Residues of the Forced Molting Agent Xylonidine in Chicken Egg Yolk and Albumen As Determined by Combined Gas-Liquid Chromatography-Mass Spectrometry

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A capillary column GLC selected ion monitoring mass spectrometric assay has been developed for the forced molting agent xylonidine [2-(2,6-xylylimino)imidazoline] in chicken egg yolk and albumen. The method employs xylonidine-d_4 as the internal standard and possesses an assay detection limit of 1.6 ppb for underivatized xylonidine in either yolk or albumen. When hens were medicated with 75 ppm of xylonidine in the feed for 2 weeks, the concentration of drug in the yolks of eggs laid before the ninth day of treatment, when production ceased, reached a maximum of ~70 ppb. The drug residues in the albumen of these eggs were correlated with the yolk residues and were ~$^1/_4$ as great. The hens resumed laying 8–17 days after xylonidine was removed from their diet, and drug concentrations of up to 11 ppb were found in the yolks eggs from those chickens that resumed production earliest after treatment. No albumen sample from any posttreatment egg contained detectable xylonidine.

Various (arylimino)imidazoline derivatives including the antihypertensive drug clonidine have marked anovulatory and antigonadal activities in laying hens (Weppelman et al., 1981). Among the most active of these is xylonidine [2-(2,6-xylylimino)imidazoline], which possesses the structure shown in Figure 1 (Weppelman and Tolman, 1982).

Because of its avian antifertility action, xylonidine was of interest as a chemical forced molting agent. Forced molting refers to the practice of forcing hens to stop laying eggs for a period of several weeks (Zeelen, 1975; Wakeling, 1977; Roland and Bushong, 1977). One result of this lull in reproductive activity is the feather loss and renewal that gives the practice its name. There are several economic advantages to forced molting. When molted hens resume production, they generally lay more eggs and produce eggs with more durable shells and at a smaller cost in feed than untreated hens. The only procedure currently available for forced molting hens is starvation, usually for about 10 days, and up to 4% fatalities can result from this drastic treatment (Swanson and Bell, 1974). In contrast, xylonidine appeared quite safe for laying hens (Weppelman and Tolman, 1982) and thus offered a desirable alternative to starvation.

For xylonidine to become a viable candidate as a forced molting agent, its concentration in eggs from treated hens had to be well characterized and this, in turn, required the development of appropriate assay procedures. Several packed-column GLC assays for substituted iminoimidazolines have been reported (Dollery et al., 1976; Timmermans et al., 1977). More recently Edlund (1980) reported an extremely sensitive assay that employs capillary column GLC and electron capture detection. This assay requires prior alkylation with pentafluorobenzyl bromide. The combination of capillary column GLC with selected ion monitoring mass spectrometry has proven to be a powerful tool for the assay of bioactive compounds (Walker et al., 1979). We report here a capillary column GLC–MS assay for xylonidine that employs tetradeuterioxylonidine (xylonidine-d_4) as the internal standard. This assay approaches Edlund's in sensitivity but does not require prior derivatization. This paper also describes the results obtained when this assay was applied to eggs from hens treated with xylonidine in the diet.

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EXPERIMENTAL SECTION

Chemicals. Xylonidine-d_4 (from ethylenediamine-d_4) and xylonidine (structures in Figure 1) were synthesized within Merck Sharp & Dohme Research Laboratories by published procedures (Rouot et al., 1976; Timmermans and Van Zwieten, 1977).

Instrumentation. A Finnigan 3200-6110 GC–MS instrument operated in the electron impact mode and utilizing selected ion monitoring was employed. Chromatographic conditions were as follows: 10 m × 0.33 mm i.d. glass capillary column coated with SE-30; oven temperature 190 °C; injection port temperature 220 °C; carrier gas (helium) flow rate ca. 2 mL/min; retention time of xylonidine and xylonidine-d_4 0.8 min. The mass spectrometer was operated by using the following conditions: ionizing potential 70 ev; emission current 0.8 mA; electron multiplier 1800 V. The M − 15 ions for xylonidine (m/e 174) and xylonidine-d_4 ($m//e$ 178) were monitored, and the peak height intensities in digital counts were obtained via computer printout.

