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Analysis of Fenbendazole Residues in Bovine Milk by ELISA

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Fenbendazole residues in bovine milk were analyzed by ELISAs using two monoclonal antibodies. One monoclonal antibody (MAb 587) bound the major benzimidazole anthelmintic drugs, including fenbendazole, oxfendazole, and fenbendazole sulfone. The other (MAb 591) was more specific for fenbendazole, with 13% cross-reactivity with the sulfone and no significant binding to the sulfoxide metabolite. The limit of detection of the ELISA method in the milk matrix was 7 ppb for MAb 587 and 3 ppb for MAb 591. Fenbendazole was administered in feed, drench, and paste form to three groups of dairy cattle. Milk was collected immediately before dosing and then every 12 h for 5 days. The ELISA indicated that residue levels varied widely among individual cows in each group. Fenbendazole levels peaked at ∼12−24 h and declined rapidly thereafter. Metabolites were detected at much higher levels than the parent compound, peaked at ∼24−36 h, and declined gradually. Residue levels were undetectable by 72 h. The ELISA data correlated well with the total residues determined by chromatographic analysis, but the use of the two separate ELISAs did not afford an advantage over ELISA with the single, broadly reactive MAb 587. The ELISA method could be used to flag high-residue samples in on-site monitoring of fenbendazole in milk and is a potential tool for studying drug pharmacokinetics.

KEYWORDS: Fenbendazole; oxfendazole; milk; ELISA; monoclonal antibody

INTRODUCTION

Fenbendazole (FBZ) is a broad-spectrum benzimidazole anthelmintic (*1*, *2*) with approved uses in four species of food animals: cattle, swine, goats, and turkeys. The Joint Food and Agricultural Organization−World Health Organization Expert Committee on Food Additives (JECFA) has recommended a single tolerance for FBZ; oxfendazole (OFZ, fenbendazole sulfoxide); and the prodrug, febantel, of 100 ppb in milk, expressed as the sum of FBZ, OFZ, and fenbendazole sulfone ($FBZSO_2$; (*3*)). In the United States, the Food and Drug Administration has established a tolerance level of 600 ppb for the marker residue, $FBZSO_2$, in bovine milk (*4*).

Although methods for FBZ residue analysis in milk by liquid chromatography (LC) have been described (*5*−*8*), rapid screening techniques, such as ELISA methodology, have not been applied. In this study, we used two ELISA methods. One was developed in our laboratory for multiresidue analysis of benzimidazoles in bovine liver (*9*); the other ELISA is more specific for the parent drug molecule, fenbendazole (Brandon et al., submitted for publication). On the basis of these methods, we present data on the time course for the appearance and decline of residues of FBZ in bovine milk and compare the results to those obtained by matrix solid-phase dispersion followed by LC analysis (*10*).

MATERIALS AND METHODS

Chemicals and Hapten Conjugates. Compounds were obtained as described previously (*9*, *11*). Hapten I (*9*) and hapten II (see **Table 1**) and their conjugates to bovine serum albumin (BSA) and horseradish peroxidase (HRP) are described elsewhere. The enzyme conjugates were stored at 1 mg/mL in a solution containing 10 mg/mL BSA and 0.02% thimerosal as preservative.

Competitive ELISA. *Assay Plates.* Assays were conducted in polystyrene wells (Immulon II 96-well plates, Dynatech, Chantilly, VA) coated with 100 μL of monoclonal mouse IgG from Clone 587 or Clone 591 at 5 μg/mL (4 h; all incubations conducted with shaking at room temperature, 20−25 °C). Wells were washed five times with distilled water, and the remaining protein-binding sites were blocked by incubation for 1 h with a solution of 10 mg/mL BSA in phosphate-buffered saline (PBS) containing Tween-20 detergent (PBS−Tween; 0.15 M NaCl, 5 mM sodium phosphate, 0.05% Tween-20, pH 7.0). This blocking solution (PBS−Tween + BSA) also contained 0.01% thimerosal as preservative. Plates were incubated with 2% sucrose in water for 30 min, then drained and dried at 37 °C for 1 h. Plates were stored desiccated at 4 °C for up to 6 months.

