Journal of Chromatography, 606 (1992) 141–146
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 304

Short Communication

Determination of diclazuril in animal feed by liquid chromatography

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(First received January 17th, 1992; revised manuscript received April 21st, 1992)

ABSTRACT

A method is described for the determination of diclazuril (Janssen Research Compound R64433; trademark Clinacox) in chicken feed at the 1 mg kg^{-1} level. Compound R062646, a structure analogous to diclazuril, was used as the internal standard. The drug was extracted from food with acidified methanol. Diclazuril was then isolated by means of solid-phase extraction with a cartridge containing a C_{18} phase. The eluate was evaporated and the residue redissolved in dimethylformamide. An aliquot was injected onto a reversed-phase high-performance liquid chromatographic column and the drug substance quantified at 280 nm by an ultraviolet detector. Extraction (absolute) recoveries of 85% for both internal standard and diclazuril were obtained. The method is suitable for diclazuril concentrations ranging from 0.1 to 1.5 mg kg^{-1}. Method validation data are presented.

INTRODUCTION

Diclazuril (I, Fig. 1) was developed as a potent and broad-spectrum anticoccidial in broiler chickens [1–3]. For the control of coccidiosis, diclazuril is admixed in the feed at a concentration of 1 mg kg^{-1}. This can be achieved by the inclusion of 200 g of the 0.5% Clinacox premix per ton of feed.

Until now no liquid chromatographic (LC) method has been published for the determination of diclazuril. We report a robust high-performance liquid chromatographic (HPLC) assay for the quantitation of the anticoccidial in feed. Analyses of the samples have shown that the medicated feed is stable for at least 6 months. A possible degradation compound (III, Fig. 1), resulting from stress-decomposition studies of the drug substance, is not found in these aged samples.

An extended clean-up procedure allows the analysis of feed samples of different origin. It is well known that, because it is dependent upon the availability of the ingredients, the composition of a feed can vary to a high degree. Hence the level of the feed excipients that are co-extracted can change.

To enhance the ruggedness of the method, an internal standard (II, Fig. 1) was taken through the whole clean-up procedure.

EXPERIMENTAL

Instrumentation

The type of equipment used for the development of the method and batch control is described below. Any instrument equivalent to that described can be used. A liquid chromatograph equipped with an autosampler and a variable UV wavelength detector

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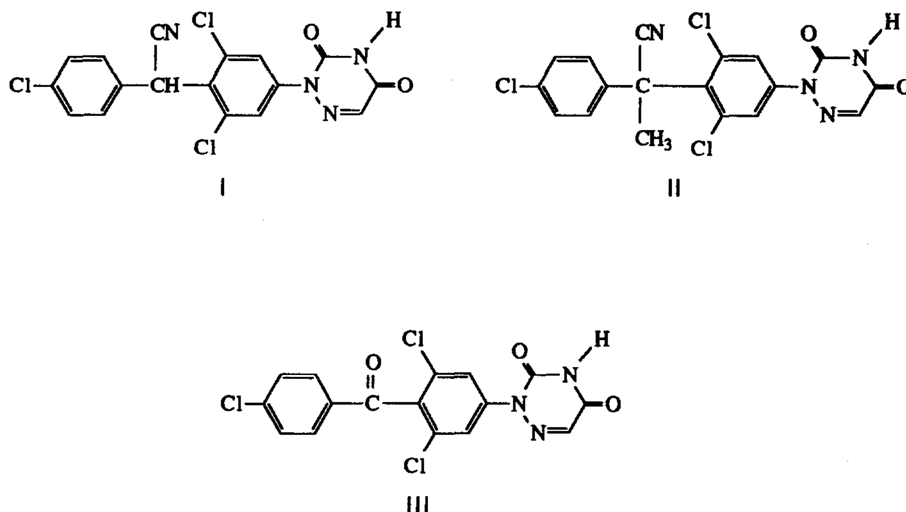

Fig. 1. Structures of dicluzaril (I), the internal standard (II) and a possible degradation product of dicluzaril (III).

(Hewlett-Packard Series 1050, Wallbron, Germany) was used. Quantitation was done at 280 nm. A flow-rate of 2 ml/min and an injection volume of 20 μl were used. The elution mode was a ternary gradient. Solvents A, B and C were, respectively, 0.5% ammonium acetate and 0.01 *M* tetrabutylammonium hydrogen sulphate in water, acetonitrile and methanol. The initial conditions were 60% A, 20% B and 20% C. These were held for 10 min and then followed by a gradient elution lasting 30 min to 45% A, 20% B and 35% C. The column was then flushed with acetonitrile for 10 min.