Extraction Procedure. The following procedure is similar to published extractions for (arylimino)imidazolines in that it exploits the wide difference in lipophilicity between ionized and unionized compounds (Dollery et al., 1976; Timmermans et al., 1977; Edlund, 1980).

For extraction of yolk, the entire sample was weighed, and 200 ng of xylonidine-d_4 (the internal standard), 35 mL of 0.4 N PCA (perchloric acid), and 3.0 mL water saturated with NaCl were added. The mixture was centrifuged in a Sorvall GSA rotor at 6000 rpm for 15 min at 4 °C, and the supernatant was extracted 3 times with 10 mL of $CHCl_3$. The three organic phases were backwashed sequentially with 5 mL of 0.4 N PCA, and this aliquot was pooled with the original aqueous phase. The pooled aqueous phases were then made alkaline by the addition of 6.25 mL of 10 N NaOH and were extracted with 20 mL toluene. The aqueous phase was removed, 10 mL of PCA–NaOH (0.4 N PCA–10 N NaOH, 8:1 by volume) was

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Table I. Regression of m/e 174/178 vs. Nanograms of Xylonidine for Standards and Spiked Extracts

samples	no. of points	regression slope ± SE,[a] ng^{-1}	eq intercept ± SE	detection limit, ng[b]	r^2
standards	22	0.005182 ± 0.00007	0.0579 ± 0.0524	21.1	0.9996
yolk isolates	6	0.004428 ± 0.00004	0.1524 ± 0.0375[c]	23.5	0.9997
albumen isolates	6	0.004589 ± 0.00004	0.1395 ± 0.0429[c]	26.0	0.9996

[a] All slopes are significantly different ($p < 0.05$). [b] The amount of xylonidine, calculated from the regression equation, that corresponds to the upper 95% confidence limit of the intercept. [c] Significantly different from zero ($p < 0.05$).

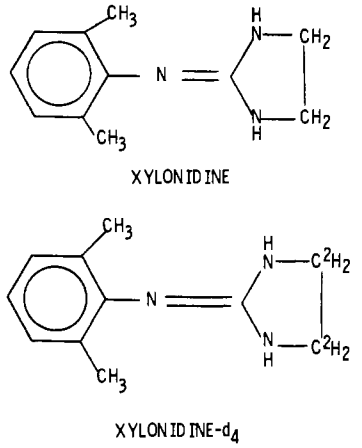

Figure 1. Structure of xylonidine and xylonidine-d_4.

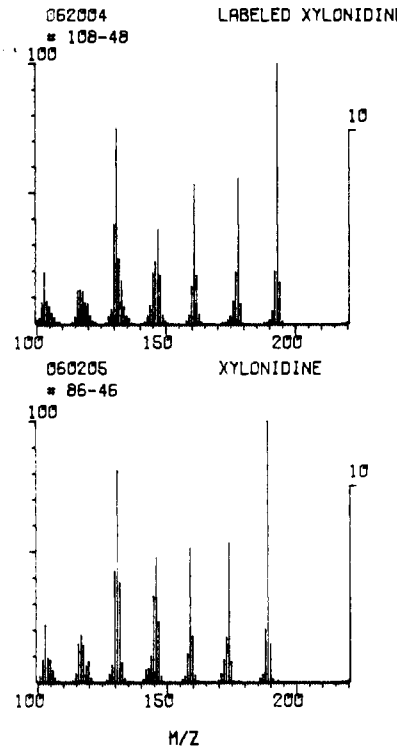

Figure 2. Electron impact mass spectra of xylonidine (bottom panel) and xylonidine-d_4 (top panel).

added to the organic phase, and the extraction was repeated. The two aqueous phases were backwashed sequentially with 5 mL of toluene, and this aliquot was added to the final organic phase. The pooled organic phases were taken to dryness with a Rotovap and resuspended in 2.0 mL of 0.1 N acetic acid and 10 mL of petroleum ether. The ether was discarded and the acetic acid was taken to dryness. At this point, on the basis of visual inspection of the mass, it was decided whether the extract appeared sufficiently clean for GLC–MS. For those extracts that were not, the dry extract was resuspended in 2 mL of $CHCl_3$ and 5 mL of 1% ammonium hydroxide. The aqueous phase was discarded, the $CHCl_3$ was taken to dryness, and the residue was then subjected to GLC–MS.

