Biochemical Pharmacology, Vol. 35, No. 22, pp. 3935–3939, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00
Pergamon Journals Ltd.

INVOLVEMENT OF LEUKOCYTES IN THE OXYGENATION AND CHLORINATION REACTION OF PHENYLBUTAZONE

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(*Received* 19 *February* 1986; *accepted* 23 *May* 1986)

Abstract—A center carbon atom of 1,3-diketone moiety of phenylbutazone was oxidized to give three metabolites—4-hydroxyphenylbutazone (metabolite I), 4-hydroperoxyphenylbutazone (metabolite II) and 4-chlorophenylbutazone (metabolite III)—by the action of enzymes present in leukocyte extract obtained from peritoneal exudate of rats. Both metabolites II and III were produced by peroxidases, while metabolite I was produced by enzymes other than the peroxidases.

The polymorphonuclear leukocyte is known to cause an inflammation reaction in Arthus phenomenon, acute nephrotoxic nephritis, necrotizing arteritis of serum sickness, etc. [1]. The inflammation process involves generation of several kinds of active oxygen species by enzyme systems in the leukocytes [2]; the toxic species not only injure host cells but also react with a wide variety of materials eventually promoting inflammation [1, 2].

According to these findings, we have considered that at least a part of anti-inflammatory activity of certain drugs is attributable to their action as a scavenger of such active oxygen species. In fact, during the course of our investigation on the mechanism of action of tenoxicam [4-hydroxy-*N*-(2′-pyridyl)-2-methyl-2*H*-thieno-⟨2,3e⟩-1,2-thiazine-3-carboxamide-1,1-dioxide], a potent anti-inflammatory drug, we successfully demonstrated that this drug was easily converted to several metabolites via oxidation reaction of the center carbon atom of 1,3-diketone moiety at the expense of the active oxygen species generated by leukocyte extract [3, 4].

Because phenylbutazone (a potent and widely used anti-inflammatory drug) also possesses a 1,3-diketone moiety in its structure, this drug seems to be a compound which acts as an active oxygen scavenger like tenoxicam. Indeed, enzymes in a leukocyte extract oxidized the center carbon atom (C-4 position) of the diketone moiety of phenylbutazone to give three metabolites.

In the present paper we elucidate the structure of these metabolites and discuss the pharmacological and toxicological significances of the oxidation reaction of phenylbutazone.

MATERIALS AND METHODS

Materials. Phenylbutazone was purchased from Sigma Chemical Co. (St. Louis, MO) and cetyltrimethylammonium bromide and guaiacol were from Wako Pure Chemical Industry Co. (Tokyo). 4-Hydroxyphenylbutazone and 4-chlorophenylbutazone were respectively synthesized from phenylbutazone by the method described by Woodruff and Polya [5] and Khaletskii *et al.* [6]. 4-Hydroperoxyphenylbutazone was enzymatically prepared by oxidation of phenylbutazone according to the following procedure: to 94.5 ml of 0.1 M potassium phosphate buffer (pH 6.0) were added 0.5 ml of 70% aqueous solution of *t*-butyl hydroperoxide, 20 mg of horseradish peroxidase (type II, EC 1.1.11.7, Sigma Chemical Co.), and 100 mg of phenylbutazone dissolved in 5 ml of ethanol. The reaction mixture was incubated at 25° for 45 min by bubbling O_2 gas and the reaction terminated by cooling the mixture in an ice-chilled bath. 4-Hydroperoxyphenylbutazone was extracted twice (each 100 ml) with diethylether. The organic layer was combined, washed with water, dried over Na_2SO_4, and evaporated under reduced pressure. Dichloromethane solution of the residue was applied to a silica gel column (17 × 2 cm) which was subsequently washed with dichloromethane. The column was eluted with a solvent mixture of dichloromethane/ethyl acetate (19:1, v/v). Evaporation of the eluate (320–360 ml) gave the desired product as white solid (about 20 mg). Proton nmr: 0.9 p.p.m. (H—δ, multiplet, 3H), 1.4 p.p.m. (H—β and γ, multiplet, 4H), 2.0 p.p.m. (H—α, multiplet, 2H), 7.3 p.p.m. (H—phenyl, multiplet, 10H), and 10.2 p.p.m. (H—4—OOH, singlet, 1H). [^{13}C]Nmr: 13.5 p.p.m. (C—δ), 22.6 p.p.m. (C—γ) 24.3 p.p.m. (C—β), 32.1 p.p.m. (C—α), 85.1 p.p.m. (C—4), 123.0 p.p.m. (C—2′ and 6′), 127.2 p.p.m. (C—4′), 135.0 p.p.m. (C—1′), and 168.3 p.p.m. (C—3 and 5).

