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Biotransformation of benzydamine by microsomes and precision-cut slices prepared from cattle liver

A. SANTI†, P. ANFOSSI‡*, N. G. COLDHAM§, F. CAPOLONGO†, M. J. SAUER§ and C. MONTESISSA†

† Istituto di Patologia e Igiene veterinaria, Università di Padova, Agripolis, I-35020 Legnaro (PD), Italy
‡ Dipartimento di Sanità Pubblica Veterinaria e Patologia Animale, Università di Bologna, Ozzano Emilia, Bologna, Italy
§ Department of Risk Research, Veterinary Laboratories Agency, New Haw, Addlestone, Surrey KT15 3NB, UK

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1. Benzydamine (BZ), a non-steroidal anti-inflammatory drug used in human and veterinary medicine, is not licensed for use in food-producing species. Biotransformation of BZ in cattle has not been reported previously and is investigated here using liver microsomes and precision-cut liver slices.

2. BZ was metabolized by cattle liver microsomes to benzydamine *N*-oxide (BZ-NO) and monodesmethyl-BZ (Nor-BZ). Both reactions followed Michaelis–Menten kinetics ($K_m = 76.4 \pm 16.0$ and $58.9 \pm 6.4\,\mu M$, $V_{max} = 6.5 \pm 0.8$ and $7.4 \pm 0.5\,nmol\,mg^{-1}\,min^{-1}$, respectively); sensitivity to heat and pH suggested that the *N*-oxidation is catalysed by the flavin-containing monooxygenases.

3. BZ-NO and Nor-BZ were the most abundant products derived from liver slice incubations, and nine other BZ metabolites were found and tentatively identified by LC-MS. Desbenzylated and hydroxylated BZ-NO analogues and a hydroxylated product of BZ were detected, which have been reported in other species. Product ion mass spectra of other metabolites, which are described here for the first time, indicated the formation of a BZ N^+-glucuronide and five hydroxylated and N^+-glucuronidated derivatives of BZ, BZ-NO and Nor-BZ.

4. The results indicate that BZ is extensively metabolized in cattle. Clearly, differences in metabolism compared with, for example, rat and human, will need to be considered in the event of submission for marketing authorization for use in food animals.

Introduction

Benzydamine (1-benzyl-3-[3-(dimethylamino)propoxy]-1*H*-indazole; BZ) is a non-steroidal analgesic anti-inflammatory drug used in humans by topical application. Although BZ has been used in veterinary medicine in cattle and pig (intramuscular administration) for the treatment of inflammatory conditions of the respiratory, gastrointestinal, genital and urinary systems and in horse by topical application for soft tissue injuries, it is not currently licensed in the EU for applications in food-producing species.

A wide range of BZ metabolites has been identified in rat, rabbit and man (Kataoka *et al*. 1971, 1973, Koppel and Tenczer 1985, Baldock *et al*. 1991). In rat, benzydamine *N*-oxide (BZ-NO) and glucuronide metabolites were found in urine

* Author for correspondence; e-mail: anfossi@vet.unibo.it

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and bile, whereas in rabbit the major metabolites were BZ-NO, a hydroxylbenzydamine derivative (BZ-OH) and its acetyl-, glucuronide and sulphate conjugates. In man, the major metabolites excreted in urine were BZ-NO, monodesmethyl-BZ (Nor-BZ), BZ-OH, the dedimethylaminopropyl-BZ, the desbenzyl-BZ and several glucuronides. Despite the use of BZ in food animals in the past and the knowledge that the drug undergoes a very complex metabolic fate in laboratory animals and man, there are no published data describing the metabolism of BZ in food species such as cattle. Establishing the extent to which the metabolite profile of the target species is representative of that evaluated in previous studies in laboratory animals provides the basis for determining whether further toxicity assessment is required for extension of licensing to a new food species. Furthermore, this provides an indication of the potential target residues for requisite pharmacokinetic and residues studies, etc.

Several *in vitro* models, including isolated hepatocytes and microsomes, are available for the assessment of xenobiotic metabolism. Recently, precision-cut liver slices have been utilized as an alternative method since the approach maintains tissue architecture and functional heterogeneity and provides a pathway for drug metabolism that is more consistent with *in vivo* metabolism than other models (Smith *et al.* 1987, Barr *et al.* 1991, Dogterom 1993, Miller *et al.* 1993, Saylers *et al.* 1994, George *et al.* 1999).

In vitro studies using rat liver and brain microsomes and hepatocytes from several species have demonstrated that the *N*-oxidation of BZ to BZ-NO is catalysed by the flavin-containing monooxygenases (FMO), whereas the *N*-demethylation of BZ to Nor-BZ involves cytochrome P450 monooxygenases (Kawaji *et al.* 1993, 1994, Ubeaud *et al.* 1999).

In vitro metabolism studies can be viewed as a means of readily enabling the generation of valuable qualitative comparative data as a basis for the initial assessment of equivalence with other target species and with model species used in toxicity studies, such as the rat. In addition, such preliminary data may better enable more effective planning of essential *in vivo* studies.

The biotransformation of BZ was investigated in the present study by using microsomes and precision-cut slices prepared from cattle liver to provide enzyme kinetic details on the production of BZ-NO and Nor-BZ as well as preliminary information on the likely pathways of BZ metabolism in the species. Metabolites were analysed by high-performance liquid chromatography (HPLC) with fluorimetric detection, and the structural identity of metabolites was investigated by mass spectrometry (MS).

