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Estimation of anthelmintic compound 81/470 in cow's milk by high-performance liquid chromatography: method development and validation[1]

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Abstract

CDRI compound 81/470 is a new broad spectrum anthelmintic agent and is being developed for veterinary use. HPLC assay method for 81/470 in cow milk was developed and validated. The sample preparation consisted of protein precipitation, followed by extraction with ether. Separation was achieved on a C_{18} column using acetonitrile–buffer (pH 6, 50 mM) mobile phase and the compound was quantitated using fluorescence detector. The recovery of 81/470 was above 90% and was consistent over the calibration range of 10–1000 ng ml^{-1}. Accuracy and precision were determined by analyzing replicate samples and were found to be within acceptable limits. Five freeze-thaw cycles of spiked milk and storage of processed dry residues at −30°C for 7 days did not have any detrimental effect on the stability of 81/470. © 1997 Elsevier Science B.V.

Keywords: Anthelmintic: 81/470; HPLC; Milk; Validation; Stability

1. Introduction

Compound 81/470 (methyl *N*-[5[[4-(2-pyridinyl)-1-piperazinyl]carbonyl]-1H-benzimidazol-2-yl] carbamate, Fig. 1) is a broad-spectrum anthelmintic [1–5], being promoted for veterinary use at CDRI, Lucknow. The compound, at present is in phase I clinical trial. In a field study, 81/470 has shown good anthelmintic efficacy in cattle (Dr J.C. Katiyar, Division of Parasitology, Central Drug Research Institute, Lucknow, April 1995, personal communication). When administered to cattle, it is essential to know whether the compound is excreted in the

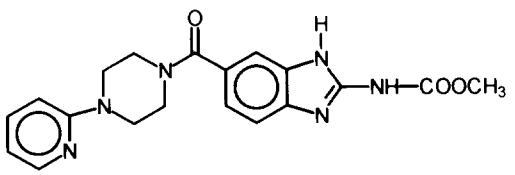

Fig. 1. CDRI compound 81/470.

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[1] CDRI communication number: 5548.

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milk and if so, to quantify it in order to establish the withdrawal period for consumption of the milk. An HPLC method for 81/470 in blood [6] has been reported, wherein, protein precipitation with acetonitrile or extraction with ether were used as alternative approaches to process the blood samples. This paper describes the development and validation of a sensitive, accurate and reproducible HPLC assay method for the quantitation of 81/470 in cow's milk, utilizing protein precipitation followed by extraction. Accuracy and precision, effect of freeze–thaw (f–t) cycles and storage of extracted residue on the stability of 81/470 were used as the parameters of validation.

2. Experimental

2.1. Materials

Pure reference standard (assay $\geq 99.9\%$) was obtained from the Pharmaceutics Division of CDRI. Acetonitrile (SD Fine Chemicals, Boisar, India), methanol (Merck, Bombay, India), and chloroform (Qualigens Fine Chemicals, Bombay, India) were of HPLC grade and used without further purification. Diethyl ether (anaesthetic grade IP, Kabra Drugs, Indore, India) was distilled before use. All other reagents were of analytical grade and were used without further purification. Normal cow's milk was purchased from the market and stored at 4°C and was used within seven days.

2.2. Instrumentation and chromatographic conditions

An HPLC pump (Kontron, Model 600, Zurich, Switzerland) was used to pump the mobile phase (acetonitrile + K_2HPO_4 buffer (50 mM, pH adjusted to 6 with orthophosphoric acid) 35:65, v/v) at a flow rate of 1 ml min^{-1}. The compound was monitored using a fluorescence HPLC monitor (Shimadzu, Model RF-535, Kyoto, Japan) set at excitation and emission wavelengths of 295 and 375 nm, respectively. A Philips computing integrator (Model PU 4811, Pye Unicam, Cambridge, UK) was used to measure the peak height. Separation of 81/470 from the endogenous milk components was achieved on a reversed-phase C_{18} column (Spheri-5, 5 μm, 220 × 4.6 mm I.D.) preceded by a guard column packed with the same material as in the analytical column (30 × 4.6 mm I.D.) (Pierce Chemical, Rockford, IL). Mobile phase was filtered and degassed before use.