Leukocyte extract was prepared as follows: male Sprague–Dawley rats (11 weeks old) were intraperitoneally given 10 ml of 4% sodium caseinate in sterilized saline and leukocytes (which contained more than 85% of polymorphonuclear leukocytes) were collected from peritoneal exudate of the rats 18 hr after administration [7]. Peroxidases in the leukocytes were solubilized with 0.3% of cetyltrimethylammonium bromide in 10 mM potassium phosphate buffer (pH 7.0) according to the procedure of Desser *et al.* [8]. The solution was applied

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to Sephadex G-25 (Pharmacia Fine Chemicals) to remove bromide and chloride ions. A unit of peroxidase activity in the extract was defined as amount of enzyme that produces an increase of 1 absorbance unit in 1 min by using guaiacol as a substrate under the conditions described in our previous report [3].

All chemicals used in the present study were of analytical special grade and were obtained commercially.

Enzymatic reaction and quantitative analysis. A typical enzymatic reaction mixture contained in a final volume of 1 ml: 50 μmol of potassium phosphate buffer (pH 6.0); 0.1 μmol of phenylbutazone; leukocyte extract containing 0.25 units of peroxidases; 0.1 μmol of hydrogen peroxide and 10 μmol of sodium chloride. After preincubation of the reaction mixture without leukocyte extract, the reaction was initiated by adding the extract. The reaction mixture was incubated at 37° for 10 min and the reaction terminated by addition of 1 ml of acetonitrile in an ice-chilled waterbath. An aliquot (20 μl) of the solution was subjected to hplc for a quantitative analysis of respective compounds: HPLC was carried out using a reverse-phase ODS column (AMW-312, 5 μm, 150 × 6 mm, Yamamura Chemical, Osaka, Japan) on a LC-4A system (Shimadzu Seisaku Co. Ltd, Kyoto, Japan) at ambient temperature. The column was eluted with a solvent mixture of acetonitrile/H_2O (3:2, v/v) at a flow rate of 1.5 ml/min. Elution of each compound was monitored at 237 nm. Amounts of each metabolite were determined by calculation of the peak area on HPLC. Linearity was obtained from 0.1–5.0 μg/ml of each metabolite.

Isolation of metabolites. In order to isolate enough respective metabolites for qualitative analysis, incubation was performed on a 20-times larger scale. The incubation conditions were the same as those described above except that: (a) incubation time was 20 min; (b) volume of the leukocyte extract was 100-times larger (containing 24 units of the peroxidases); and (c) sodium chloride was omitted from the reaction mixture in the case of isolation of metabolite I (4-hydroxyphenylbutazone) and II (4-hydroperoxyphenylbutazone). After termination of the reaction by ice-chilling, metabolites were extracted twice with 20 ml of diethyl ether. The organic layer was evaporated under reduced pressure. The residue was redissolved in a small volume of chloroform and applied to a glass plate pre-coated with silica gel 60 F_{254} (0.25 mm thick, Merck, Darmstadt, F.R.G.). Solvent systems used were chloroform/ethyl acetate (4:1, v/v) for metabolite I and II and n-hexane/diethyl ether (2:1, v/v) for metabolite III. Bands of silica gel corresponding to these metabolites were separately scraped from the plate under u.v. light and each compound was extracted with diethyl ether. R_f values of metabolite I, II, and III were 0.45, 0.58, and 0.50 respectively.

Instruments. Electron impact (EI) mass spectra (30 eV) were obtained on a JMS-DX300 high resolution mass spectroscope (JEOL Co. Ltd, Tokyo, Japan) with a JMA 3500 data acquisition system (JEOL Co. Ltd). Both [^{1}H]- and [^{13}C]nmr spectra were obtained in $CDCl_3$ with a JEOL JNM-FX-100 spectrometer (JEOL Co. Ltd). Ultra-violet spectral data were recorded in methanol with a UV-190 spectrophotometer (Shimadzu Seisaku Co. Ltd, Kyoto).