For extraction of albumen, each sample was vigorously mixed and a 16-g aliquot was removed. PCA and xylonidine-d_4 were then added and the mixture was centrifuged as described above. The supernatant was filtered through a 0.45-μm nalgene filter to remove the fine precipitate that remained. The rest of the extraction was as described above for yolk. Recoveries of drug for these procedures averaged 13% for both yolk and albumen samples.

Animal Handling Procedures. SexSal laying hens were obtained through Kerr Hatcheries, Frenchtown, NJ, and were housed in individual compartments of laying hen battery cages from Bussey Products, Chicago, IL. The hens were offered ad libitum water, oyster shell, and Layena (G) N (Ralston Purina Co., St. Louis, MO) that had been milled to a mash. Lighting was on from 6 a.m. until 8 p.m. local time, and temperature was maintained at 70 °F.

Nine hens, 63 weeks of age, and producing at least eight eggs in 10 days were randomly allocated to the experimental group (six hens) or to the control group (three hens). The experiment started when the ration of the experimental hens was switched to Layena (G) N containing xylonidine at 75 ppm. After 2 weeks, the hens were switched back to unmedicated ration, and the experiment was continued until each of the six treated hens had resumed production and had laid a minimum of three eggs. All eggs laid during medication (and the first three eggs laid postmedication) were collected daily and separated into yolk and albumen, which were stored at −20 °F until extracted. Control eggs were obtained from the three hens that had been treated identically but had never been medicated with xylonidine.

Statistics. Regression equations and correlation coefficients were calculated with the RS/1 computer system (Bolt, Beranek and Neuman, Inc., Cambridge, MA).

RESULTS AND DISCUSSION

Mass Spectra of Drug. The mass spectra of xylonidine (bottom panel) and xylonidine-d_4 (top panel) are shown in Figure 2. The M − 15 ions of m/e 174 (xylonidine) and m/e 178 (xylonidine-d_4) were chosen for selected ion monitoring since extracts contained material that interfered with the molecular ion of xylonidine at m/e 189.

Selected Ion Monitoring. Figure 3 presents the results obtained when two control yolk samples were monitored at m/e 174 and at m/e 178. One of these had been spiked before extraction with 200 ng of xylonidine-d_4 (left panel) and the other with 200 ng of xylonidine-d_4 and 20 ng of xylonidine (right panel). Twenty nanograms of xylonidine is slightly less than the limit of detection of xylonidine in yolk (23.5 ng), which was estimated statistically from the regression equation in Table I. Comparison of the response due to 20 ng at m/e 174 with the corresponding response for the sample containing no xylonidine suggests that 23.5 ng is an appropriate and conservative estimate of the detection limit. Since the yolks ranged from 15 to

Table II. Xylonidine Levels (ppb) in Eggs (Yolks and Albumen[a]) from Chickens Dosed with Drug in Feed at 75 ppm

chicken no.	days on drugs								days off drug							
	1	2	3	4	5	6	7	8	8	9	10	11	12	17	18	20
79	0[b] (0)[a]					8.4 (5.6)			8.3 (0)	0 (0)	0 (0)					
80	0 (0)	4.4 (1.6)					26.4 (6.8)							2.7 (0)	0 (0)	0 (0)
81	0 (0)		12.5 (2.7)					61.7 (14)			2.4 (0)	0 (0)	0 (0)			
88	0 (0)				72.5 (15.9)					7.7 (0)	7.5 (0)		5.0 (0)			
89									10.9 (0)	10.9 (0)	5.0 (0)					
90			16.9 (2.8)								0 (0)	c (0)	0 (0)			

[a] Albumen values given in parentheses. [b] 0 to <1.6 ppb for yolk and albumen. [c] Sample lost.