Material and methods

Chemicals

Benzydamine hydrochloride (purity 99.8%), benzydamine *N*-oxide hydrochloride (97.3%) and norbenzydamine hydrochloride (99.7%) were supplied by Angelini Ricerche S.p.A. (Società per Azioni). NADPH and bovine serum albumin (BSA) were from Boehringer Mannheim Italia S.p.A. (Milan, Italy). Earl's balanced salt solution (EBSS) without phenol red, RPMI 1640 w/GLUTAMAX II with 25 mm HEPES and glutamine were supplied by Gibco BRL (Life Technologies, Paisley, UK). Methionine, hydrocortisone, insulin, foetal calf serum, gentamycin and amphoteracin B were supplied by Sigma Chemical Co. (St Louis, MO, USA). All other reagents, salts (analytical grade) and solvents (hplc grade) were purchased from Mallinchrodt Baker S.p.A. (Deventer, The Netherlands) and Carlo Erba (Milan, Italy).

Metabolism of BZ by liver microsomes

Samples of liver tissue (caudate lobe) from four healthy steers (550–600 kg b.w.) were collected at the slaughterhouse immediately *post mortem*. Samples of liver (~50 g) were cut into small pieces and washed three times with ice-cold 1.15% (w/v) KCl, suspended in 2 vols 0.1 M phosphate buffer (pH 7.4) and 1.15% (w/v) KCl solution (1:1 v/v) and homogenized in a Potter apparatus with a Teflon pestle (Heavy Stirrer, Apparecchi per Laboratori Chimici s.r.l., Milan, Italy). The homogenate was centrifuged at 9000*g* at 4°C for 20 min and the supernatant retained and further centrifuged at 105 000*g* for 60 min to obtain the microsomal fraction. The microsomes were suspended in 0.1 M phosphate buffer (pH 7.4):glycerol 80:20 (v/v) containing 0.1 M EDTA and stored under liquid N_2 pending biotransformation studies. Immediately before use, the microsomal fraction was thawed at 37°C and suspended in 0.1 M phosphate buffer (pH 7.4) or 0.1 M Tris-HCl buffer (pH 8.5) for evaluation of CYP- and FMO-catalysed reactions, respectively. The protein concentration of the microsomal preparation was determined using bovine serum albumin (BSA) as a calibration standard (Lowry *et al.* 1951). Total microsomal cytochrome P450 was determined by measuring the CO spectra of dithionite reduced P450 (Omura and Sato 1964).

Biotransformation was studied in triplicate by incubating 0.2 mg ml^{-1} microsomal protein, 1 mM NADPH, 2.5 mM $MgCl_2$, and substrate (BZ; concentrations from 2.5 to 1000 μM) in a final volume of 0.4 ml, with either 0.1 M phosphate buffer (pH 7.4) or 0.1 M Tris-HCl (pH 8.5) for 10 min at 37°C. Blank samples without NADPH or BZ were used to control for non-enzymatic transformations.

The effects of microsomal protein concentration and of incubation time on the formation of metabolites was evaluated as above over the range 0.1–1 mg ml^{-1} microsomal protein and from 5 to 30 min of incubation. The reactions were stopped by addition of acetonitrile (0.8 ml). After vortexing for 1 min, samples were centrifuged at 13 000*g* for 5 min (Microfuge E, Beckman, San Ramon, CA, USA), and the supernatant diluted from 1:1 to 1:20 v/v with the HPLC mobile phase.

To evaluate the contribution of CYP- and FMO-catalysed reactions, further assays were performed with substrate concentrations of 1.0 and 2.5 μM in a similar manner ± heat treatment (47°C for 3 min) to inactivate FMO (Ziegler 1988).

Metabolism of BZ by precision-cut liver slices

Samples of liver tissue were obtained from three healthy calves Angus/Friesian (cross steer, 7–10 months old) slaughtered by captive bolt and exsanguination. Livers were perfused within 10 min of slaughter with ice-cold Earl's balanced salt solution (EBSS, previously equilibrated with 95% O_2/5% CO_2) and transported to the laboratory on ice.

Cylindrical tissue cores were prepared using a 10-mm diameter motor-driven tissue-coring tool. Tissue slices (200–300 μm thick) were cut from the cylinders with a Krumdieck tissue slicer (Alabama Research and Development Corp., Munford, AL, USA) in oxygenated EBSS containing 25 mM glucose at 4°C. Liver slices were floated into 12-well plates (one slice per well) and incubated with culture medium (1 ml) employing a continuously submerged incubation system. The culture medium was prepared from RPMI 1640 medium containing 5% (v/v) foetal calf serum, 1 μM insulin, 0.5 mM L-methionine, 0.1 mM hydrocortisone-21-hemisuccinate and 50 μg ml^{-1} gentamycin (Coldham *et al.* 1995).

Culture plates were incubated at 37°C in a humidified atmosphere of 95% O_2/5% CO_2. After 30-min incubation, treatment was commenced by replacing the culture medium with fresh medium and a range of BZ concentrations (0, 100, 500, 1000 μM) was added to the appropriate wells.

Following incubations of 0, 2, 4, 6, 8 and 24 h, the medium was removed for analysis of metabolites. Liver slices were washed in 1.15% KCl, containing 50 mm Tris-HCl (1 ml), homogenized in this medium with a IKA-Labotechnic Ultra Turrax 25 and sonicated with an MSE Soniprep 150 Ultrasonic Disintegrator (MSE Scientific Instruments, Crawley, UK).

The liver slice homogenates and samples of culture medium were stored at −80°C before analysis. Whole homogenates of liver slices were assayed for protein content with BSA as calibration standard (Lowry *et al.* 1951). Samples of culture medium were diluted and analysed by HPLC and HPLC-MS.

