Journal of Pharmaceutical & Biomedical Analysis
Vol. 11, No. 10, pp. 947–954, 1993
Printed in Great Britain

0731–7085/93 $6.00 + 0.00

Analytical method development and preformulation stability studies of L-665,871 (a β-agonist) in swine feed

PIERRE DeMONTIGNY* and K.J. DAVE

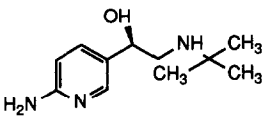
Merck Research Laboratories, P.O. Box 2000 RY123-22, Rahway, NJ 07065-0900, USA

Abstract: An analytical procedure for the determination of the β-agonist L-665,871 by LC with fluorescence detection has been developed. The mobile phase consists of acetonitrile–phosphate buffer (pH 3.0) with ion-pairing reagent hexanesulphonic acid sodium salt for the analysis of swine feed samples with 1 and 20 μg g^{-1} L-665,871. Storage of feed samples at room temperature, however, indicated that the stability L-665,871 and its analogue L-674,239 were greatly compromised by the presence of moisture in the feed. Acidification with maleic acid and/or freezing the feeds were found to significantly improve drug stability.

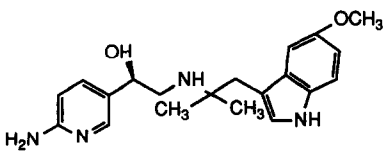
Keywords: *β-Agonist; liquid chromatography; fluorescence detection; stability studies.*

Introduction

The β-agonist, L-665,871, an heteroalkylamine (Fig. 1) and a potential swine growth promoter is being tested to determine its safety and residue levels in swine. Also, preformulation studies were initiated for the development of a stable medicated feed with L-665,871 for swine, in the knowledge that analogues of this compound, tested previously, were found to be unstable in feed stored at ambient temperature. This report describes the preformulation studies and the analytical procedure to determine the stability of medicated feeds with L-665,871 at low μg g^{-1} concentrations. Additional preformulation data are reported for its analogue L-674,239 (Fig. 1).



L-665,871



L-674,239

Figure 1
Chemical structures of L-665,871 and its analogue L-674,239.

Materials and Methods

Chemicals and reagents

HPLC-grade methanol, acetonitrile and chloroform were obtained from Fisher Scientific. Water was deionized and filtered (0.1 μm). Hexanesulphonic acid sodium salt and maleic acid were received from Aldrich. Sulphuric acid (fuming) and potassium phosphate were obtained from Fisher.

Apparatus

The chromatographic system consisted of an ABI Analytical Spectroflow 400 pump, a Waters model 712 autosampler, an ABI Analytical Spectroflow 980 fluorescence detector and a Spectra Physics SP-4290 integrator.

Sample preparation

Extraction of the feed samples with L-665,871 was accomplished by adding 25 ml (1% sulphuric acid in water) to 5.0 g of feed in a 50-ml centrifuge tube. After sonication (15 min) and thorough mixing (30 min) the feed extracts were centrifuged at 2500 rpm for 15 min. A 2.0 ml aliquot of the supernatant was then transferred into a 15-ml centrifuge tube to

*Author to whom correspondence should be addressed.

which was added 5 ml chloroform. After mixing for 30 s, the clear aqueous supernatant (50 μl) was diluted with mobile phase (1.0 ml) for analysis by LC.

Standard preparation

Standard solutions of L-665,871 were prepared by dissolving the drug in methanol (1 mg ml^{-1}). This was followed by dilution with 1% sulphuric acid in water to concentrations equivalent to the extracts of feed samples with 0.25, 1.0, 15, 20 and 25 μg g^{-1} L-665,871. Aliquots of the aqueous solutions were then diluted with mobile phase as described in the sample preparation section.

Extraction recovery determination

Drug-free feed samples (5 g) were fortified in a 50-ml centrifuge tube with stock standard solutions of L-665,871 in 1% sulphuric acid to obtain concentrations equivalent to 0.25, 1.0, 15, 20 and 25 μg g^{-1} in feed. These samples were then extracted by the procedure described in sample preparation. Extraction recoveries were determined by comparing peak height measurements of the sample extracts and the standard solutions.