2.3. Stock and standard solutions

A 100 μg ml^{-1} solution of 81/470 was prepared in a solvent mixture of chloroform and methanol (60:40, v/v). Standard solutions were prepared in the mobile phase from the stock solution in the range 10–1000 ng ml^{-1} by serial dilution method. Calibration standards of 81/470 were prepared in milk by adding specific volumes of stock solution to dry tubes and evaporating the solvent under a stream of nitrogen before adding the required volumes of milk to get the concentrations 10—1000 ng ml^{-1}. Care was taken not to vortex-mix milk samples vigorously to avoid the separation of the fatty layer. All the solutions were stored at 4°C and spiked milk samples at −30°C.

The degradation product of 81/470 formed by base hydrolysis, its *N*-decarboxylate derivative, was spiked along with 81/470 in the mobile phase and the milk and milk samples were processed as described in Section 2.4, for HPLC analysis.

2.4. Sample preparation

Blank or spiked milk (0.2 ml) was added with 0.6 ml of acetonitrile in a glass tube, vortex-mixed for 1 min and kept at 4°C for 30 min with occasional vortex-mixing. Then the samples were centrifuged at 2000 × *g* for 10 min at 0°C, and 0.4 ml supernatant was transferred into another glass tube and evaporated to dryness under vacuum in a SVC-200H Speed Vac concentrator (Savant Instruments, NY). The residue was vortex-mixed with 0.2 ml of K_2HPO_4 buffer (50 mM, pH 8) and was vortex-mixed for 1 min with 2 × 2 ml diethyl ether. After centrifuging the samples at 2000 × *g* for 10 min at 0°C the ether layer was separated by snap-freezing the aqueous layer. Ether was evaporated under vacuum and the residue was reconstituted in 0.2 ml mobile phase for injection into the

HPLC through a fixed 50 μl loop injector (Rheodyne, Model 7125, Cotati, CA).

2.5. Validation

2.5.1. Calibration curve

A calibration curve in milk in the range 10–1000 ng ml^{-1} was constructed. Standard curve data drawn from 5 different batches were pooled and both individual and pooled data sets were used to select the model. PCNONLIN software [7] was used to fit the data and study the effect of weighting the data with $1/x$ and $1/x^2$ on the residuals.

2.5.2. Accuracy and precision

Accuracy and precision were studied at low (lowest limit of quantitation, LLOQ, 10 ng ml^{-1}), medium (100 ng ml^{-1}) and high (1000 ng ml^{-1}) concentration levels. Four samples of each concentration were prepared and analyzed, and four such batches were assessed. Accuracy was expressed as %bias and precision as percent relative standard deviation (%R.S.D.). Acceptance limits of $\pm 20\%$ at LLOQ and $\pm 15\%$ at other concentrations in the calibration range [8] were used for the validation.

2.5.3. Effect of freeze–thaw (f–t) cycles on the stability of 81/470 in milk

Low (LLOQ), medium and high concentration samples (0.2 ml) were stored in sealed glass tubes at -30°C. One set of three concentrations containing four samples each was analyzed on day 1 (no f–t cycle) and other similar sets were analyzed after 1, 2 and 4 f–t cycles. Thawing was achieved by keeping the sealed tubes at room temperature for 30 min. The results of day 1 were taken as standard (100%) and the rest were compared with them as %deviation.

2.5.4. In-process stability of 81/470 in the dry extract at -30°C

The effect of storing the dry residue after extraction at -30°C on the stability of 81/470 was studied at low, medium and high concentration levels as mentioned above. One set (four samples of each concentration) was reconstituted with mobile phase and analyzed on the same day of preparation (day 1) and other tubes containing dry residue were sealed and stored at -30°C. Other similar sets were reconstituted and analyzed on day 2, 3, and day 7 after preparation. Data analysis was as described in Section 2.5.3.