RESULTS

Metabolism of phenylbutazone by enzymes in leukocyte extract

Under the incubation conditions described in Materials and Methods, phenylbutazone was enzymatically converted to approximately equal amounts (5–7 nmol) of the three metabolites, which were designated as metabolite I (R_t = 5.2 min, 4-hydroxyphenylbutazone), II (R_t = 6.0 min, 4-hydroperoxyphenylbutazone), and III (R_t = 14.0 min, 4-chlorophenylbutazone) in the order of the elution time on the HPLC (Fig. 1).

In a control incubation mixture run simultaneously with heat denatured enzymes (100°, 5 min), none of these three metabolites was produced. Omission of hydrogen peroxide likewise abolished the formation of metabolites and the parent compound was recovered without significant loss. Omission of chloride ion completely inhibited the formation of metabolite III without any influence on metabolite I and II. On the other hand, incubation under nitrogen atmosphere caused a marked decrease of amount of metabolite I and II but not that of metabolite III.

Sodium azide (0.1 mM), a potent inhibitor of peroxidases, completely inhibited the formation of metabolite III, whereas incomplete inhibition was observed for the other two metabolites (metabolite I and II were respectively generated at a ratio of 0.7 and 0.2–0.3 of the control reaction).

Identification of metabolites

Metabolite I. The EI mass spectrum of phenylbutazone showed a molecular ion peak at m/z 308, while metabolite I gave the peak at m/z 324 ($C_{19}H_{20}N_2O_3$) (Fig. 2). The result indicates the attachment of one oxygen atom to phenylbutazone to form metabolite I. The presence of an unchanged

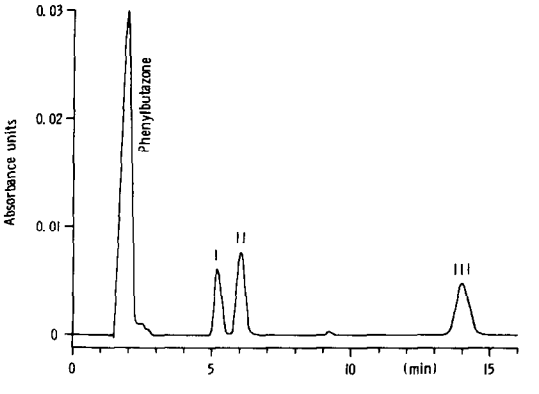


Fig. 1. HPLC analysis of metabolites of phenylbutazone by enzymes in leukocyte extract. The reaction mixture contained in a final volume of 1 ml: 50 μmol of potassium phosphate buffer (pH 6.0); 0.1 μmol of phenylbutazone; leukocyte extract containing 0.25 units of peroxidases; 0.1 μmol of hydrogen peroxide; and 10 μmol of sodium chloride. The reaction mixture was incubated at 37° for 10 min. HPLC was carried out using a reverse-phase ODS column. The column was eluted with a solvent mixture of acetonitrile/H_2O (3:2, v/v) at a flow rate of 1.5 ml/min.

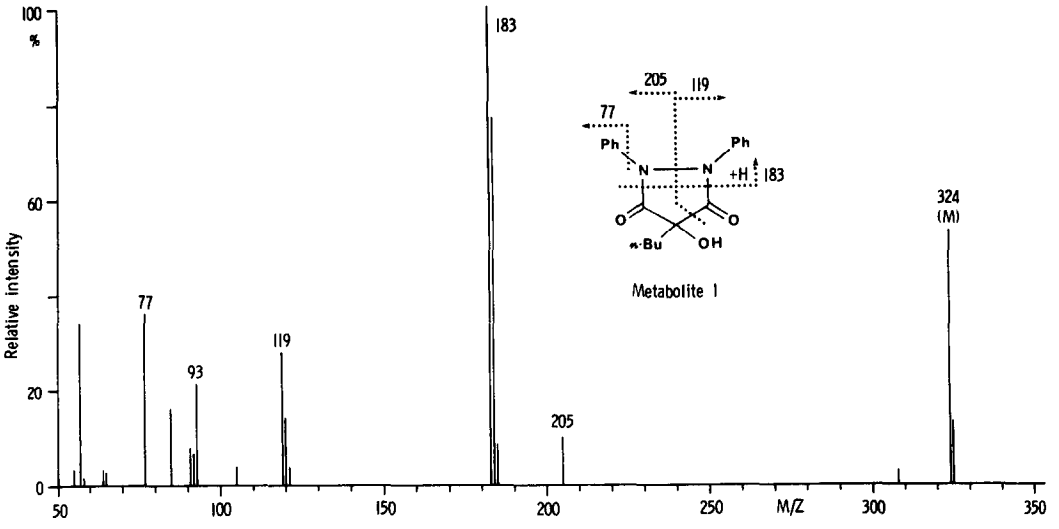
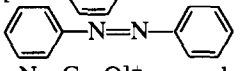
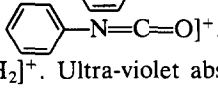
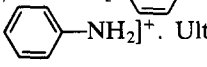