Medicated feed stability studies

Premixes of L-665,871 on toasted soy flour were homogeneously mixed with swine feed ration B-30 in our feed mixing laboratories. Feed samples with 1 and 20 μg g^{-1} L-665,871 were then taken and stored frozen, at room temperature and at 50 or 60°C in the dark to determine the stability of L-665,871 in feed.

pK_a determination of L-665,871

The pK_a of the aminopyridine moiety of L-665,871 was determined by measuring the absorbance at 310 nm of buffered solutions of L-665,871 (25 μg ml^{-1}) at pH 2.2, 5.40, 5.70, 6.00, 6.31 and 8.7 with phosphate buffer (0.01 M) at ambient temperature (ionic strength not adjusted).

Chromatographic conditions

The mobile phase consisted of acetonitrile–phosphate buffer (pH 3.0, 0.01 M) (25:75, v/v) containing 1-hexanesulphonic acid sodium salt (0.05 M) pumped at 1.0 ml min^{-1} through a Zorbax ODS column (Dupont, 4.6 mm i.d. × 250 mm) thermostated at 30°C, with fluorescence detection at an excitation wavelength of 230 nm and an emission cut-off wavelength of 320 nm.

Radiolabelled L-665,871 study

C-14 labelled L-665,871 (10 μg ml^{-1}) was added to aqueous feed extract and aged at 60°C for 15 days in the dark. Samples were taken for analysis by LC with fluorescence detection. Fractions were collected every minute for 10 min and then mixed with scintillation cocktail for radioactivity measurements. A 15-day drug-free feed extract (control) and a reference standard solution with C-14 labelled L-665,871

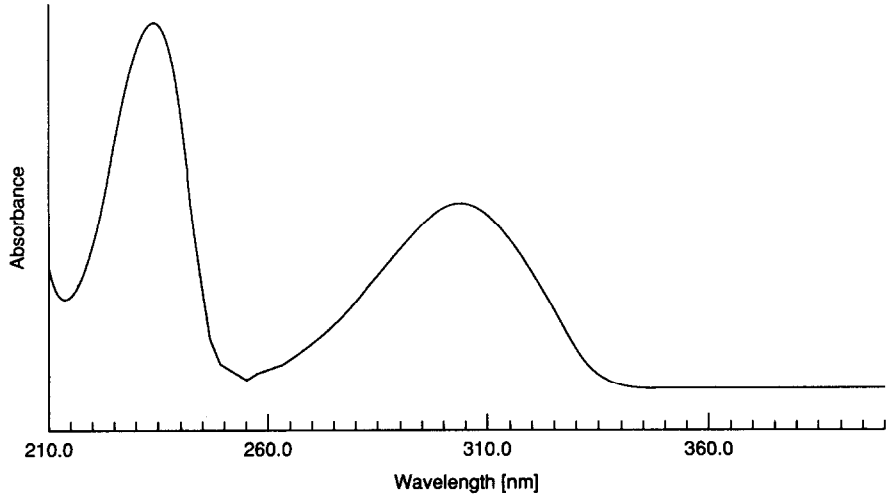


Figure 2
HPLC-diode array UV spectrum of L-665,871 in acetonitrile–potassium phosphate (0.01 M, pH 3.0) (25:75, v/v) with hexanesulphonic acid sodium salt.

were also injected into the LC for fraction collections and radioactivity measurements.

Results and Discussion

Analytical method development for L-665,871 in swine feed and validation

Physico-chemical properties of L-665,871. The spectroscopic properties of L-665,871 were evaluated. Two absorption maxima were detected at 230 and 305 nm (Fig. 2). In addition, it was found that this compound was fluorescent at low pH in aqueous solution. Spectrofluorometric titration of L-665,871 as shown in Fig. 3 revealed that fluorescence efficiency was optimal only at pH less than 4 to fully protonate the aminopyridine moiety of L-665,871. Subsequently the pK_a value of L-665,871 was determined spectrophotometrically to be 5.86. Consequently, for optimal sensitivity to determine L-665,871 in feed by HPLC, the mobile phase should be acidic for detection of the analyte by fluorescence. An excitation wavelength 230 nm was chosen to achieve the best response with a detector equipped with a deuterium lamp (note the differences between absorption spectrum on Fig. 2 obtained with a deuterium lamp source and the excitation spectra on Fig. 3 obtained with a xenon lamp source).