2.6. Degradation of 81/470

Acid and base hydrolyses of 81/470 were carried out to identify the degradation products or putative metabolites of 81/470. For acid hydrolysis, the compound (100 mg) was dissolved in 1 M hydrochloric acid and heated at 60°C for 2 h. An aliquot of the reaction mixture, after adjusting the pH to 6 was diluted with mobile phase and injected into HPLC. For base hydrolysis, 100 mg of 81/470 was refluxed with 5% w/v potassium hydroxide in methanol for 24 h. After adjusting the pH to 6, the reaction mixture was diluted and injected into HPLC.

3. Results and discussion

The representative chromatograms of 81/470 and its *N*-decarboxylate derivative in mobile standard, blank and spiked milk and milk samples from a cow dosed with a solution formulation of 81/470 (20 mg kg^{-1}, oral route) are presented in Fig. 2. Chromatograms F and G represent a milk sample taken at 24 h and milk output from 48–60 h post-dose, respectively. Initially, the calibration standards were chromatographed with a mobile phase containing acetonitrile and buffer (25:75, v/v). Retention times were 11.0 ± 0.3 and 7 ± 0.2 min for 81/470 and the *N*-decarboxylate, respectively. Recovery of *N*-decarboxylate after sample processing was 50%. As the *N*-decarboxylate was not observed in any of the milk samples of one treated cow (in the pilot study), an assay method to estimate 81/470 alone (acetonitrile and buffer 35:65, v/v) was developed and validated. An increase in the sensitivity due to peak sharpening and a decrease in analysis time were the advantages gained by this optimization. Peaks of 81/470 and the decarboxylate were partially merged (retention times 5 ± 0.2 and 4.3 ± 0.1 min, respec-

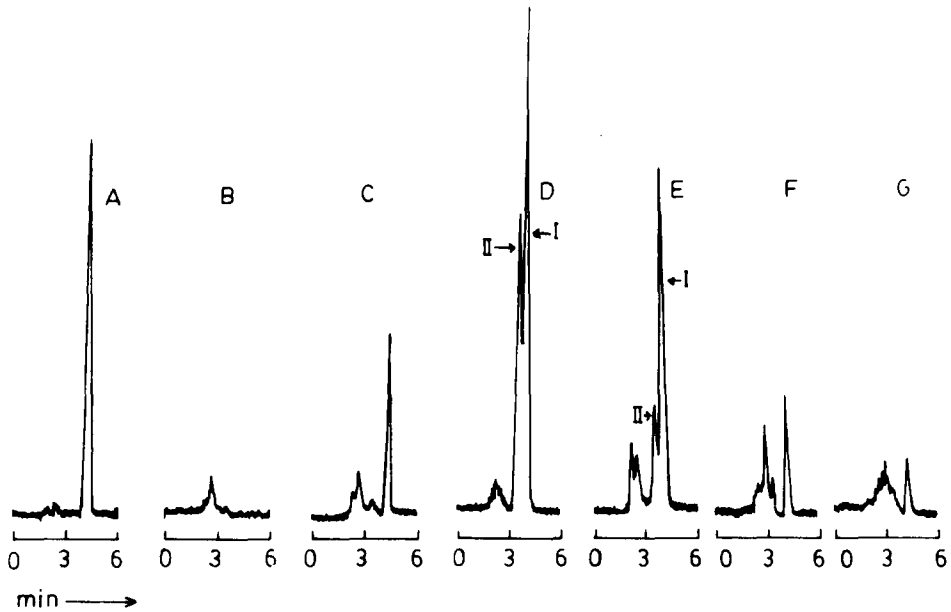

Fig. 2. Representative chromatograms of (A) mobile standard containing 100 ng ml^{-1} of 81/470; (B) blank milk; (C) milk spiked with 81/470 (100 ng ml^{-1}); (D) mobile standard and (E) milk containing 200 ng ml^{-1} each of 81/470 and its *N*-decarboxylate; (F) 24 h milk sample and (G) 48–60 h milk collected from a cow after an oral dose of 81/470.

tively) under these chromatographic conditions (Fig. 2D and E).

Sample injected after protein precipitation with acetonitrile showed an interfering peak at the retention time of 81/470 (5 min) and hence further extraction of the compound with ether was necessary. The resulting chromatograms of milk were clean and no interfering endogenous peaks were observed.