Assay Procedure. Dilution series of milk samples (1:2 to 1:256) were prepared in PBS−Tween + BSA unless otherwise noted. Standard solutions of FBZ and OFZ were prepared as 10 ppm in dimethylfor-

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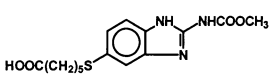
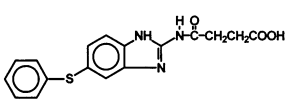
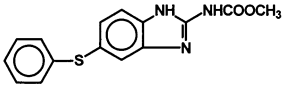
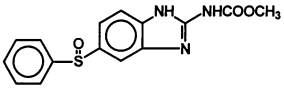
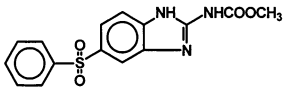
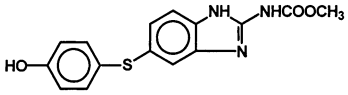
Table 1. Haptens and Specificity of MAbs in ELISAs for Fenbendazole and Metabolites

Compound	Antibody 587	Antibody 591
	 Hapten I	 Hapten II
	IC_{50} (ppb)	IC_{50} (ppb)
 Fenbendazole	3.8	2.5
 Oxfendazole (Fenbendazole sulfoxide)	0.62	420
 Fenbendazole sulfone	8.3	19
 4'-Hydroxyfenbendazole	5.3	16

mamide and stored at 4 °C. They were diluted in PBS−Tween + BSA to 100 ppb and then in a 5-fold dilution series to cover the working range of the assay (0.16−100 ppb). Diluted samples or standard solutions (50 μL/well) and HRP conjugate dissolved in PBS−Tween + BSA (50 μL/well) were added sequentially to the assay wells and then mixed. After incubation for 1 h, assay wells were washed and rinsed as described above, and bound HRP conjugate was detected by reaction with substrate, as described previously (*9*). In the MAb 587 ELISA, the HRP conjugate of hapten I was employed at 0.3 μg/mL. In the FBZ-specific MAb 591 ELISA, the HRP conjugate of hapten II was used at 0.08 μg/mL.

Incurred Residue Samples. The samples were produced for a study (School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA) that was previously described (*10*). In brief, fenbendazole was administered to three groups of 10 cows as a feed top dressing, a drench, or as a paste, with a target dose of 5.0 mg/kg. Each group also contained an undosed control. Milk samples were collected at the time of dosing (0 h) and at 12 h intervals for 72 h and were frozen until analyzed by LC (*5*). Samples were refrozen and maintained frozen until analyzed by ELISA at the Agricultural Research Service, Albany, CA.

ELISA Analysis of Milk Containing Incurred Residues of FBZ. To prevent bias, all of the samples were analyzed without decoding the sample labels. Samples were thawed at room temperature and mixed by shaking immediately before analysis. Serial dilutions (1:2 to 1:256) were prepared using a Biomek 1000 workstation (Beckman Instruments, Fullerton, CA). A FBZ standard was also diluted robotically. The same diluted samples and standard were applied to each of the two assay plates, and the assay was then conducted as described above. To analyze the data, FBZ was determined from the ELISA using MAb 591. Because MAb 587 measures a group of compounds, the ELISA result was expressed as "OFZ equivalents." To estimate the concentration of OFZ that would give the equivalent ELISA value determined using the FBZ standard, the calculated FBZ concentration was divided by a factor: (50% inhibitory concentration [IC_{50}] for FBZ/IC_{50} for OFZ) = (3.8/0.62) = 6.1. This calculation assumed that other metabolites of fenbendazole made a relatively small contribution to the ELISA value. Because antibody 587 binds fenbendazole sulfone weakly (7.5% cross-reactivity relative to OFZ), the assumption seemed reasonable.