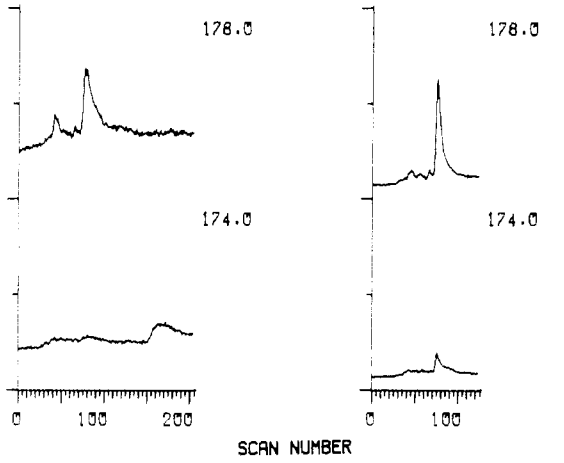

Figure 3. Selected ion monitoring plots from the analysis of isolates from control yolks spiked with either 200 ng of xylonidine-d_4 (left panel) or 20 ng of xylonidine and 200 ng of xylonidine-d_4 (right panel).

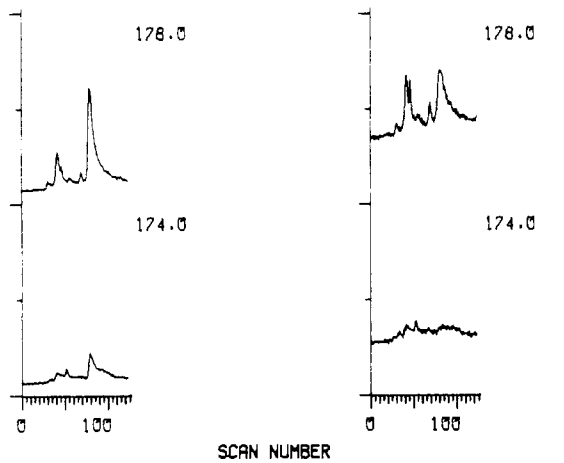

Figure 4. Selected ion monitoring plots from the analysis of yolk isolates from hen no. 81 10 days after treatment (left panel) or 11 days after treatment (right panel). Xylonidine concentration was determined to be 2.4 ppb in the 10-day sample and <1.6 ppb in the 11-day sample.

18 g, this detection limit corresponds to 1.6 ppb.

Albumen samples spiked with 20 ng of xylonidine and 200 ng of xylonidine-d_4 yielded results similar to those in Figure 3. This supports the detection limit of 26 ng presented in Table I. Since all albumen samples weighed 16 g, this corresponds to 1.6 ppb.

Figure 4 presents the results obtained with yolk isolates from a hen 10 days (left panel) or 11 days (right panel) off drug. The 10-day sample was determined to contain 2.4 ppb of xylonidine and the 11-day to contain <1.6 ppb.

Assay. When 22 samples containing 30 ng of xylonidine-d_4 and amounts of xylonidine between 0 and 30 ng were subjected to GLC–MS, the ratios of the responses at m/e 174 to those at m/e 178 were a linear function of the amount of xylonidine as shown by the regression equation in Table I. The intercept of this equation is not significantly different from zero, and its coefficient of determination, r^2, is 0.9996.

For admixture experiments, six yolk or six albumen samples from control eggs were mixed before extraction with 200 ng of xylonidine-d_4 and with 0–200 ng of xylonidine. When these samples were assayed, the ratios of the responses at m/e 174 to those at m/e 178 were also linear functions of the amount of xylonidine as shown by the regression equations for yolk and albumen samples in Table I. The coefficients of determination for these equations were greater than 0.999, and their intercepts were both significantly different from zero. Since the slopes of all three regression lines in Table I were different from each other, the amounts of xylonidine in experimental yolk and albumen samples were determined from the homologous standard curve.

For each of the three regression equations, the detection limit in Table I was defined as the amount of xylonidine that would be expected from the equation to yield a response equal to the upper 95% confidence limit of the intercept. Thus, on the average, this amount of xylonidine would yield a response greater than that yielded by 97.5% of the samples containing no xylonidine.

Egg Residue Studies. When six hens were treated with 75 ppm of xylonidine in the diet for 14 days, egg production ceased after 8 days of the treatment and did not resume until 8 days after the drug was withdrawn (Table II). The hens molted for a period of about 1 week beginning during the second week of treatment.

All four eggs laid after 1 day of treatment (18–30 h after the start of medication) contained negligible residue (<1.6 ppb). This is probably due to the fact that approximately 20 h of shell deposition immediately precede oviposition (Sturkie and Mueller, 1976) and the egg's interior probably becomes inaccessible to systemic drugs shortly after shell deposition starts.