HPLC analysis

HPLC consisted of a Jasco Pump PU 980 equipped with an LG 980 02 Ternary Gradient Unit and a GASSTORR GT-103 degasser, a Jasco Autosampler AS 950, and a Jasco fluorometric detector FP 920 (Japan Spectroscopic, Tokyo, Japan) set at 303 nm excitation and 350 nm emission.

Samples of microsomal supernatant and culture medium (20 μl) were chromatographed on a Prodigy ODS(3) column (5 μm; 250 × 4.6 mm i.d.) (Phenomenex, Torrance, CA, USA) using an isocratic binary mobile phase consisting of water 0.1% trifluoracetic acid (TFA):acetonitrile 0.082%TFA 65:35 (v/v) at a flow rate of 0.8 ml min^{-1}. Under these chromatographic conditions reference compounds eluted (retention time ± SD, min) in the order: Nor-BZ (11.8 ± 0.3), BZ (13.1 ± 0.3) and BZ-NO (15.7 ± 0.3).

Calibration curves of BZ, BZ-NO and Nor-BZ in the range 0.01−1 μm were prepared in the mobile phase starting from aqueous standard solutions (1 mM). The concentration of analyte in unknown samples was determined by interpolating from the appropriate calibration curve.

LC-MS analysis

Culture medium (20 µl) from precision-cut liver slices was analysed by LC-MS using an HP1050 HPLC system (Hewlett Packard, Bracknell, UK) and an ion-trap mass spectrometer (LCQ, Finnigan, Hemel Hempstead, UK) in series with a Shimatzu RF-535 fluorescence detector set at 303 nm excitation and 350 nm emission.

BZ metabolites were chromatographed on the same Prodigy RP column and eluted using a binary mixture of (A) water 2% formic acid:(B) acetonitrile 2% formic acid. Metabolites were eluted at flow rate of 0.8 ml min^{-1} with a linear gradient from 90% A to 65% A over 10 min, followed by an isocratic period at 65% A for 15 min.

Under these chromatographic conditions, the reference compounds were resolved (retention time ± SD, min) in the order Nor-BZ (16.4 ± 0.2), BZ (17.1 ± 0.3) and BZ-NO (18.2 ± 0.4).

MS was optimized by infusion of a BZ standard (1 µg ml^{-1}). Positive-ion electrospray provided the greatest sensitivity. The probe settings included a heated capillary temperature of 271.5°C and voltage of 23.6 V.

Mass spectra were collected in either full scan (m/z 100–550) or product ion modes, the latter being derived from the molecular ion $[M + H]^+$. MS experiments on BZ and metabolites were performed with CID energies of 30% and mass isolation width of 2 amu.

Analysis of BZ, Nor-BZ and BZ-NO fragmentation by MS was determined by direct infusion of 10 µM solutions into mobile phase at a flow rate of 10 µl min^{-1}.

Potential metabolites were investigated by selecting masses at m/z 236, 326, 342, 486, 488, 502 and 518 for further MS experiments (taking account of published literature and fragmentation pathways of BZ, Nor-BZ and BZ-NO), at elution times corresponding with peaks detected by fluorescence.

Results

Metabolism of BZ by liver microsomes

The content of cytochrome P450 in bovine microsomes was 0.61 ± 0.05 nmol mg^{-1} protein (mean ± SD, $n = 4$). After incubation of BZ with microsomes, three metabolites were observed in the supernatant (figure 1), the first unidentified product eluting at an earlier retention time (~9.9 min) than BZ (12.8 min) and two further metabolites co-eluting at retention times corresponding with

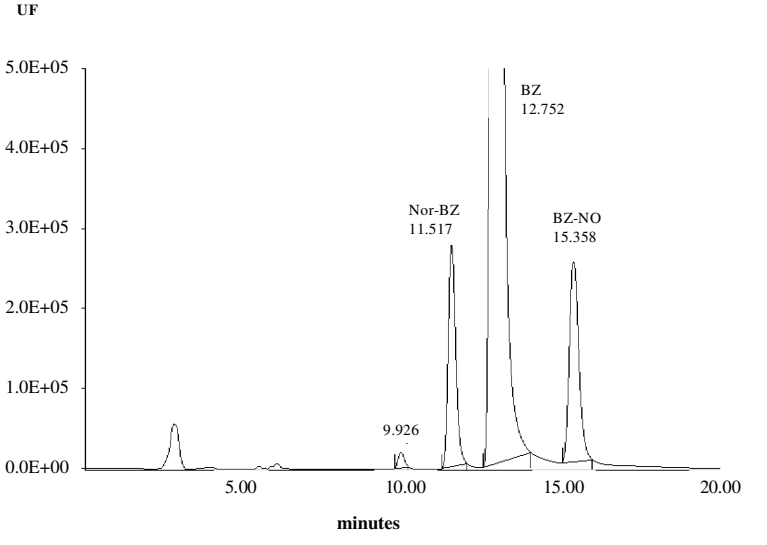

Figure 1. HPLC chromatogram obtained from cattle liver microsomes incubated with BZ (100 µm). The HPLC conditions were: Prodigy ODS(3) column (5 µm; 250 × 4.6 mm i.d.) (Phenomenex, Torrance, CA, USA); isocratic binary mobile phase consisting of water 0.1%TFA:acetonitrile 0.082%TFA 65:35 (v/v); flow rate 0.8 ml min^{-1}; fluorometric detection (303 nm excitation, 350 nm emission). UF, units of fluorescence.