Statistical Analysis. Correlation coefficients, fitted curves, and limits of detection were computed using Excel (Microsoft, Bellingham, WA), SlideWrite Plus (Advanced Graphics Software, Encinitas, CA), and Statgraphics Plus (Manugistics, Rockville, MD), respectively. A "measurement error model" (*12*) was used to define the relationship between ELISA and LC measurements. Both variables were expressed as log to stabilize their variances, which increased with larger numerical readings, and to linearize the relationship. Each method of application (feed, drench, paste) was handled separately after determining that differences existed among them. Initial estimates of variances and

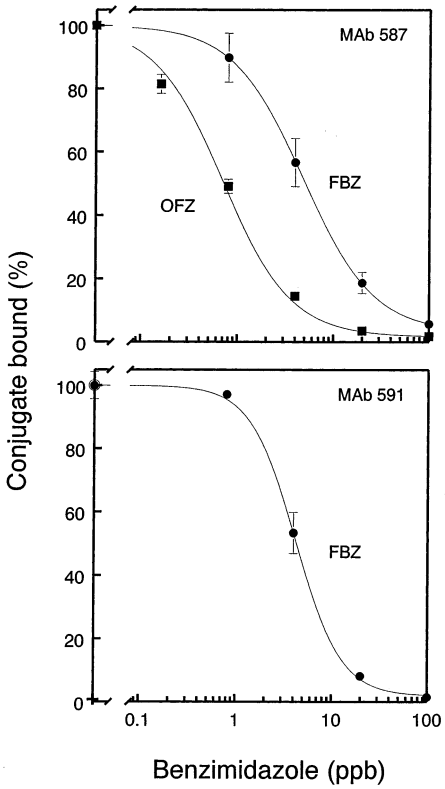


Figure 1. Standard curves for ELISA with antibody 587 and antibody 591.

covariances, obtained using SAS PROC MIXED with cows as a random factor and PROC CORR (*13*), were supplied to the computer program EV CARP (Statistical Laboratory, Iowa State University, Ames, IA). A specialized computer program was written that used regression coefficients and their covariances and estimated mean square error output from EV CARP to obtain confidence intervals for values of interest.

RESULTS AND DISCUSSION

ELISA. Typical standard curves for the competitive ELISAs are shown in **Figure 1**. The working range of the assays (defined operationally as the IC_{80} to IC_{20}) for fenbendazole is ~1−20 ppb for MAb 587 and 2−10 ppb for MAb 591. A comparison between specificities of the two ELISAs for fenbendazole and its metabolites is shown in **Table 1**. MAb 587 recognizes common features of the methyl benzimidazolecarbamates, with reduced binding to the bulkier substituents present on $FBZSO_2$ and 4′-OH-FBZ. MAb 591 is much more selective for FBZ, although there is significant binding to the hydroxy and sulfone metabolites.

Limits of Detection and Recovery of FBZ and OFZ. The lower limit of detection (LLD), defined by the 95% one-sided lower confidence limit, was determined for fenbendazole in both ELISAs and for oxfendazole using Ab 587. Whole milk samples were spiked with known amounts of analyte then diluted for analysis. **Table 2** shows that these assays detected less than 10 ppb with high recovery rates. Qualitative results could be obtained without the aid of a microplate reader, with a lower limit of detection of about 20 ppb FBZ.

Matrix Effects. To determine whether milk interfered with the assay, oxfendazole standards were prepared and diluted in buffer, raw milk, homogenized milk, or nonfat milk. Standard curves were obtained, and three representative curves are shown in **Figure 2**. The curve obtained using homogenized milk as

Table 2. Limits of Detection and Recovery of Fenbendazole in Milk

	antibody 587		antibody 591	
compd	LLD (ppb)	% recovery (mean ± SD)	LLD (ppb)	% recovery (mean ± SD)
fenbendazole[a]	7	95 ± 23	3	95 ± 25
oxfendazole[b]	3	122 ± 23	n.d.[c]	n.d.

[a] Recoveries determined at 10, 20, 50, and 100 ppb ($n = 4$). [b] Recoveries determined at 5, 10, and 20 ppb ($n = 3$). [c] Not determined; MAb 591 binds OFZ weakly.