Reagents, materials and eluent

The model compounds (I, II and III) used in this study were reference standard grade from Janssen Pharmaceutica (Beerse, Belgium). Deionized water was purified through a Milli-Q4 System (Millipore, Brussels, Belgium). Acetonitrile and methanol were of HPLC quality (Janssen Chimica, Geel, Belgium). Inorganic compounds were all of p.a. quality.

The solid-phase extraction cartridges (Mega Bond C_{18}) were obtained from Analytichem (Harbor City, CA, USA). The analytical column was a stainless-steel tube (100 mm × 4.6 mm I.D.) packed with Hypersil ODS or BDS (3-μm particles, Shandon Scientific, Astmoor, UK).

Standard and sample preparations

An internal standard solution of 0.05 mg of II per ml of dimethylformamide was used. A reference solution was prepared containing 2 μg of diclazuril and II each per ml of a water–dimethylformamide mixture (60:40, v/v). Both solutions were prepared preferably in amber-coloured flasks and stored in the refrigerator at −4°C.

The extraction solvent (acidified methanol) was prepared by addition of 5 ml of hydrochloric acid to 1000 ml of methanol.

Meal (mash) and pellets were ground in order to be homogenized. From the ground food about 50 g were accurately weighed into a suitable glass container. Pellets were ground before sampling. Exactly 1 ml of the internal standard solution and 200 ml extraction solvent were added. The mixture was stirred overnight. The supernatant (20 ml) was transferred into a suitable vessel and diluted with 20 ml of water. This solution was put onto a Mega-Bond (C_{18}) column. Vacuum was applied with a waterjet and the cartridge was washed with 25 ml of acidified methanol–water (65:35, v/v). The fraction collected was discarded and the model compounds were eluted from the Mega-Bond column with 25 ml of acidified methanol–water (80:20 v/v). The eluate was evaporated to dryness at 60°C. The residue was

redissolved in 1 ml of dimethylformamide and 1.5 ml of water.

It is recommended the final sample solution be filtered over a 0.45-μm chemically resistant filter (Gelman) before injection.

Sample analysis

Before injection of a sample sequence the analytical column was flushed with 100% acetonitrile for at least 15 min followed by equilibration with the initial elution solvents for 10 min. During the sequence the gradient composition described in the *Instrumentation* section was followed.

The amount of diclazuril present in the feed sample, expressed as weight percentage, was calculated from:

$$\text{Diclazuril}\,(\%) = \frac{H_{c,s}}{H_{i,s}} \times \frac{H_{i,r}}{H_{c,r}} \times \frac{C_{c,r}}{C_{a,s}} \times 100$$

where $C_{c,r}$ is the concentration of diclazuril in the reference solution, $C_{a,s}$ is the concentration of the theoretical amount of diclazuril analyte in the final sample solution and $H_{c,r}$, $H_{i,r}$, $H_{c,s}$ and $H_{i,s}$ are the peak heights measured for diclazuril (c) and internal standard (i) in the reference (r) and sample (s) solutions.

Absolute recovery

A set of two blank feed samples (50 g) were taken; one was spiked with internal standard and diclazuril, the other with internal standard only. Spiking amounts were at the 1 mg kg^{-1} level. Both sets were then subjected to the whole work-up procedure. At the end diclazuril was added (1 mg kg^{-1} level) to the samples containing only internal standard. The recovery was calculated from the peak height ratios measured on the chromatograms. The same procedure was repeated for the recovery of the internal standard (one set of samples contained internal standard only).

Linearity

The linearity for diclazuril was determined in the 50–150% range with the nominal batch level of 1.0 mg kg^{-1}.

Blank feed (50 g) was spiked three times with 50 μg of internal standard and 25, 50 and 75 μg of diclazuril (corresponding to 50, 100 and 150%) and analysed according to the method described.

Accuracy and precision

Blank feed was spiked with diclazuril in various amounts (concentrations between 0 and 1.5 mg kg^{-1}) and analysed according to the method described.

Precision (repeatability and reproducibility) studies were performed on a broiler feed sample produced by a commercial feed mill in Brazil at the 1 mg kg^{-1} level.