Chromatography of L-665,871. The chromatographic properties of L-665,871 were then evaluated to achieve separation from the feed components following extraction. It was found that the retention of L-665,871 on Zorbax ODS was strongly affected by the concentration of ionic mobile phase modifiers. Increasing the concentration of potassium phosphate (pH 3.0) decreased substantially the elution time (Fig. 4) and improved greatly the peak symmetry (Fig. 5) presumably by masking the residual silanol groups on the silica-based reversed-phase HPLC column. With the mobile phase consisting of methanol–potassium phosphate (pH 3.0, 0.01 M) (10:90, v/v) at a flow rate of 1.0 ml min^{-1} on Zorbax ODS the elution time of L-665,871 was approximately 4 min but insufficient to achieve baseline resolution from feed components. Consequently, to increase the retention time of L-665,871 and maintain peak symmetry, the hydrophobic ion-pairing reagent 1-hexanesulphonic acid sodium salt was added to the

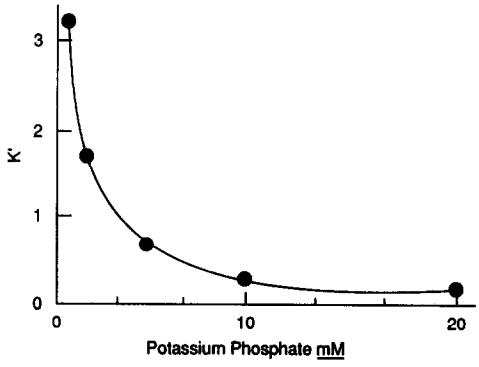


Figure 4
Plot of the effect of potassium phosphate concentration in the eluent on the retention parameter (k') for L-665,871 on Zorbax ODS in a mobile phase consisting of (10:90, v/v) methanol–potassium phosphate (pH 3.0).

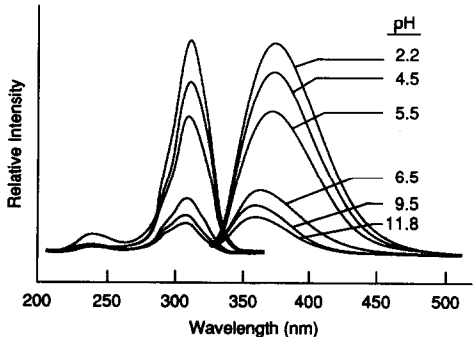


Figure 3
Fluorescence (uncorrected) spectra of L-665,871 (2 μg ml^{-1}) in buffered solutions at pH 2.2, 4.5, 5.5, 6.5, 9.5 and 11.8.

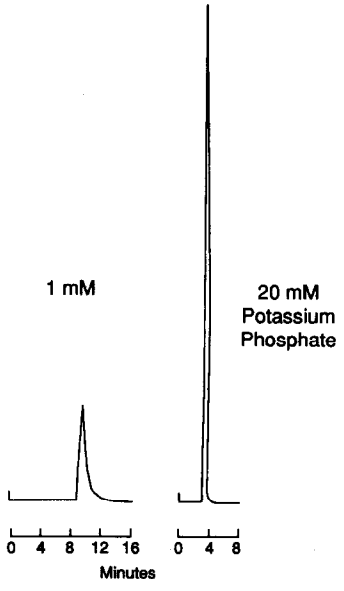


Figure 5
Chromatograms of L-665,871 eluted on Zorbax ODS with 10% methanol–potassium phosphate (pH 3.0) (10:90, v/v) at a flow rate of 1.0 ml min^{-1}.

mobile phase. Feed sample extracts with and without added drug were then compared to determine the optimal ion-pairing reagent concentration to be added to the mobile phase (Fig. 6). Clearly, greater than 0.05 M hexanesulphonic acid sodium salt was required to achieve baseline resolution between the closest interfering unidentified feed component and L-665,871. Consequently, the mobile phase for the determination of L-665,871 in feed consisted of 25% acetonitrile–potassium phosphate (pH 3.0, 0.01 M) (25:75, v/v) with 0.05 M 1-hexanesulphonic acid sodium salt. Chromatograms depicting the separation achieved for the determination of L-665,871 extracted from swine feed samples are shown in Figs 7 and 8.