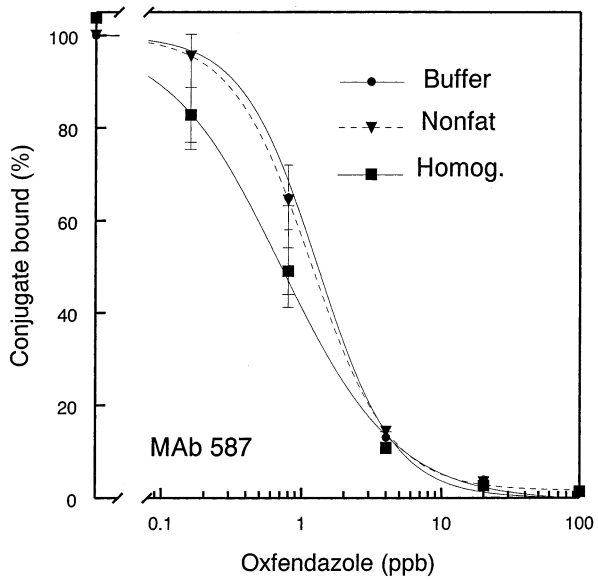


Figure 2. Milk matrix effects in analysis of oxfendazole using antibody 587. The oxfendazole standard was diluted in buffer, nonfat milk, or homogenized milk and analyzed by ELISA.

diluent had a significant matrix effect, with the standard curve shifted to the left. The results obtained for the raw milk matrix (data not shown), were identical to those for homogenized milk. This result suggests that fat caused the observed matrix effect. Dilution of milk samples at least 10-fold was sufficient to eliminate significant matrix effects with either antibody.

Analysis of Milk from FBZ-Treated Cattle. The data in **Figure 3** show the average values obtained from the ELISA data. There was considerable variation among the animals of each of the groups. For the "feed" and "paste" groups, the highest level of FBZ appeared in the first milk sample after dosing (12 h), and the highest level of OFZ equivalents was found in the 24 h samples. In the "drench" group, the highest levels of both analytes were measured in the 24 h sample. At all times after the 12 h time point, the residue comprises 75% or more metabolites of FBZ. In all three sets, the residue levels are near background by 72 h. **Figure 4** shows the results for two representative animals. The residue kinetics are similar to the averages, with OFZ equivalents far exceeding the FBZ ELISA value and the highest levels of residues measured with MAb 587 occurring one sample (12 h) later than the peak FBZ levels (MAb 591 ELISA).

Correlation of ELISA and LC Data. Figure 5 shows the correlation between ELISA and LC results, on the basis of the analysis of samples from all 30 animals. **Figure 5A** shows individual data points ($r = 0.80$); **Figure 5B** shows data averaged for each time point ($r = 0.98$). **Table 3** summarizes the correlation coefficients obtained in this manner for various combinations of LC and ELISA data. The correlations using

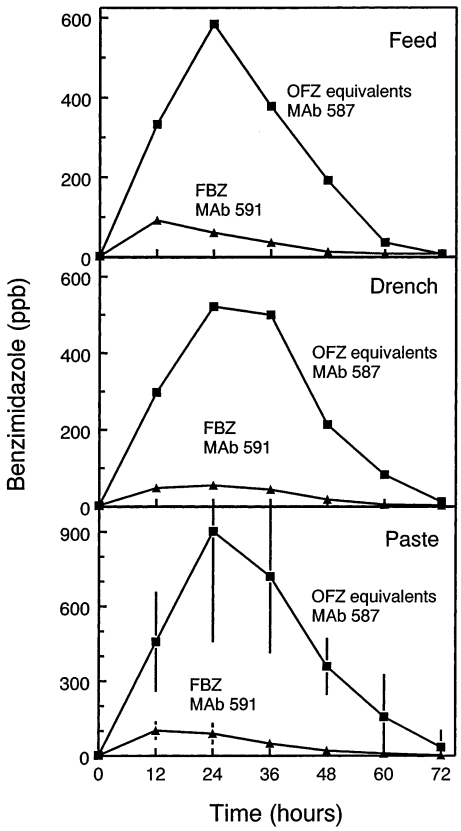


Figure 3. Analysis of milk from cows dosed with three formulations of fenbendazole (feed, drench, paste). Data are averages of analyses of 10 animals. FBZ values were computed from the ELISA using MAb 591; OFZ equivalents, from the ELISA using MAb 587. Cow-to-cow variation was similar for the three groups, but for clarity, error bars are shown only for the paste group.