Fig. 2. EI mass (30 eV) spectrum of metabolite I.

phenyl moiety in the metabolite was confirmed by several fragment ion peaks at m/z 308 [M − OH + H]$^+$, m/z 205 [M −  —N=C=O]$^+$, m/z 183 (base peak) [ + H]$^+$, m/z 119 [ —N=C=O]$^+$, and m/z 93 [ —NH_2]$^+$. Ultra-violet absorption spectrum of phenylbutazone in methanol gave an absorption maximum peak at 239 nm with a shoulder peak around 275 nm, which were respectively attributable to an aminobenzene moiety and an enolic form of 1,3-diketone moiety. In contrast, metabolite I showed only one absorption maximum at 237 nm derived from an aminobenzene moiety. On the basis of these data, the 1,3-diketone moiety is incapable of giving an enolic form in metabolite I due to the presence of a hydroxyl group at the center carbon of 1,3-diketone moiety (i.e. C-4 position). Metabolite I was finally identified as 4-hydroxyphenylbutazone by comparison of u.v. and mass spectral data and the behaviour on both TLC and HPLC under the conditions described above with those of the authentic 4-hydroxyphenylbutazone.

Metabolite II. The EI mass spectrum of metabolite II (Fig. 3) showed a weak but definite molecular ion peak at m/z 340 and a closely similar fragmentation pattern with that of metabolite I, the results being indicative of an attachment of two oxygen atoms to phenylbutazone to yield metabolite II. Metabolite II was positive to a specific detection reagent for peroxides (Merckoquant®, Peroxide Test, Merck, Darmstadt, F.R.G.) and was quantitatively converted to metabolite I with a weak reductant like dithiothreitol. Together with the identical u.v. spectrum of this metabolite with that of metabolite I, these results strongly indicate the presence of a hydroperoxy moiety on the C-4 position in metabolite II. Structure of metabolite II was conclusively

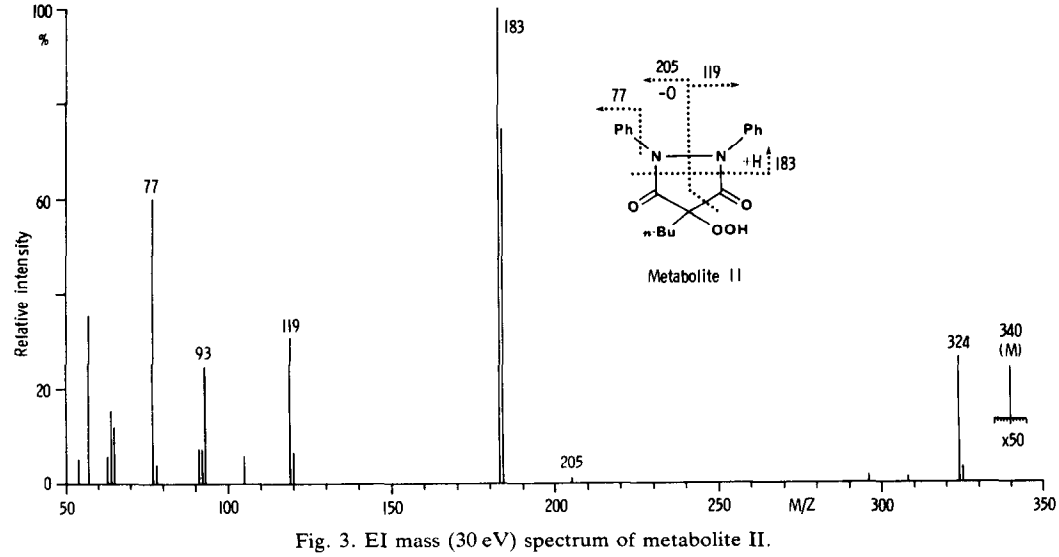


Fig. 3. EI mass (30 eV) spectrum of metabolite II.