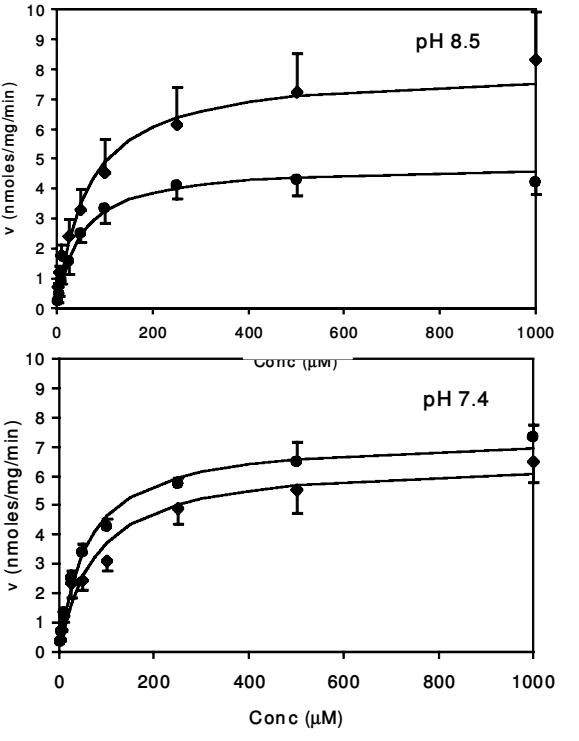

Figure 2. Production of Nor-BZ (●) and BZ-NO (◆) as a function of substrate concentration in cattle liver microsomes. Values are the mean ± SD of data derived from samples from four animals. A non-linear regression software (Fig.P 2.0, Biosoft, Cambridge, UK) was used to provide curves of best fit to data derived from the Michaelis–Menten equation.

Table 1. Kinetic parameters derived from the Michaelis–Menten equation for Nor-BZ and BZ-NO formation from BZ by cattle liver microsomes.

Product	K_m(μM)	V_{max} (nmol mg^{-1} min^{-1})
Nor-BZ		
pH 7.4	58.9 ± 6.4 (52.7–67.5)	7.4 ± 0.5 (6.9–7.9)
pH 8.5	46.7 ± 4.2 (41.2–51.4)	4.8 ± 0.5 (4.1–5.2)
BZ-NO		
pH 7.4	76.4 ± 16.0 (64.6–100.0)	6.5 ± 0.8 (5.6–7.5)
pH 8.5	63.6 ± 14.0 (46.8–79.6)	8.0 ± 1.6 (6.4–10.3)

Values are mean ± SD ($n = 4$) (range).

authentic Nor-BZ (11.5 min) and BZ-NO (15.4 min). The production of metabolites was linear up to 0.5 mg ml^{-1} of microsomal protein and for incubation times up to 15 min (data not shown).

The formation of Nor-BZ and BZ-NO followed Michaelis–Menten kinetics either when the incubations were performed at pH 8.5 or at pH 7.4 (figure 2). K_m and V_{max} (mean ± SD and ranges) are provided in table 1. The production of Nor-BZ and BZ-NO was greatest at pH 7.4 and 8.5, respectively, although the small number of animals weakened the statistical analysis.

The formation of Nor-BZ was not reduced by heat treatment of the microsomal protein, whereas that of BZ-NO decreased to 29–33% of the control samples.

Metabolism of BZ by precision-cut liver slices

Figure 3 shows HPLC chromatograms obtained from medium after incubation of precision-cut liver slices with BZ for 2, 6 and 24 h compared with a blank sample (liver slices incubated for 24 h). After 2 h of incubation, the main component was parent compound (95%), with only traces of Nor-BZ (1%) and BZ-NO (4%) evident. The percentage abundance of BZ-NO increased between 6 and 24 h