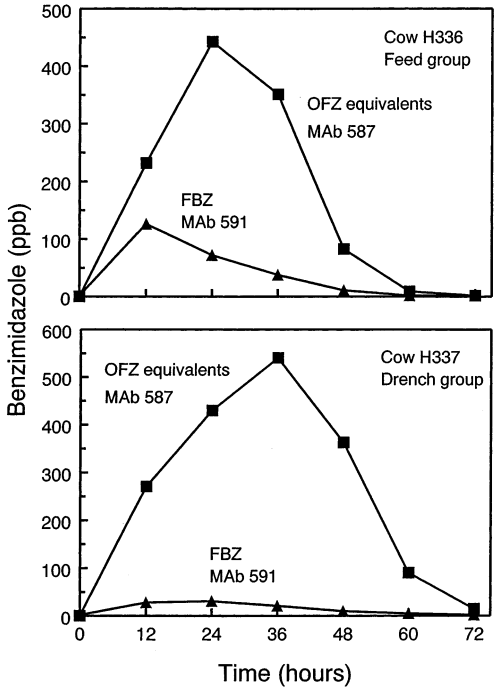


Figure 4. Typical analysis of milk from individual cows dosed with fenbendazole as medicated feed (upper panel) or drench (lower panel).

results calculated from the two ELISAs were nearly identical to those using only the MAb 587 ELISA. Therefore, a single

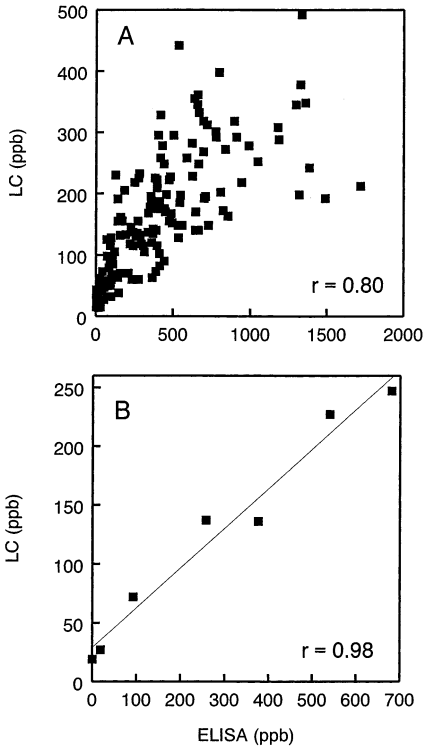


Figure 5. Correlation of LC and ELISA data. Residues were calculated for each method (LC: FBZ + OFZ + $FBZSO_2$; ELISA: MAb 587 assay, calculated as OFZ equivalents). (A) Available data for all 30 animals at all time points are plotted ($n = 207$). (B) Data for each time point are averaged and the fitted straight line is plotted.

Table 3. Correlation between ELISA and LC Data

data selected			corr coeff (r) groups			
ELISA	LC analysis	data type[a]	feed	drench	paste	all
total[b]	total[c]	individual	0.83	0.81	0.82	0.81
total	total	mean	0.96	0.99	0.96	0.97
MAb 587	total	individual	0.82	0.80	0.82	0.80
MAb 587	total	mean	0.97	0.99	0.97	0.98
metabolites[d]	metabolites[e]	individual	0.82	0.80	0.79	0.79
metabolites	metabolites	mean	0.97	0.99	0.96	0.98

[a] Values from individual animals or mean values for each time point. For mean data, $n = 7$ for each group, $n = 21$ for all groups. For individual points, $n = 68$ for feed group, $n = 69$ for drench group, and $n = 70$ for paste group, $n = 207$ for all groups. [b] ELISA (MAb 591) + [ELISA (MAb 587) − ELISA (MAb 591)]/6.1. [c] FBZ + OFZ + $FBZSO_2$. [d] [ELISA (MAb 587) − ELISA (MAb 591)]/6.1. [e] OFZ + $FBZSO_2$.

MAb would be the preferred ELISA method for practical use. Because measurement errors exist in both the ELISA and LC analyses, a measurement error model (*12*) was applied to the data. The results of this analysis, using only nonzero points and eliminating outliers, are illustrated in **Figure 6**.