The yolks of eggs laid after 2 or more days of treatment had higher xylonidine concentrations. For eggs laid during treatment, yolk drug content increased with duration of treatment, and the greatest concentrations of ~70 ppb were found in the yolks of those eggs laid after 5 and 8 days of medication. The albumen and yolk residues of the on-drug eggs were correlated (Spearman's rank correlation

coefficient = 0.89; $p < 0.05$), presumably because both reflected the systemic drug levels around the time of ovulation. For those on-drug eggs in which yolk and albumen both contained detectable xylonidine, the average ratio of yolk to albumen concentrations was 4.0 (95% confidence limits: 2.6–5.4). Thus, the xylonidine in yolk was about 4 times as concentrated as that in albumen.

As shown in Table II, the yolk residues after drug withdrawal depended both on the day laid and on whether the egg was the first, second, or third laid posttreatment. Highest yolk residues were seen in the first or second egg from those hens that resumed production the earliest. No residues were detected in any posttreatment albumen samples, and thus albumen and yolk residues were totally uncorrelated for posttreatment eggs. This is probably due to the different time courses of yolk and albumen synthesis. Albumen is synthesized de novo during an approximately 3-h period beginning 18 h before oviposition, while yolk synthesis requires considerably more time, with the rapid growth phase consuming the final 5–11 days that precede ovulation (Sturkie and Mueller, 1976). Thus the yolks from posttreatment eggs probably reflected systemic drug concentrations that were earlier and presumably higher than those reflected by the corresponding albumens.

These results demonstrate the suitability and sensitivity of capillary column GLC–MS for determining xylonidine concentrations in eggs. The data that resulted when these procedures were applied to eggs from hens forced molted by this drug will be used to determine the possible human health implications posed by xylonidine egg residues.

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Storage Stability of Pesticide Residues

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The stability of residues of 19 plant protection agents or plant regulators in different substrates at −20 °C were determined as were the hydrolysis half-life times in neutral solutions at +50 and +70 °C. The following correlations between these two properties were found: (1) residues are stable for at least 1 year if half-life times are above 10 days at 70 °C; (2) residues are unstable if half-life times are below 1 day at 50 °C, especially in crops with a high water content; (3) residue stabilities need examination if half-life times lie in between. On the basis of this, it is proposed that residue stabilities can be derived from hydrolysis data and a residue stability study should be run only in doubtful cases. It is shown that such studies can be performed with fortified samples.

In residue analytical practice samples often cannot be analyzed immediately after sampling. They therefore have to be stored. Although samples usually are deep frozen, the question arises whether residues are sufficiently stable during storage. Several papers dealing with this question have been published up to now: Kawar et al. (1973) gave a comprehensive review on storage stability. Since then papers on the degradation during storage of metribuzin (Webster and Reimer, 1976) and of atrazin (Swain, 1979) in soil appeared. Storage conditions used by the authors differed and so did the stabilities reported. Thus, it is common practice to check the stability of residues of each compound in each substrate under investigation, if samples are to be stored. There is no generally accepted methodology for performing residue stability studies, but several points have to be considered in order to end up with reliable results: What should be done if no field-treated samples with finite residues are available? Do "artificial residue samples", i.e., fortified samples, show the same degradation behavior as do real field samples? How should artificial samples be prepared? What can be done if residues are not stable? To answer these questions is one of the goals of the present paper.

Factors determining the rate of degradation are the rates of hydrolysis, of photolysis, and of oxidation. (Enzymatic degradation by these or other pathways is considered to be of minor importance at low temperatures.) If stored in the dark, residues are not photodegraded; oxidation could sometimes be an important process (e.g., thio compounds), but generally the rate of oxidation of organic compounds is slow. Hydrolysis, however, is suspected to be a main route of degradation. This assumption is supported by observations of several authors (Hamaker, 1972; Kawar et al., 1973; Minett and Belcher, 1970; Swain, 1979) who report data on the dependence of residue stability on the water content of the substrate. Therefore, the second goal of the work presented in this paper is to investigate the suspected correlation of the hydrolytical behavior of a number of chemically different compounds with the storage stabilities of their residues. The existence of such

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