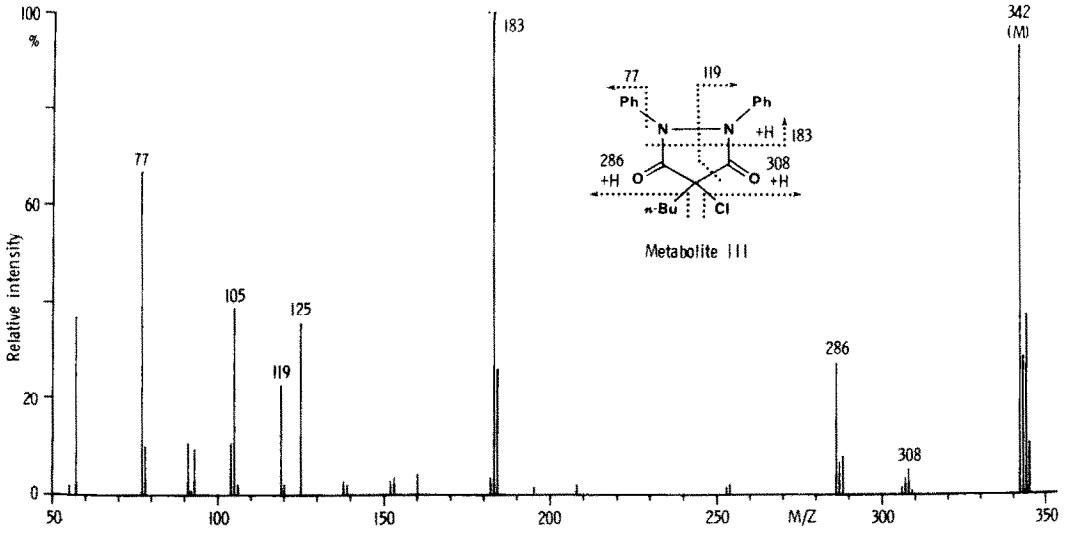
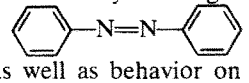


Fig. 4. EI Mass (30 eV) spectrum of metabolite III.

determined as 4-hydroperoxyphenylbutazone on the basis of the closely similar physicochemical data and chromatographic behavior of metabolite II with those of the authentic compound.

Metabolite III. The EI mass spectrum of metabolite III (Fig. 4) gave a molecular ion peak at *m/z* 342 ($C_{19}H_{19}N_2O_2Cl$) with an isotope ion peak at *m/z* 344, the result indicating an attachment of one chlorine atom to phenylbutazone to form metabolite III. The presence of both the unchanged butyl and phenyl moieties in the metabolite was respectively confirmed by the fragment ion peaks at *m/z* 286 $[M - CH_2{=}CH{-}CH_2{-}CH_3]^+$ probably yielded by MacLafferty rearrangement and *m/z* 183 (base peak) [ + H]$^+$. The mass spectra as well as behavior on the TLC and HPLC were identical with those of authentic 4-chlorophenylbutazone, hence, we concluded that metabolite III was 4-chlorophenylbutazone.

DISCUSSION

Leukocyte extract, which possessed a high activity of peroxidases, enzymatically converted phenylbutazone to 4-hydroxyphenylbutazone (metabolite I), 4-hydroperoxyphenylbutazone (metabolite II), and 4-chlorophenylbutazone (metabolite III), the results indicating the susceptibility of a C-4 carbon atom (a center carbon atom of 1,3-diketone moiety) of phenylbutazone to an oxidation reaction. Despite numerous reports concerning the metabolism of phenylbutazone not only *in vivo* [9–12] but also *in vitro* [13, 14] systems, an attachment of a chlorine atom or hydroperoxyl group to this compound has not yet been reported to our knowledge.

Although a minor amount of metabolite I was nonenzymatically produced by a reduction reaction of metabolite II by phenylbutazone, none of three metabolites was converted to another metabolite in significant amounts under the present incubation conditions (data not shown). Coupled with the results that a molecular oxygen and chlorine atom were respective essential factors for formation of metabolite II and III, this finding suggests that the present metabolites are generated via different routes as is shown in Fig. 5.