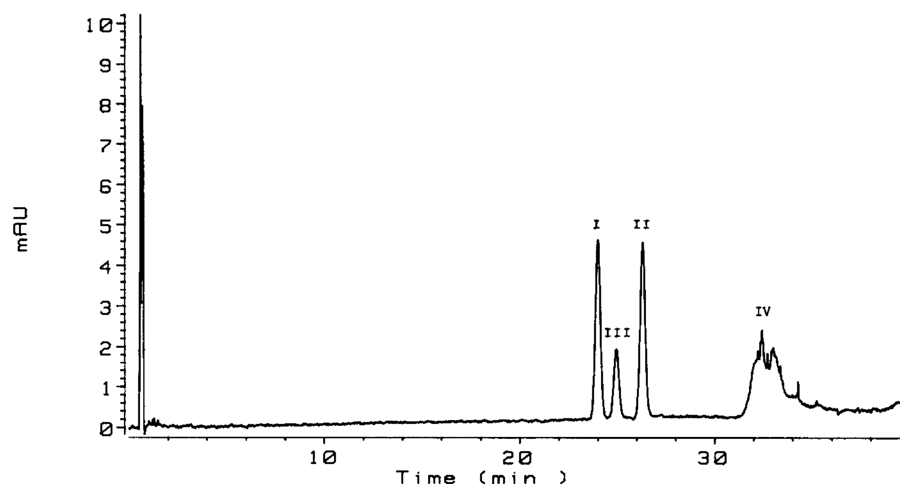

Fig. 2. Chromatogram demonstrating the selectivity of the method. Peaks: I = diclazuril; II = internal standard; III = possible degradation compound (each 2 μg ml^{-1}); IV = mobile phase impurities.

RESULTS AND DISCUSSION

Selectivity

Stress-decomposition studies on the drug substance revealed the formation of compound III. Because of the similar structure of this compound, a gradient elution is necessary to obtain a separation between the three model compounds. In Fig. 2 a specimen chromatogram demonstrating the selectivity is shown. Furthermore, the gradient enhances the selectivity against possible feed excipients. It was noted during development of the assay that proper adjustment of the gradient for some samples is necessary to eliminate interference from the excipients.

Determination at wavelength 280 nm gave a good signal-to-noise ratio.

Extraction procedure and absolute recovery

In the laboratory for gas chromatography (GC) [4] in our analytical department, similar studies were performed. The results indicated that the extraction procedure described gives the highest recovery. Stirring overnight is not necessary, but because the