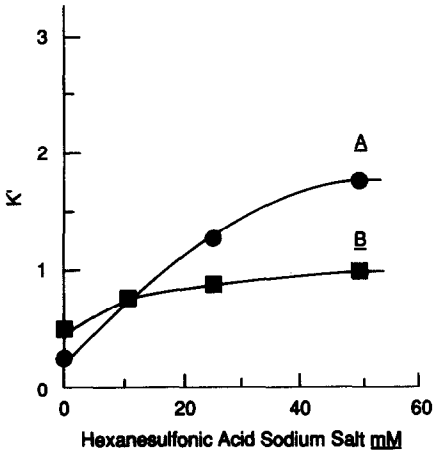


Figure 6
Plot of the effect of hexanesulphonic acid sodium salt on the retention parameter (k') for L-665,871 (A) and a co-extracted feed component (B) on Zorbax ODS in an eluent consisting of acetonitrile–0.01 M potassium phosphate (pH 3.0) (25:75, v/v).

Extraction of L-665,871 from feed and method validation

The isolation of L-665,871 from feed (5 g) was achieved following a single (30 min) extraction of L-665,871 with 1% sulphuric acid in water (25 ml) followed by a liquid–liquid extraction clean-up with chloroform to precipitate the unwanted feed components from the aqueous phase. With drug-free feed samples fortified with L-665,871 at 0.25, 1, 15, 20 and 25 μg g^{-1} (Table 1) the extraction recovery (compared with direct standard solutions) was 97.0% (RSD 3.9%, $n = 23$). Correlation coefficient for linearity determination exceeded 0.999. Limit of quantitation (LOQ) was 0.25 μg g^{-1} ($S/N = 5$).

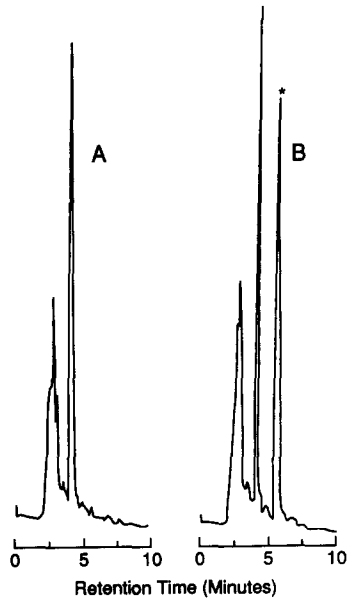


Figure 7
Chromatograms of a blank feed extract (A) and a medicated feed (20 μg g^{-1}) extract (B) obtained on Zorbax ODS with acetonitrile–potassium phosphate (0.01 M pH 3.0) (25:75, v/v) and 0.05 M hexanesulphonic acid sodium salt at a flow rate of 1.0 ml min^{-1} with fluorescence detection. L-665,871 is labelled with an asterisk.

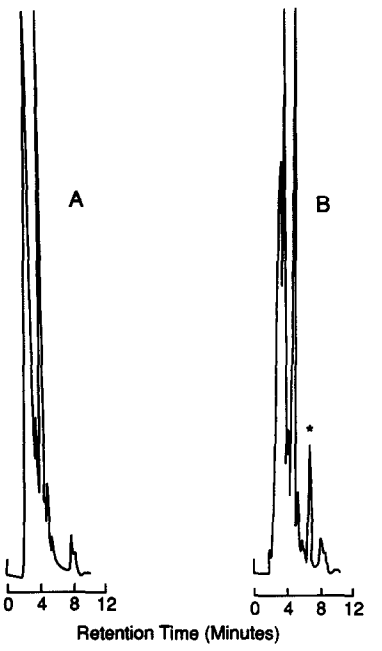


Figure 8
Chromatograms of a blank feed extract (A) and 1 μg g^{-1} (B) obtained by HPLC on Zorbax ODS and fluorescence detection (range 6X), L-665,871 is labelled with an asterisk.

Table 1
Calibration and extraction recovery data for the determination of L-665,871 (μg g^{-1}, free base) in swine feed ration B-30

Feed sample	Conc. added (μg g^{-1})	Conc. found (μg g^{-1})	Extraction recovery (%)
1	0.25	0.24	96
2	0.25	0.24	96
3	0.25	0.22	88
4	0.25	0.24	96
5	0.25	0.24	96
6	1.00	0.94	94
7	1.00	0.94	94
8	1.00	0.97	97
9	1.00	0.94	94
10	1.00	0.94	94
11	4.00	3.80	95
12	4.00	3.80	95
13	4.00	4.00	100
14	4.00	3.80	95
15	4.00	3.80	95
16	15.00	15.30	102
17	15.00	15.30	102
18	15.00	14.90	99
19	20.00	20.00	100
20	20.00	20.00	100
21	20.00	20.40	102
22	25.00	26.20	105
23	25.00	24.30	97
Mean			97.0
RSD			3.9