CONCLUSIONS

The ELISA results agree with those of previous studies of the pharmacokinetics of FBZ in cattle (*10*, *14*, *15*), which reported peak levels of FBZ in blood at 28−30 h after administration and a half-life of 15 h. In a study of lactating cows (*16*), FBZ administered as a drench (10 mg/kg body weight) resulted in peak milk residue levels at the second and third milkings, dropping to <10 ppb within one week. A second study (*17*), with FBZ administered as a drench or in feed, found

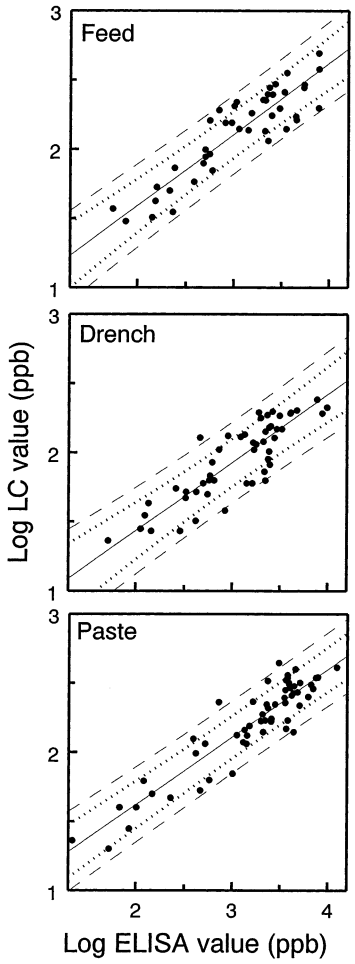


Figure 6. Relationship of LC and ELISA data using measurement error model. Outliers and zero data were eliminated. LC (FBZ + OFZ + $FBZSO_2$) and ELISA results (OFZ equivalents from MAb 587 assay) are plotted separately for the three treatments (feed, $n = 43$; drench, $n = 51$; paste, $n = 58$). The prediction equation and the 95% confidence interval for a single observation (dashed lines) and for the mean of three observations (dotted lines) are plotted.

that FBZ peaked at 24−32 h after administration. Large variations were noted between animals, but the oxfendazole level generally exceeded the FBZ level. Residues became undetectable after 4 d. Both the study of [^{14}C]FBZ depletion (*15*) and the LC study of FBZ residues (*10*) concluded that residues of FBZ become undetectable close to 72 h. The present ELISA results show a similar time course.

In addition to the regulatory concern about residues occurring in milk, the use of veterinary drugs in dairy cattle raises the issue of transfer of drug from cow to calf. In the case of fenbendazole, a study has shown that FBZ is transferred from treated cows to their calves (*18*); however, it is not clear whether the oxidized metabolites found in calf tissues were transferred to the calf or were formed by the metabolism of transferred FBZ. The levels did not appear to be of regulatory concern, but if desired, could be monitored in calf liver by the MAb 587 ELISA (*9*).

The monoclonal antibody-based ELISAs described in this report provide a simple means of evaluating fenbendazole residues in milk. The MAb 587 ELISA offers sensitivity in the order OFZ > FBZ > $FBZSO_2$ (**Table 1**), an appropriate specificity for detecting the major detectable polar metabolite, oxfendazole. No sample preparation is involved, and only aqueous buffers are used. In its present form, the test requires minimal equipment, and it could readily be expedited by formatting it as a tube or membrane assay. Such a method could be used as a "cowside" test or for checking tanker loads of milk. Our experience is that FBZ residues as low as 20 ppb can be readily assessed as positive by eye. The sensitivity could be adjusted by use of a suitable dilution to achieve the desired cutoff.

There was a substantial bias in the ELISA data compared to the LC, with the ELISA values consistently higher. Some of the bias could be due to matrix effects in samples assayed at relatively low dilution (e.g., 1:4−1:8). In addition, some of the discrepancy could have resulted from protein-bound residues that might not have been detected by LC. For example, protein-bound residues form when cultured hepatocytes metabolize thiabendazole, a related benzimidazole anthelmintic (*19*).

Although there were quantitative differences between ELISA and LC results, the data in this paper (**Figures 5** and **6**) show that the ELISA could be used as a broad indicator of residue levels or to flag high samples. Averaging several samples (e.g., dotted lines, **Figure 6**) could improve assay utility, in view of the wide confidence intervals based on individual samples (dashed lines). Although further work is needed to explain the differences between ELISA and LC results, it seems likely that the assay would perform best on pooled samples of milk.

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