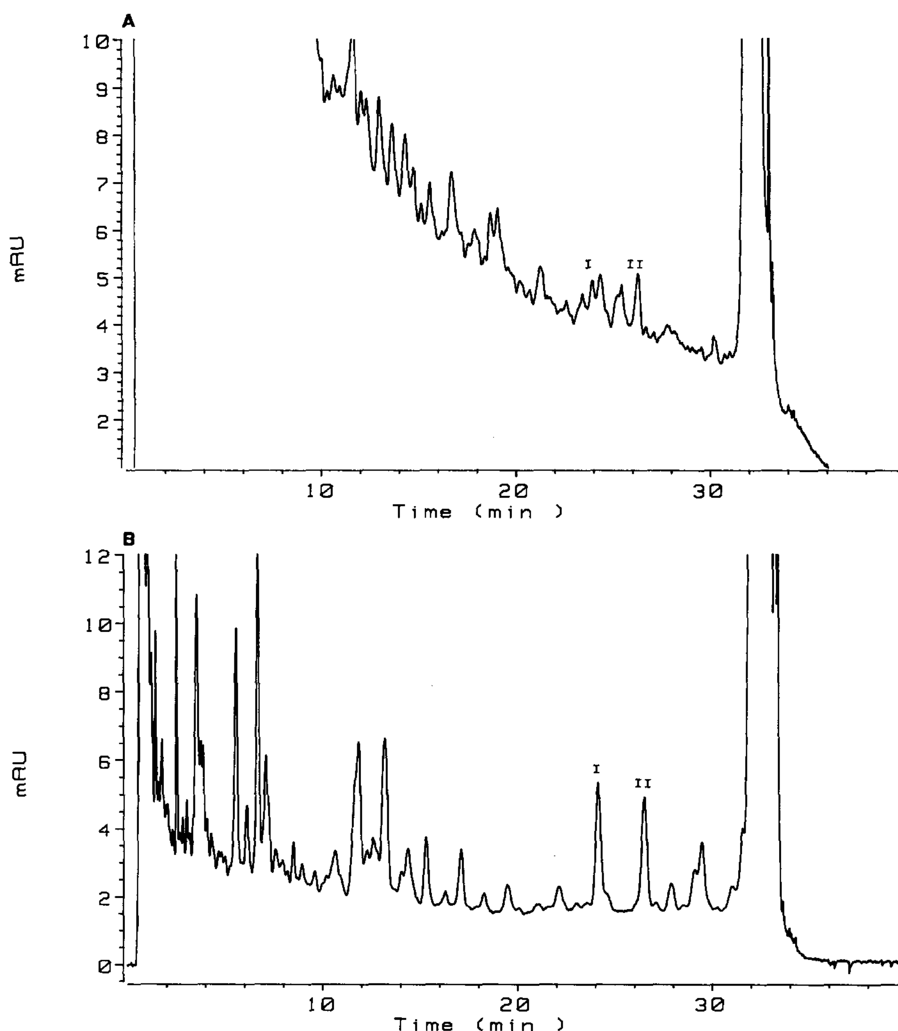

Fig. 3. (A) Chromatogram before the solid phase extraction. (B) Chromatogram after clean-up with a C_{18} cartridge. Peaks: I = diclazuril; II = internal standard.

TABLE I

REPEATABILITY, REPRODUCIBILITY ACCURACY AND LINEARITY AT THE 1 mg kg^{-1} LEVEL

	n	Concentration found (mean ± S.D.)	R.S.D.[a] (%)
Repeatability	6	1.00 ± 0.02 mg kg^{-1}	2.3
Reproducibility	6	0.95 ± 0.05 mg kg^{-1}	5.0
Accuracy[b]	14	92.7 ± 9.7%	10.4
Linearity (0.5–1.0–1.5 mg kg^{-1})			
Regression: $y = -0.33 + 1.26x$ $r^2 = 0.997$			

[a] R.S.D. = relative standard deviation.
[b] Diclazuril concentrations between 0 and 1.5 mg kg^{-1}.

liquid extraction period is at least 6 h it can be a time-saving step. The solid phase extraction step is necessary to remove most of the excipients (see Fig. 3). No loss of model compound was noted during the first wash step of the clean-up procedure. The liquid extraction and the solid-phase clean-up procedure gave for both model compounds I and II an absolute recovery of 85%.

Validation of the method

The linearity was checked at the anticipated level of 1 mg kg^{-1}. In the 50–150% range a good relationship between the peak-area ratio of diclazuril and internal standard and the injected amount of diclazuril was obtained. The equation of the linear regression line together with the correlation is given in Table I.

The accuracy can be evaluated through the analysis of samples spiked with known amounts of diclazuril. The tolerance for anticoccidials at this low level is 20%. The results in Table I show that the method is accurate at an extended level of the nominal concentration of 1 mg kg^{-1}.

From the point of view of system suitability and quality assurance it is advisable to analyse a control sample before each sample sequence. This sample may be a laboratory-made sample or a production batch containing a known concentration of diclazuril, preferably at the 1 mg kg^{-1} level.

The precision (repeatability) of the entire analytical method was measured by six replicate determinations of diclazuril by one analyst using the same liquid chromatograph. The reproducibility study was performed over a 4-week period by different analysts in different laboratories with different chromatographs. The results and statistical evaluation of this study are given in Table I.

In order to demonstrate the reliability of the method a comparative study was set up. Samples from different origins were analysed with a GC method [4] and with the LC method described. The results expressed in mg kg^{-1} are shown in Table II. The results of both methods were obtained by one single analysis of the sample.

The data of the precision and accuracy studies together with the results of the comparative test prove the ruggedness of the method.

TABLE II

ASSAY OF DICLAZURIL IN FEED SAMPLES; A COMPARATIVE STUDY OF LC AND GC

Sample origin	LC result (mg kg^{-1})	GC result (mg kg^{-1})
Columbia	0.88	0.89
Yugoslavia	0.20	0.20
Australia	0.69	0.71
Austria	1.04	1.01
Brazil	1.11	0.93
Venezuela	0.20	0.18
South Africa	1.54	1.53

CONCLUSION

A reliable method has been developed for the determination of diclazuril in animal feed at a concentration of 1 mg kg^{-1}. An extended extraction procedure is necessary to cover a wide range of feed samples.

ACKNOWLEDGEMENT

The authors are grateful to the quality assurance laboratory of the analytical research department for the follow-up of the method validation.

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