Effect of Cooking on Diazepam and Its Metabolites in Liver of Bulls

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The stability of residues of diazepam and its metabolites (oxazepam, temazepam, and demethyldiazepam) in the liver of treated bulls when subjected to a heating procedure (100 °C in water for 1 h) was studied. Compounds were quantified by reversed-phase high-performance liquid chromatography using ultraviolet detection. The most unstable of the compounds studied was, under those conditions, oxazepam, which was degraded to approximately 50% of the original quantities. The other compounds were more stable and, after treatment, were present in 70–89% of the initial quantity.

INTRODUCTION

Diazepam is a tranquilizer used as a growth promoter and ansiolytic agent in various animal species. In the fattening of bulls, this drug has been shown to be of significant value in improving carcass weight (Bastos, 1988). In a previous study, the biotransformation of diazepam in bulls was evaluated, its major metabolites were identified, and all compounds were quantified in edible tissues after treatment of animals for 30 days and slaughtering at different times thereafter (Bastos, 1990).

As meat is usually submitted to heat treatment prior to human consumption, it is therefore important to evaluate the degree of alteration of drug residues. There are already some published papers on this subject, but the compounds studied are mainly antimicrobians (DePaolis et al., 1977; Honikel et al., 1978; Epstein et al., 1988; Hasset et al., 1990).

In the present study, we intend to evaluate the stability of diazepam, oxazepam, temazepam, and demethyldiazepam after boiling the liver samples of treated animals at 100 °C for 1 h. The liver was the sample assayed because it had been previously found to be the organ which had mainly accumulated those compounds.

EXPERIMENTAL PROCEDURES

Materials. In this study, the liver used was from six bulls orally treated with diazepam (0.3 mg/kg of body weight per day, for 30 days) and slaughtered 12 or 24 h after the administration of the drug was suspended. A 1-kg liver sample from each animal was minced and frozen at −40 °C in separate portions. The liver of an untreated animal was used to study the recovery of the drugs.

A standard solution of diazepam, oxazepam, temazepam, and demethyldiazepam (5 μg/mL each) was also used to study the stability of these compounds subjected to the same boiling procedure.

All chemicals were of analytical grade, from Merck (Lisbon, Portugal). Methanol used in liquid chromatography was Lichrosolv, Merck. Oxazepam and temazepam were kindly supplied by Wyeth Laboratories (Lisbon, Portugal) and diazepam and demethyldiazepam by Bial Laboratories (Oporto, Portugal). Clonazepam was supplied by Hoffmann-La Roche (Basel, Switzerland). β-Glucuronidase/arylsulfatase was purchased from Sigma Chemical Co. (St. Louis, MO, type H-2, activity, 130 000 Fishman units/mL.

Acetate buffer for enzymatic digestion was prepared by dissolving 4.102 g of anhydrous sodium acetate in exactly 1000 mL of water. The pH was ajusted with hydrochloric acid or sodium hydroxide to 5.5.

Apparatus. The sample homogenizer used was an Ultra-Turrax (Ika-Werk). A Varian 5000 liquid chromatograph with a variable UV detector and a Varian 4290 integrator was also used.

Processing. Two portions of each frozen liver sample (10 g each) were taken: one was directly submitted to enzymatic digestion for subsequent extraction of the drugs; the other was suspended in 50 mL of distilled water in a glass beaker and subjected to boiling for 1 h. After this treatment, the cooked product was also submitted to enzymatic digestion and subsequent extraction of the drugs.

One milliliter of a 5 μg/mL standard solution of diazepam and metabolites was added to 50 mL of water and heated in a similar procedure.

Enzymatic Digestion. Raw liver (10 g) was suspended in 50 mL of distilled water, homogeneized, diluted with 50 mL of acetate buffer (pH 5.5), and, after 0.2 mL of enzyme solution was added, agitated by rotatory movements in a water bath at 37 °C, during 24 h. The cooked liver, after homogenization in the broth water, was diluted in the same volume of acetate buffer, added with the enzyme, and submitted to the enzymatic procedure.

Recovery Study. To evaluate the recovery of the drugs, 1 mL of a 5 μg/mL standard solution (corresponding to 0.5 μg of each drug/g of liver) was added to 10 g of liver (n = 5) of an untreated animal. These spiked samples were submitted to the whole procedure (enzymatic digestion and extraction), and the final extracts were chromatographically analyzed. Results of the obtained recoveries and respective coefficients of variation are presented in Table III.

Analytical Methods. Diazepam and metabolites were quantified in liver extracts by high-performance liquid chromatography (Bastos, 1989). The compounds were extracted according to a similar previously published procedure (Bastos, 1990). With the objective of improving the extracts' purity and shortening the analysis time, some changes to the initial procedure were introduced. Briefly stated, after enzymatic digestion with β-glucuronidase in acetate buffer (pH 5.5), internal standard (1 mL of clonazepam solution, 5 μg/mL) was added to the liver suspension and was then alkalinized with 2 mL of a 25% ammonia solution. The drugs were extracted two times with 50 mL of chloroform, and the organic extracts were added together and evaporated to a reduced volume. The chloroform layer was partitioned with 2 × 1 mL of 5 M HCl. The acidic layer was washed with n-hexane and, after alkalinizing, was poured into an Extrelut[3] column and the drugs were extracted thoughout with 15 mL of diethyl ether. After drying, the residue was reconstituted with 1 mL of methanol. The internal standard used was clonazepam, which elutes just before oxazepam and is free of impurities.