Preformulation stability studies of L-665,871 in feed. Stability of L-665,871 in solution and in feed

L-665,871 was dissolved in 10% aqueous methanol buffered at pH ranging between 2 and 12 and maintained at 25 and 50°C for at least 2 weeks for stability assessment. As expected, no sign of instability caused by hydrolysis was observed. Also, no thermal decomposition was detected for solid L-665,871 for 15 days at 25, 40 and 50°C. However, for medicated feed (20 μg g^{-1}) with L-665,871, instability was observed with half-life of 18 and 10 days at 25 and 50°C, respectively (Fig. 9). Extraction of the feed samples for as long as 16 h with 1% sulphuric acid (instead of 30 min) produced the same results. Attempts were then made to improve the stability of L-665,871 in feed. Preliminary testing with the addition of ascorbic acid (0.1%), sodium bisulphite (0.5%), and fumaric acid (1%) to medicated swine feeds revealed that the antioxidants were ineffective but that fumaric acid seemed to improve slightly the stability of L-665,871 in feed. This observation led to further investigations to determine the effect of acidification of the feed to stabilize L-665,871.

Effect of acidification on stability of L-665,871 in feed

Measurements of the pH of 15% feed slurries in water with no additive, 0.5% sodium bisulphite, 0.1% ascorbic acid, and 1% fumaric acid were 6.0, 5.8, 6.0 and 4.9, respectively. Acidification of the feed would protonate the aminopyridine moiety of L-665,871 (pK_a 5.86) dissolved in the residual moisture of the feed.

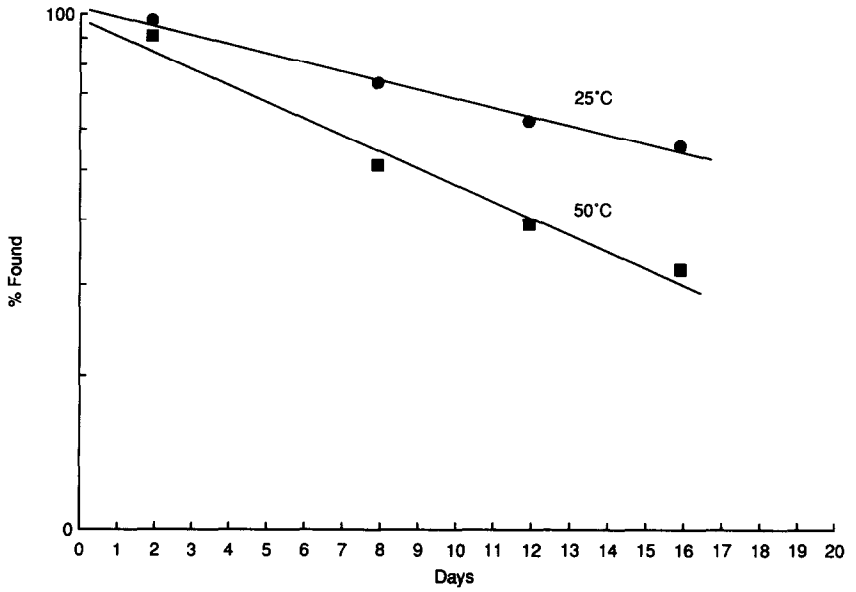


Figure 9
Stability profiles of medicated (20 μg g^{-1}) swine rations B-30 with L-665,871 stored at 25 and 50°C for 16 days.

