isoniazid analogues VI, Boll. Chim. Farm. 137 (1998) 267–276.
[10] J.R. Dimmock, G.B. Baker, W.G. Taylor, Arylhydrazones — Part II. The ultraviolet spectroscopy and antimicrobial evaluation of some substituted aroylhydrazones, Can. J. Pharm. Sci. 7 (1972) 100–103.
[11] J.W. Gorrod, N.J. Gooderham, Improved preparation of α,*N*-diphenylnitrones and *N*-benzyl-*N*-phenylhydroxylamines by direct oxidation of secondary anilines, Arch. Pharm. (Weinheim) 319 (1986) 261–265.
[12] J.B. Schenkman, D.L. Cinti, Preparation of microsomes with calcium, Methods Enzymol. 52 (1978) 83–89.
[13] C.M. Low, M. Ülgen, J.W. Gorrod, The recognition of a diarylimine as a metabonate produced during incubation of *N*-benzyl-4-chloroaniline with hepatic microsomal preparations, J. Pharm. Pharmacol. 46 (1994) 585–590.
[14] M. Ülgen, J.W. Gorrod, D. Barlow, Stucture–activity relationships in the formation of amides from substituted *N*-benzylanilines, Xenobiotica 24 (1994) 735–748.