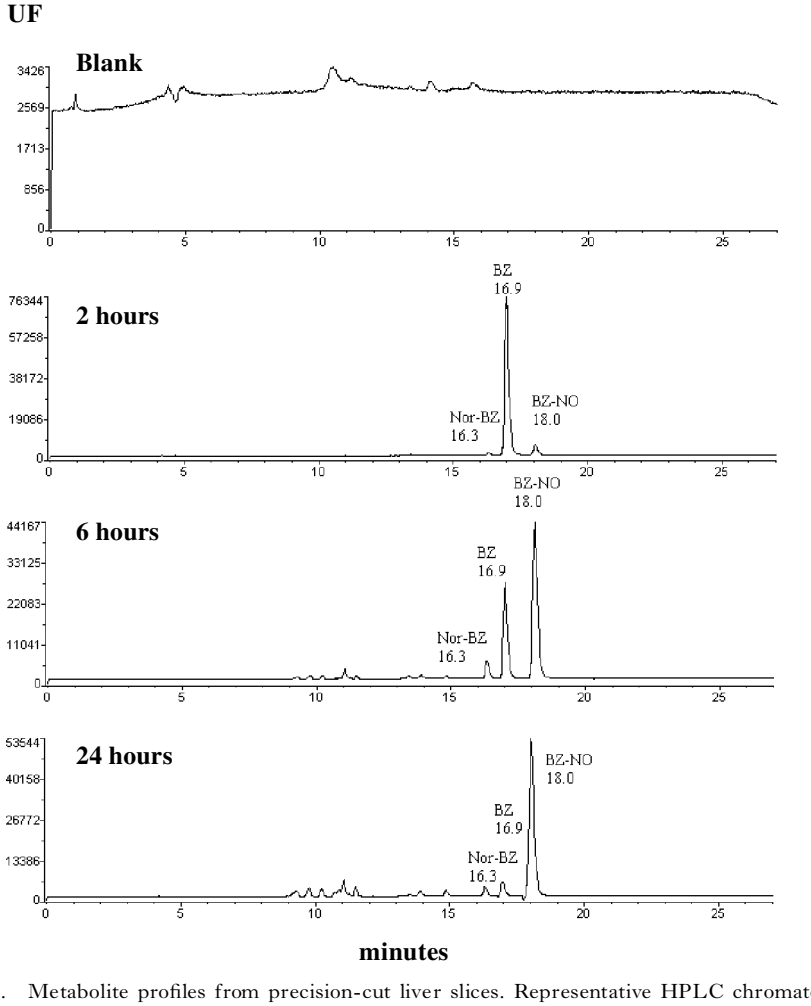

Figure 3. Metabolite profiles from precision-cut liver slices. Representative HPLC chromatograms have been selected to illustrate metabolites present in extracts of medium following incubation of precision-cut liver slices with BZ for 2, 6 and 24 h. The HPLC conditions were: Prodigy ODS(3) column (5 µm; 250 × 4.6 mm i.d.) (Phenomenex); elution by binary mixture of (A) water 2% formic acid; (B) acetonitrile 2% formic acid with a gradient from 90% A to 65% A over 10 min, followed by an isocratic period at 65% A for 15 min; flow rate of 0.8 ml min^{-1}; fluorometric detection (303 nm excitation, 350 nm emission). UF, units of fluorescence.

Table 2. Summary of major product ions formed following mass spectral analysis of BZ, Nor-BZ and BZ-NO $[M + H]^+$ parent ions (m/z 310, 296 and 326, respectively).

Compound	MS^n n	[Molecular ion], precursor ion (m/z)	Product ions (m/z)
BZ	2	[310]	265
	3	[310], 265	237, 187, 174*, 91
	4	[310], 265, 174	146*
	4	[310], 265, 237	209
Nor-BZ	2	[296]	265*, 225
	3	[296], 265	237, 209, 187, 174*
BZ-NO	2	[326]	265, 209, 102*
	3	[326], 265	237, 174*

* Base product ion in the spectra.

from 33 to 56%. In contrast, Nor-BZ production was maximal (5%) by 6 h. At 24 h several other peaks eluting before Nor-BZ were also evident.

LC-MS analysis of metabolites

Product ions generated from the molecular ions ($[M + H]^+$) of BZ (m/z 310), Nor-BZ (m/z 296) and BZ-NO (m/z 326) are summarized in table 2. A product ion of m/z 265 was evident in the spectrum of BZ, Nor-BZ and BZ-NO, whereas those at m/z 225 and 102 were only evident in the spectrum of Nor-BZ and BZ-NO, respectively.

Further LC-MS experiments were performed on ions that co-chromatographed with unidentified fluorescent entities found in the medium at 6- and 24-h incubations. Figure 4 shows selected ion chromatograms corresponding to the $[M + H]^+$ of eight putative metabolites (labelled M1–8 in order of elution) obtained from a representative 24-h medium sample; retention times, molecular and product ions for each are provided in table 3.

Two metabolites eluting at 13.3 and 17.9 min (M9 and BZ-NO respectively) were evident from the total ion current trace for the parent ion of m/z 326. The retention time and product ion spectrum of the latter were consistent with BZ-NO. The former metabolite was not detected by fluorescence but generated a single product ion of m/z 281, which was also evident as a product ion of other metabolites (table 3).

Discussion

The different roles of FMO and cytochrome P450 in the *N*-oxidation of BZ and *N*-demethylation of BZ, respectively, were previously established by Kawaji *et al.* (1993, 1994) using rat liver microsomes. From that model, K_m and V_{max} for the formation of Nor-BZ were produced that were ~50 times greater and 2000 times lower, respectively, than those for BZ-NO.

In the present study using liver microsomes from cattle, BZ-NO production increased at pH 8.5 and was sensitive to heat treatment, suggesting that FMOs may be largely responsible for catalysing this reaction. However, the closeness of