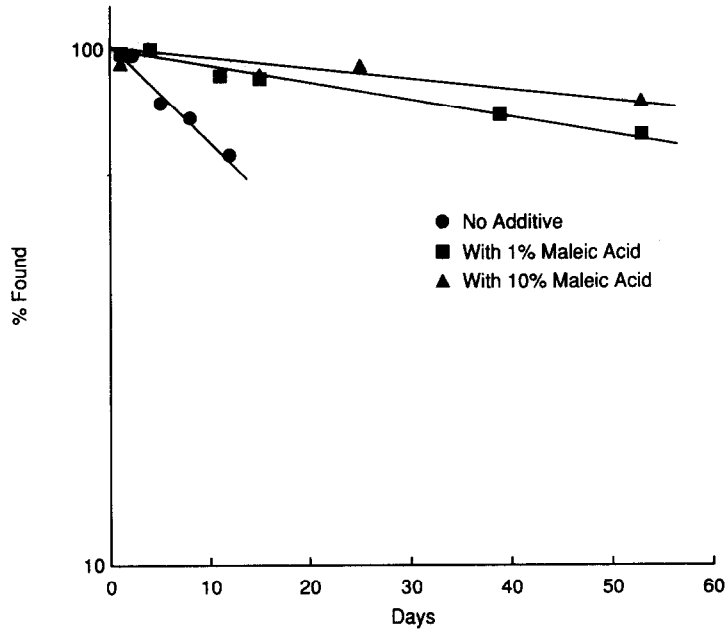


Figure 10
Stability profiles of L-665,871 (20 μg g^{-1}) in swine feed stored at 25°C with/without the addition of 1 or 10% maleic acid to the feeds.

Knowing that oligosaccharides react with 2-aminopyridine in water to produce fluorescent derivatives [1–3], acidification of the feed could inhibit the formation of L-665,871 derivatives with feed components such as oligosaccharides. To test this hypothesis, medicated swine feeds were acidified with 1 and 10% maleic acid (pH of 15% w/v aqueous feed slurry were 4.9 and 2.3 respectively, compared to 6.0 for unaltered feed). The results are presented in Fig. 10. Significant stabilization of L-665,871 was observed for the acidified feeds, especially with 10% maleic acid added, suggesting that protonation of the aminopyridine moiety decreases significantly the rate of drug loss in the feed.

Effect of moisture (and temperature) on stability of L-665,871 in feed

Further stability investigations with the addition of moisture (10%) to selected feed samples were then done. The results presented in Fig. 11 clearly indicate that for feed samples with 1 μg g^{-1} drug, the rate of disappearance of L-665,871 increased significantly with the addition of water. Figure 11 also depicts the best fit lines for the data gathered from this study. At low moisture content (<1% w/w) in the feed, the kinetics of disappearance of L-665,871 were pseudo-zero order, while at high moisture content (10% w/w), first-order

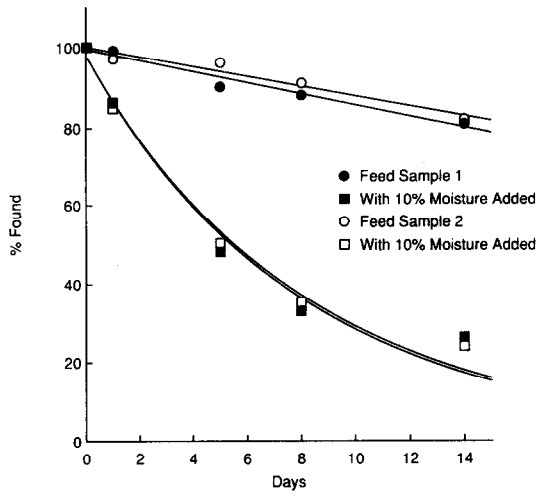


Figure 11
Stability profiles of C-14 labelled L-665,871 (1 μg g^{-1}) in swine feed at 25°C with and without the addition of water (10% w/w).

kinetics were observed with a half-life less than 6 days. Since L-665,871 was not susceptible to hydrolysis, these results further support the hypothesis that a large concentration of water soluble feed component(s) is available to react with the limited concentration of L-665,871 (1–20 μg g^{-1}) in the aqueous phase of the feed. This was verified by adding L-665,871 to drug-free feed extracts in water (Fig. 12).

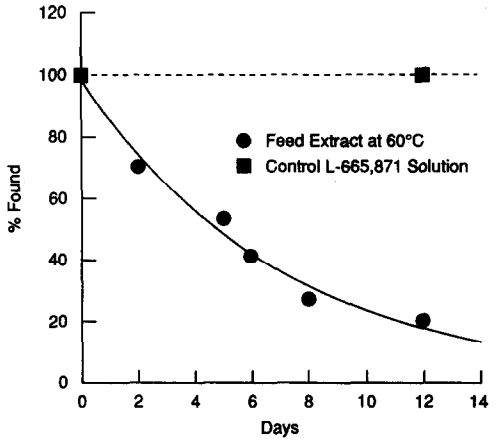


Figure 12
Stability of L-665,871 (1 μg ml^{-1}) added to aqueous feed extract and stored at 60°C for 12 days.