Several lines of evidence clearly indicate that both 4-hydroperoxyphenylbutazone (metabolite II) and 4-chlorophenylbutazone (metabolite III) were produced by an action of peroxidases; firstly, hydrogen peroxide was required as an essential factor for the

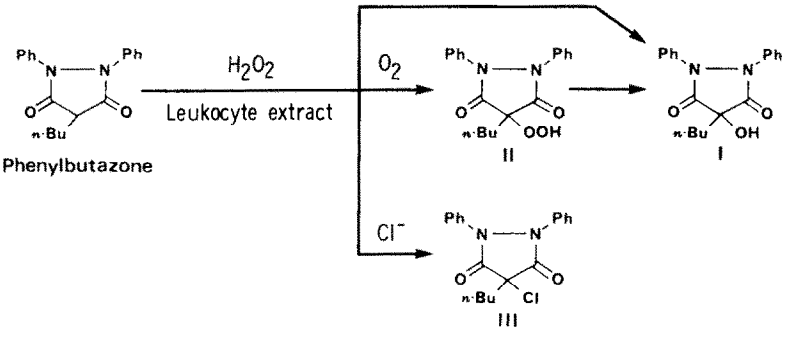


Fig. 5. Metabolic conversion of phenylbutaxone by enzymes in leukocyte extract.

formation of these two metabolites, secondly, sodium azide, which is a potent inhibitor of heme-containing enzymes such as peroxidases, significantly inhibited the formation of both metabolites at the concentration of 0.1 mM, and thirdly, peroxidases are well known to mediate the chlorination or oxygenation reaction under acidic conditions (i.e. pH 5–6) and, indeed, amounts of formation of these two metabolites were higher in such a pH range.

In the generation of metabolite III, preferential participation of myeloperoxidase is quite plausible, because myeloperoxidase in polymorphonuclear leukocytes chlorinate several kinds of organic compounds such as fluorescein [15] and 1,3,5-trimethoxybenzene [16]; a supportive finding for the implication of myeloperoxidase in the chlorination was that phenylbutazone was converted to 4-chlorophenylbutazone (metabolite III) with a high yield with hypochlorous acid (which is known to be derived from chloride ion by means of myeloperoxidase and acts as an active species for chlorination [1]) (data not shown).

Opposite to metabolite II and III, the large amounts of 4-hydroxyphenylbutazone (metabolite I) present in the reaction mixture were likely to have been formed by the action of enzyme(s) other than the peroxidases described above, because sodium azide showed only slight inhibitory effect on the formation of this compound and neither metabolite II nor III was enzymatically converted to 4-hydroxyphenylbutazone.

In our previous report we described how the center carbon atom of 1,3-diketone moiety of tenoxicam [4-hydroxy-*N*-(2′-pyridyl)-2-methyl-2*H*-thieno-(2, 3e)-1,2-thiazine-3-carboxamide-1,1-dioxide], a potent anti-inflammatory drug, easily reacts with active oxygen species derived from hydrogen peroxide by Fe^{2+} (Fenton reaction) [4] or by leukocyte peroxidases [3]. Based on this result, we have postulated that a part of anti-inflammatory activity of tenoxicam was ascribed to its scavenging activity towards the active oxygen species (a possible promoting factor for inflammation and preferentially generated at the inflammatory site) and that the diketone moiety was essential for the action. The present results suggest that the center carbon atom of the 1,3-diketone moiety in phenylbutazone likewise plays an important role in exerting the anti-inflammatory activity.

Although both tenoxicam and phenylbutazone showed the same type of oxidation reaction (i.e. oxidation reaction of the center carbon of 1,3-diketone moiety) by peroxidases, a large and conspicuous difference between these two drugs was that metabolites having hydroperoxyl substituent at the center carbon could be successfully isolated for phenylbutazone but not for tenoxicam [3, 17]. In tenoxicam such oxidized metabolite(s) are decomposed immediately after the formation because of the markedly low stability imparted by the presence of nitrogen atom adjacent to the C-3 carbon atom. The higher stability of 4-hydroperoxyphenylbutazone (metabolite II) appears to explain the high incidence of side effects of phenylbutazone: 4-hydroperoxyphenylbutazone is likely to act as an active oxidant *per se* and cause a serious damage to living cells.

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