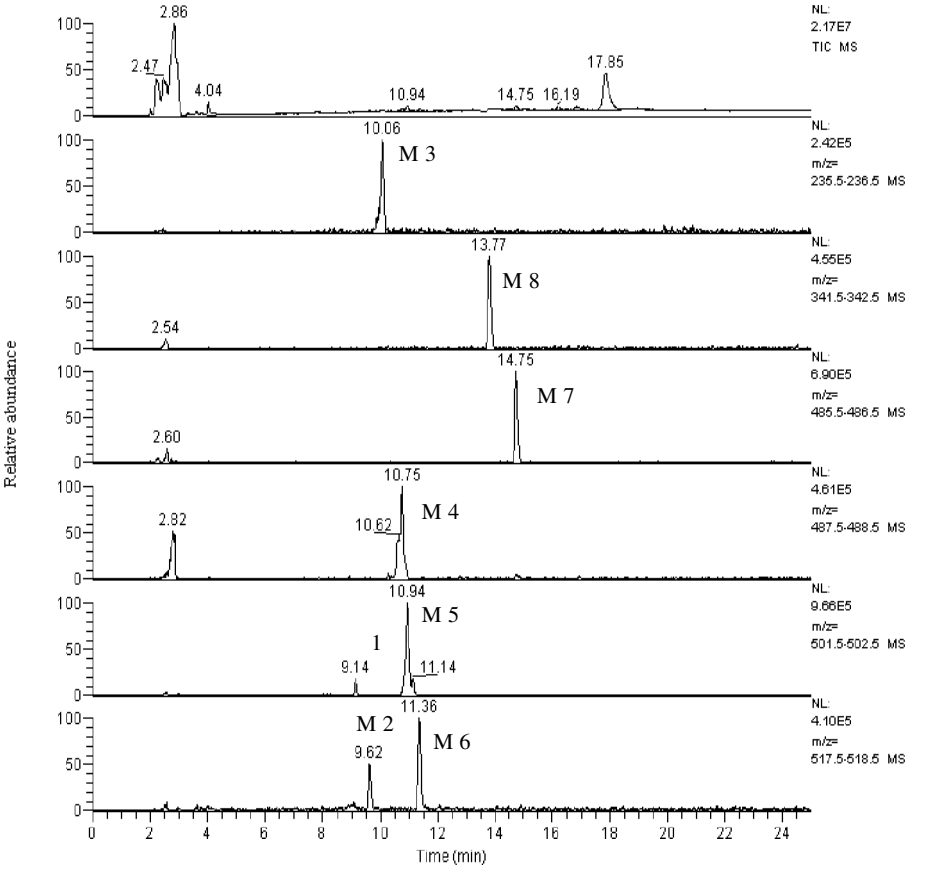

Figure 4. Identification of potential [M + H^+] molecular ions (*m/z* 236, 342, 486, 488, 502 and 518) of metabolites from precision-cut liver slices by LC-MS by extraction of ions from full-scan data. Precision-cut liver slices were incubated with BZ for 24 h and the incubation medium extracted and subjected to analysis by full scan (*m/z* 200–600) HPLC-MS.

Table 3. Fragmentation of precursor ions of metabolites found in the medium following incubation of precision-cut liver slices for 24 h.

Metabolite	Retention time (min)	Precursor ions (*m/z*)	MS^n *n*	Product ions (*m/z*)
M1	9.1	502 ($M + H^+$)	2	457*, 326, 281
		502, 457	3	281*, 191
M2	9.6	518 ($M + H^+$)	2	457*, 281
M3	10.1	236 ($M + H^+$)	2	175, 102*
M4	10.8	488 ($M + H^+$)	2	457, 312*, 281
		488, 312	3	281*, 175
M5	10.9	502 ($M + H^+$)	2	457, 326*, 281
		502, 457	3	281*, 175
M6	11.4	518 ($M + H^+$)	2	457, 342*, 281
M7	13.8	342 ($M + H^+$)	2	281, 102*
M8	14.7	486 ($M + H^+$)	2	441, 310, 265*
M9	13.3	326 ($M + H^+$)	2	281

* Base product ion in the MS spectra.

K_m and V_{max} of Nor-BZ and BZ-NO formation indicated the involvement of either the same enzyme or different enzymes with similar substrate affinity.

In addition to BZ, Nor-BZ and BZ-NO, only one further minor peak was evident in the HPLC chromatograms of microsomal incubations (figure 1). In contrast, chromatograms from the media of liver slices gave rise to several peaks that eluted before reference standards for BZ, Nor-BZ and BZ-NO (figure 3); these were present in sufficient quantity to enable direct identification by LC-MS analysis.

The concentration of BZ-NO in liver slice culture medium exhibited a striking increase from 2 to 24 h compared with the other metabolites, and this feature of BZ biotransformation could modify BZ residue kinetics. Back reduction to the parent compound is a mechanism already described for *N*-oxides and hydroxylamines in biological fluids (Bickel 1969, Kaibaf *et al*. 1992, Blake *et al*. 1995, Montesissa *et al*. 1998). Previous studies have demonstrated that BZ-NO can also form a reservoir of the parent compound, since it can be *N*-deoxygenated back to BZ, both *in vitro* by rat liver microsomes and *in vivo* after oral administration of BZ to rat (Kataoka *et al*. 1979a, b). *In vivo*, the reduction of *N*-oxidated metabolites is often related to the inability to detect these labile derivatives in biological fluids and to the increased half-life of their parent compounds.

The previously undetected BZ metabolites produced in cattle liver slices were tentatively identified as follows (figure 5): M1 (*m*/*z* 502), 1-benzyl-3-[3-(dimethylamino)propoxy]-1H-hydroxy-indazole N^+-glucuronide; M2 (*m*/*z* 518), 1-benzyl-3-[3-(dimethylamino)propoxy]-1H-hydroxy-indazole *N*-oxide N^+-glucuronide; M3 (*m*/*z* 236), 3-[3-(dimethylamino)propoxy]-1H-indazole *N*-oxide; M4 (*m*/*z* 488), 1-hydroxy-benzyl-3-[3-(methylamino)propoxy]-1H-indazole N^+-glucuronide; M5 (*m*/*z* 502), 1-hydroxy-benzyl-3-[3-(dimethylamino)propoxy]-1H-indazole N^+-glucuronide; M6 (*m*/*z* 518), 1-hydroxy-benzyl-3-[3-(dimethylamino)propoxy]-1H-indazole *N*-oxide N^+-glucuronide; M7 (*m*/*z* 342), hydroxylated analogue of BZ-NO; M8 (*m*/*z* 486), benzydamine N^+-glucuronide; and M9 (*m*/*z* 326), hydroxylated analogue of BZ. These products are presented in a proposed hepatic biotransformation pathway in figure 6.

Only three of the previously unidentified metabolites (M3, M7 and M9) (figures 5a and 6) found in the incubation medium of cattle liver slices were unconjugated compounds. The metabolite M3 (*m*/*z* 236) could be produced by either the loss of the benzyl group from BZ-NO or by debenzylation of BZ followed by an oxidation. Desbenzyl-BZ was first identified in rabbit urine by Kataoka *et al*. (1971) and subsequently in human urine by Koppel and Tenczer (1985).