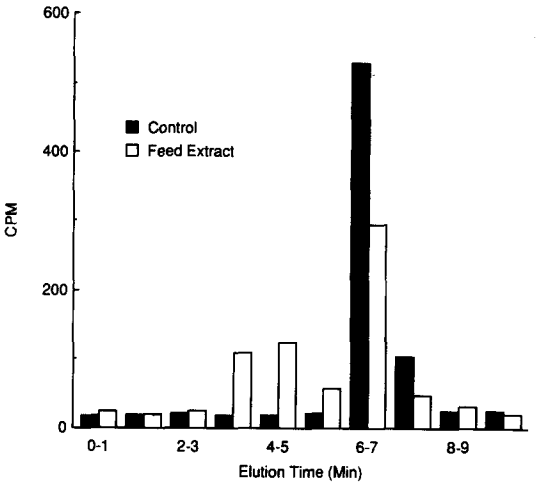


Figure 13
Radiochromatogram of C-14 labelled L-665,871 obtained from fraction collections of the eluate from the LC of an aqueous feed extract after 15 days at 60°C and of the injection of a reference standard solution of C-14 L-665,871.

Studies with radiolabelled L-665,871 in feed extract

Separation of the reaction product(s) in the feed extract with radiolabelled L-665,871 revealed the presence of a polar and non-fluorescent (at excitation 230 nm) product (Fig. 13) eluting prior to L-665,871 in a feed sample aged for 15 days at 60°C. The lack of fluorescence (at excitation 230 nm) of the radiolabelled product detected in the feed extract is further evidence that the amino-pyridine moiety had reacted with a feed component to produce a new chemical species with different spectral properties.

Stability of feed of analogue of L-665,871: L-674,239

Additional studies were conducted with L-674,239, an heteroarylalkylamine analogue of L-665,871, which also showed the same degradation pattern at room temperature (Fig. 14) with and without the addition of moisture. This study also demonstrated that feed samples with L-674,239 stored frozen for 28 days were significantly more stable than feed samples stored at room temperature. The data obtained with L-674,239 suggest that the reaction of these types of growth promoters with feed in the presence of moisture may be a more general problem.

Conclusion

Swine feed containing L-665,871 and analogues are highly susceptible to degradation

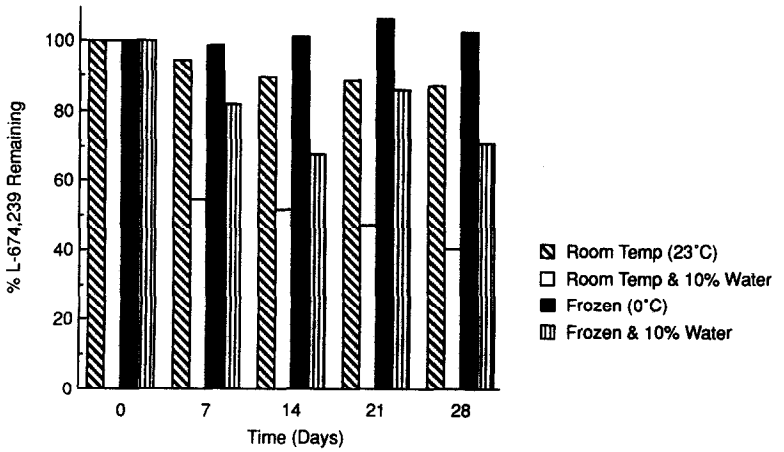


Figure 14
Bar graph showing the stability of L-674,239 in feed at 23 and 0°C in the presence and absence of added water.

if not properly stored before use. Results from the studies presented herein clearly suggest that excess moisture promotes the reaction of the aminopyridine with feed components and that optimal storage conditions should be dry and/or frozen until consumption. However, use of an acidic feed, if available, would provide the most stable condition.

References

[1] S. Hase, T. Ikenaka and Y. Matsushima, *Biochem. Biophys. Res. Comm.* **85**, 257–263 (1978).
[2] N.O. Maness, E.T. Miranda and A.J. Mort, *J. Chromatogr.* **587**, 177–183.
[3] K. Ohara, M. Sano, A. Kondo and I. Kato, *J. Chromatogr.* **586**, 35–41 (1991).

[Received for review 4 February 1993; revised manuscript received 7 April 1993]