The extraction of heated standard compounds was achieved by alkalinizing the aqueous solution with ammonia solution and extracting two times with 50 mL of chloroform. After drying, the residue was reconstituted with 1 mL of methanol.

Chromatographic Conditions. Separation of compounds was achieved with a Perkin-Elmer stainless steel C_{18} column (25 cm × 4.5 mm i.d.), 10 μm particle size, attached to an Alltech C_{18} precolumn, 30–40-μm particle size. The mobile phase used was a mixture of methanol/water (68 + 32) with a flux of 0.7 mL/min. The wavelength detector was set at 228 nm.

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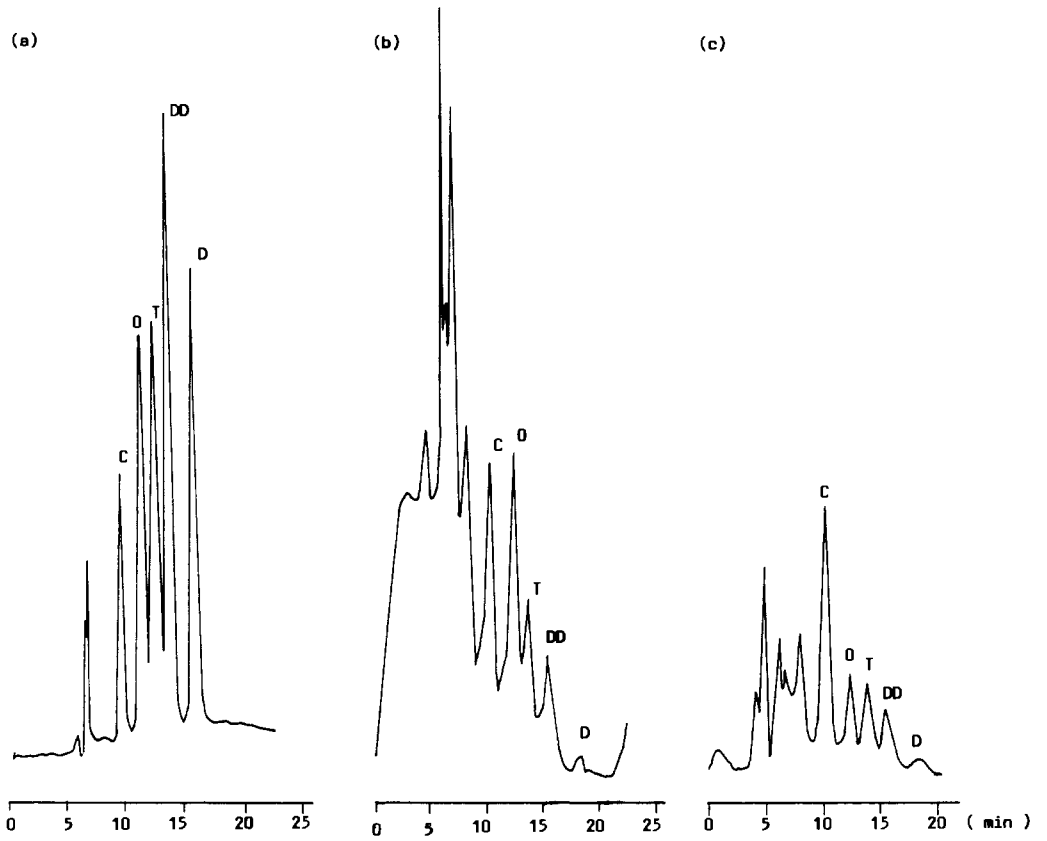

Figure 1. Typical chromatograms of diazepam and metabolites: (a) standard solution (100 ng each); (b) organic extract of bull liver treated with diazepam, without heating procedure; (c) organic extract of bull liver treated with diazepam and heated in water at 100 °C for 1 h. C, clonazepam (internal standard); O, oxazepam; T, temazepam; DD, demethyldiazepam; D, diazepam.