The exact position of the hydroxyl group for the metabolites M7 (*m*/*z* 342) (figure 5a) and M9 (*m*/*z* 326) (figure 6) can not be assigned from the product ion spectra. However, Kataoka *et al*. (1971) described a hydroxy-benzyl derivative in rabbit urine with the site of hydroxylation at the 4-position of the benzyl ring, whereas Koppel and Tenczer (1985) suggested the 5-position of the indazole ring for a hydroxylated metabolite found in human urine.

The fragmentation patterns of the other metabolites (M1, M2, M4–6 and M8) were consistent with fragments formed from N^+-glucuronides. The peak for M8 (*m*/*z* 486) (table 3, figure 5b) gave rise to a product ion at *m*/*z* 441, which correspond to the sum of the major fragment of BZ (*m*/*z* 265, table 2) and glucuronic acid (*m*/*z* 176). This suggests that M8 is a novel N^+-glucuronide of

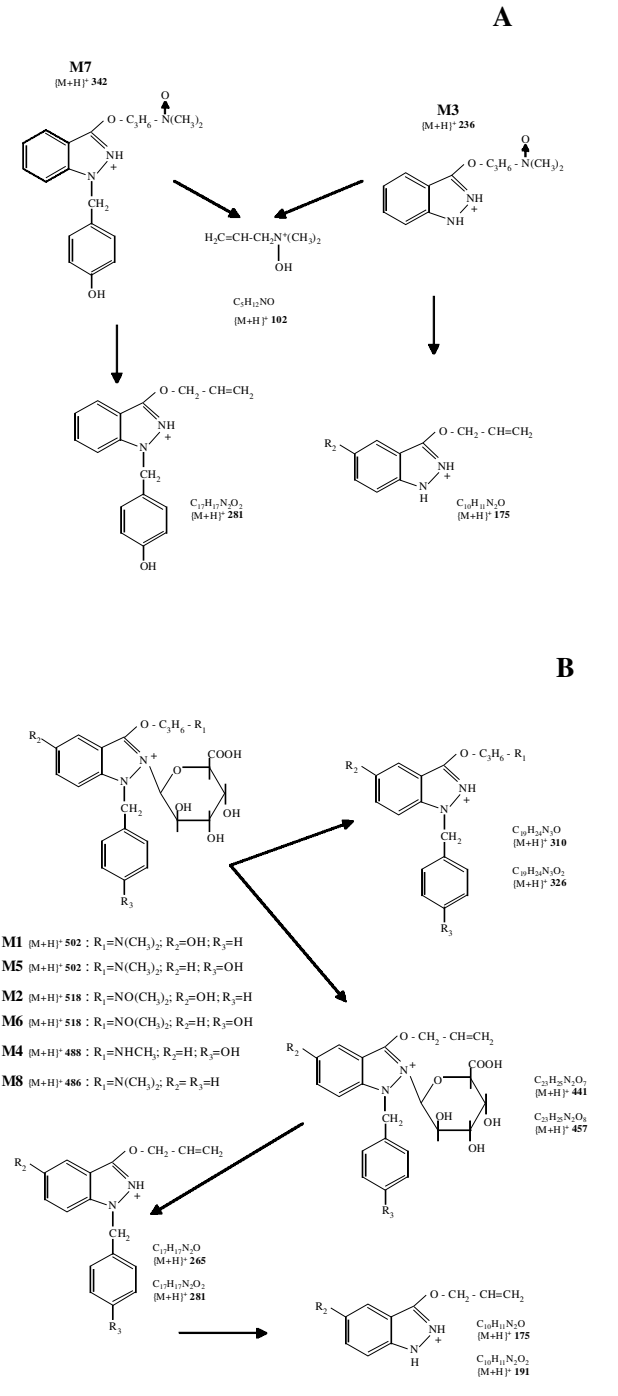

Figure 5. Proposed structures of precursor and product ions of BZ metabolites. Part A shows the unconjugated derivatives M3 (m/z 236, debenzyl BZ-NO) and M7 (m/z 342, hydroxylated BZ-NO). Part B shows the N^+-glucuronated derivatives M1 and M5 (m/z 502, hydroxylated BZ N^+-glucuronide), M2 and M6 (m/z 518, hydroxylated BZ-NO N^+-glucuronide), M4 (m/z 488, hydroxylated Nor-benzydamine N^+-glucuronide) and M8 (m/z 486, benzydamine N^+-glucuronide). The position of the hydroxyl group is suggested in every metabolite, but cannot be definitely stated based on product ion spectra.

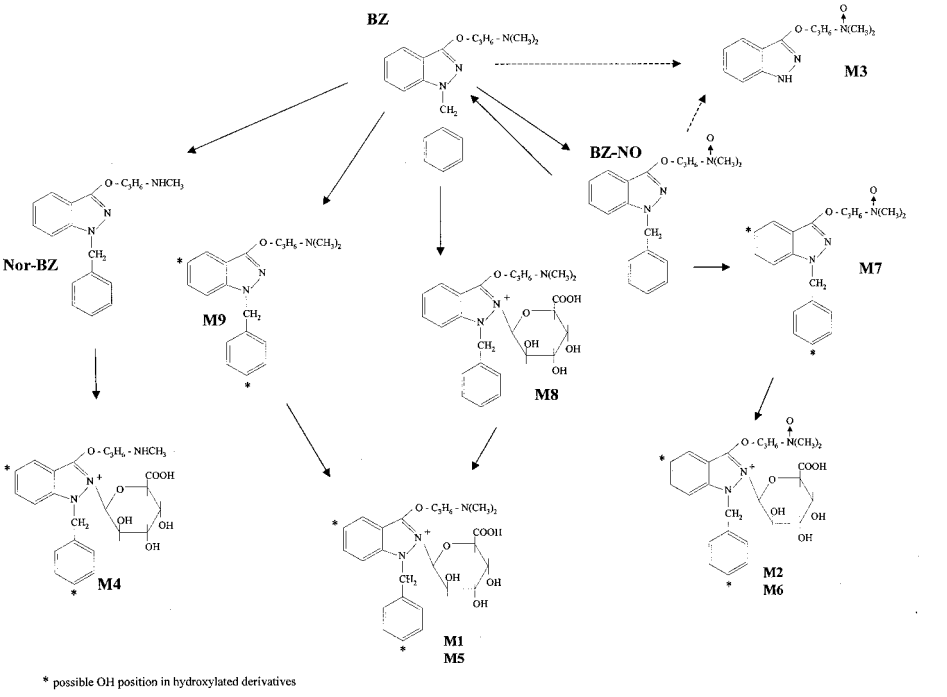

Figure 6. Proposed pathways for hepatic BZ metabolism in cattle.

BZ which to date has not been described in the literature. M4 (m/z 488), M1 and M5 (both m/z 502), M2 and M6 (both m/z 518) (table 3, figure 5b) generated a common fragment at m/z 457 which is an oxidated (+16 amu) fragment analogous to the one at m/z 441 originated from M8. The mass for the putative $[M + H]^+$ ion of M8 is consistent with benzydamine N^+-glucuronide; thus it is reasonable to propose analogous N^+-glucuronide structures for metabolites M1, M2 and M4–6 (figures 5b and 6). Maggs *et al*. (1997) reported that the *in vivo* glucuronidation of OH-carbamazepine (the main derivative of carbamazepine, an analgesic and anticonvulsant agent) gave rise to both *O*- and *N*-glucuronides. Their mass spectra have been compared and the product ion found to differ: the *O*-glucuronides (m/z 429) yielded only the aglycone fragment (m/z 253), whereas the *N*-glucuronide conjugate produced a fragment still bearing the glucuronic acid moiety (m/z 411). Thus, the ion at m/z 457 in the spectra of BZ metabolites M1, M2 and M4–6 is evidence of the *N*-conjugated moiety still bearing the glucuronic acid. This is confirmed in their MS spectra by the presence of a main fragment at m/z 281 corresponding to the loss of the glucuronic group. Further evidence for the N^+-glucuronide structural assignment was provided by metabolites such as M1/5 and M2/M6, which have identical masses but not HPLC retention times due to different molecular sites of hydroxylation and their polarities.

The unsaturated nitrogen of the imidazole moiety of BZ and BZ-OH is the most likely site for conjugation with glucuronic acid. In ring systems of aromatic tertiary amines, such as imidazole (where one ring nitrogen is substituted), pyridine, 1,2,4-triazine and polycyclic heterocycles, where N^+-glucuronidation

can occur at more than one site, conjugation has been observed to occur at the nitrogen with the less bulky substituent(s) (Hawes 1998).

The exact structures of the metabolites of BZ produced by precision-cut slices from cattle liver cannot be definitely stated without other analysis by NMR or synthesis of reference compounds. Nevertheless, the assignment of N^+-glucuronides at both the parent compound and its hydroxylated derivatives is strongly supported by the mass spectra and chromatographic data.

N^+-glucuronidation has been widely observed in man as a pathway in the Phase II metabolism of tricyclic drugs with an aliphatic tertiary amine group. Examples include imipramine, clomipramine, clozapine, chlorpromazine, trazodone, loxapine and trimipramine (Luo *et al.* 1995, Dain *et al.* 1997), but there are no reports in the literature describing N^+-glucuronidated products from hydroxylated BZ or reports on the ability of cattle to perform N^+-glucuronidation. Until now, only the rabbit, unlike the other laboratory species (dog, rat, cat) is reported to share this metabolic pathway with humans. In both species, N^+-glucuronides of ketotifen and cyproheptadine were produced during *in vivo* and *in vitro* studies, and N^+-glucuronides of tioconazole and croconazole were found in both rabbit and human urine (Hawes 1998).

As expected from published studies carried out in other animals and in man, the findings of the present study indicate that the metabolism of BZ is very complex in cattle. In the case of drugs adopted both for human and veterinary therapy, liver slices are an appropriate *in vitro* model to elucidate the metabolites to which humans can be re-exposed following consumption of animal tissues. To support the process of licensing of BZ in the bovine, and in food species in general, it is essential to define the composition of tissue residues after administration of the compound and to know whether the metabolites found in the target species are representative of those 'tested' in laboratory animals (as part of toxicity testing) and those found in humans. Glucuronidation is generally regarded as a means of detoxification and the clinical importance of N^+-glucuronides has not been thoroughly investigated. Nevertheless, a few studies described their effects on histamine release, as well as the competition for glucuronic substrate between tertiary amines and testosterone (Chiu and Huskey 1998).

The results of this study suggest that BZ metabolites in cattle are qualitatively different from those described in humans and laboratory animals, and further toxicity tests on metabolites in laboratory species should be required to enable marketing authorization of BZ for food species.

In view of the understanding of the mass balance of BZ in cattle and to highlight a pathway of detoxification that has never been reported in the bovine, the metabolism of BZ in cattle is worthy of further investigation.

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