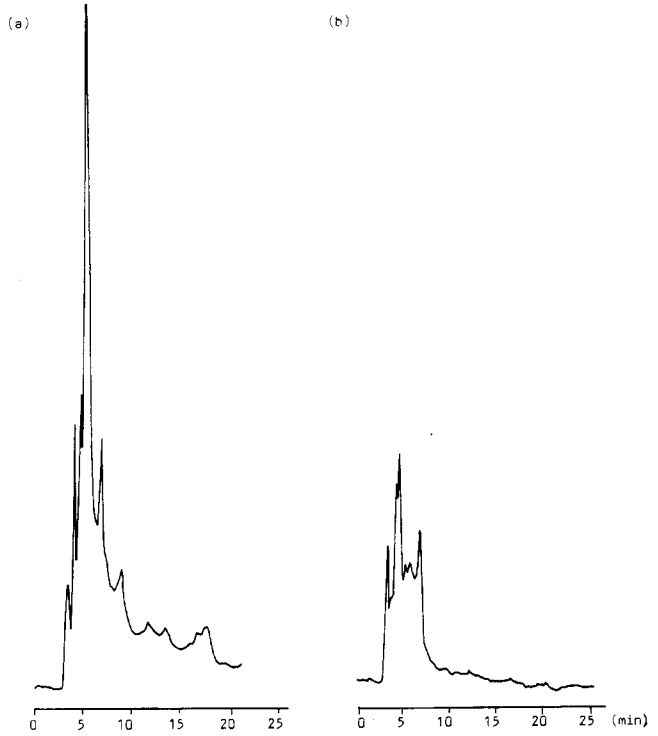

Figure 2. Chromatograms of blank liver extracts: (a) without heating procedure; (b) after heating procedure.

RESULTS AND DISCUSSION

Figure 1 presents the chromatogram of a standard solution of the compounds (a), of an extract of liver without heating (b), and of an extract of liver after boiling for 1 h (c). Figure 2 presents the chromatograms of blank liver extracts without the heating procedure (a) and after the heating procedure (b); no interfering peaks at the retention times of the benzodiazepines were observed in control liver extracts in both cases, especially relating to diazepam metabolites, which are the drugs mainly present in the analyzed organ.

Table I. Effect of Heat (100 °C, 1 h) on Diazepam and Metabolites in Liver of Bulls

drug	residue,[a] μg/100 g before heating	residue,[a] μg/100 g after heating	% stable to heat	CV, %
diazepam	4.0 (0–8.6)[b]	3.6	89	15
oxazepam	85.6 (21.6–217)[b]	43.6	51	17
temazepam	15.3 (0–34.3)[b]	12.4	81	11
demethyldiazepam	28.0 (4.7–50.0)[b]	19.6	70	14

[a] Mean value of six analyzed liver samples. [b] Value intervals of residue drugs found in six analyzed liver samples.

The changes introduced in the extraction procedure improved the extracts' purity, making possible the separation of all drugs in a reduced-time analysis, free of endogenous interferences.

After chronic oral treatment of bulls with diazepam, it had already been found that oxazepam is the principal drug in liver (Bastos, 1990); this metabolite is also the compound most altered after the boiling procedure (100 °C, 1 h), but it remains approximately 50% stable. The other metabolites are more stable, and results are presented in Table I. Diazepam is also stable to heat but, as can be seen in Table I, in some samples it was impossible to detect before the boiling procedure. It was the drug present in the lowest quantity in all samples.

Table II. Effect of Heat (100 °C, 1 h) on Standard Solution of Diazepam and Metabolites (5 μg/mL)

drug	found,[a] μg/mL	% stable to heat	CV, %
diazepam	5.35	107	4
oxazepam	0.20	4	12
temazepam	4.85	97	8
demethyldiazepam	5.30	106	2

[a] Mean of six assayed samples.

Table III. Recovery of Drugs from Spiked Blank Liver with 0.5 μg/g of Each Drug

drug	found,[a] μg/g	mean rec, %	CV, %
diazepam	0.495	99	13
oxazepam	0.405	81	13
temazepam	0.440	88	9
demethyldiazepam	0.465	93	6

[a] Mean of five assayed samples.

When we heated a standard solution of the drugs in water under the same conditions, oxazepam, more unstable than when it occurs in liver, is almost completely destroyed. The other metabolites and diazepam are very stable and not affected by the treatment (Table II). The greater stability of oxazepam in liver may be related to the linkage of the compound to endogenous molecules such as glucuronic acid or proteins.

The instability of oxazepam to heat has already been referred to when it was analyzed by gas chromatography at a temperature of 200 °C (Frigerio et al., 1973). After this treatment, the author verified the stability of diazepam, demethyldiazepam, and temazepam and the degradation of the structure of oxazepam. In the present study, although at a lower temperature, the alteration of oxazepam is total when the standard aqueous solution is heated.

The method of boiling (100 °C for 1 h) is a drastic one compared to some procedures like frying or broiling; the time of heating is also long, compared to other stability studies made on antibiotics (Katz et al., 1978). In normal cooking procedures, perhaps the quantities of stable residues of these drugs will be greater; therefore, the exposure of consumers to them will be greater.

Previously published results on this subject show that, under the same assay conditions, heat stability of different veterinary drug residues is variable, depending on the structure of the compounds (Moats, 1988). Since meat is normally consumed in the cooked state, it is advisable that residues of veterinary drugs be evaluated after heating by planned protocol treatments.

The samples used in this study were minced liver, the exposure of drug residues to heat being direct; in the usual household procedures, residues of the inner part of tough cuts of liver will be more protected and the destruction by heat will be less.

ACKNOWLEDGMENT

We thank INIC (Project 89/SAD/4) for the financial support of this study.

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Received for review November 21, 1991. Revised manuscript received March 26, 1992. Accepted March 8, 1993.