Both individual and pooled peak height versus concentration data in the mobile phase and milk were treated for linear regression analysis by the Nelder-Mead (simplex) method in the PCNONLIN package [7]. The convergence was achieved without any intercept on the y axis. Weighting on the concentration (x) with $1/x$ and $1/x^2$, though marginally minimizing the residuals at lower concentrations, destabilized the fitting at higher concentrations. Hence, the simple model $y = mx$ (where m is the slope of the line) was considered as the straight line equation to explain the generated data. A typical standard curve could be represented by the equation $y = 74.5x$. Linearity was observed in the range 10–1000 ng ml^{-1} with a coefficient of correlation $r > 0.999$).

3.1. Recovery

Recovery of 81/470 from the milk was calculated by comparing the peak heights obtained by the milk calibration standards with the calibration curve in the mobile phase. Recovery of 81/470 from milk at all the concentration levels studied was more than 90% (Table 1).

3.2. Accuracy and precision

These parameters were assessed by studying inter/intra-branch variations in concentrations of spiked milk samples calculated from the calibration curve in milk. Percent bias was calculated by the formula [8]

(overall mean calculated concn. – theoretical concn.)/theoretical concn. × 100.

Precision was measured as %R.S.D. by the formula [8]

Table 1
Recovery, overall accuracy and precision of the assay method for 81/470 in cow's milk

Concentration (ng ml^{-1})	Recovery (%)		%Bias ($n = 4$)	Precision (%R.S.D.) ($n = 4$)
	($n = 5$)	S.D.		
10	96.7	7.6	+6.13	18.78
25	103.8	7.3	—	
50	97.2	7.0	—	—
100	98.0	4.1	−6.5	6.31
500	93.4	5.7	—	—
1000	106.1	5.6	−2.11	9.93

$$\frac{\text{S.D.}}{\text{mean}} \times 100.$$

Overall %bias and precision at the three concentrations are presented in Table 1. The results show that the method is accurate and the bias lies within the acceptance limits of ±20% at LLOQ and ±15% at other concentrations. Similarly the precision was within ±15% at all the three concentration levels studied.

3.3. Effect of f–t cycles on the stability of 81/470 in milk

In-process stability and f–t stability of 81/470 in the milk matrix were assessed [9]. Concentration obtained from the spiked milk samples not subjected to f–t was considered as 100% and the concentrations calculated on analysis after subsequent f–t cycles were compared with the initial concentration. Results showed that 81/470 is stable upon 4 f–t cycles at all the three concentration levels (Fig. 3A). No extra peaks of degradation products, if any, were observed. The variations observed in these results were of the same order as inter/intra-batch variations in accuracy and precision and hence, it could be judged that the deviations observed in this study were not due to degradation of the compound but to the errors inherent within the analysis. No trend was observed in the calculated concentrations after different f–t cycles, indicating the stability of the compound in the milk matrix under these conditions.

3.4. In-process stability of 81/470 in the dry extract at −30°C

Results showed that 81/470 was stable in the dry extract form at −30°C even up to 7 days after sample preparation at all the three concentrations (Fig. 3B). No trend was observed and observed variations were interpreted to be due to inter/intra-batch variations which were within acceptance limits.

3.5. Effect of acid/base hydrolysis

The compound was stable upon acid hydrolysis and no additional peaks were observed after injecting the diluted reaction mixture. The degradation product of 81/470 by base hydrolysis was its *N*-decarboxylate derivative. By using the described sample processing method, *N*-decarboxylate could be detected in the spiked milk samples but, however, was absent in the treated cow's milk samples.

The HPLC method presented in this paper is simple, sensitive, reproducible and reliable. This method was applied to monitor the excretion of 81/470 in cow's milk after a single oral dose of 81/470. This demonstrates the efficiency of the method in monitoring the concentrations of 81/470 in cow's milk after dosing with 81/470, studying the pharmacokinetics and establishing the withdrawal period, based on parent compound concentrations, for the consumption of the milk.

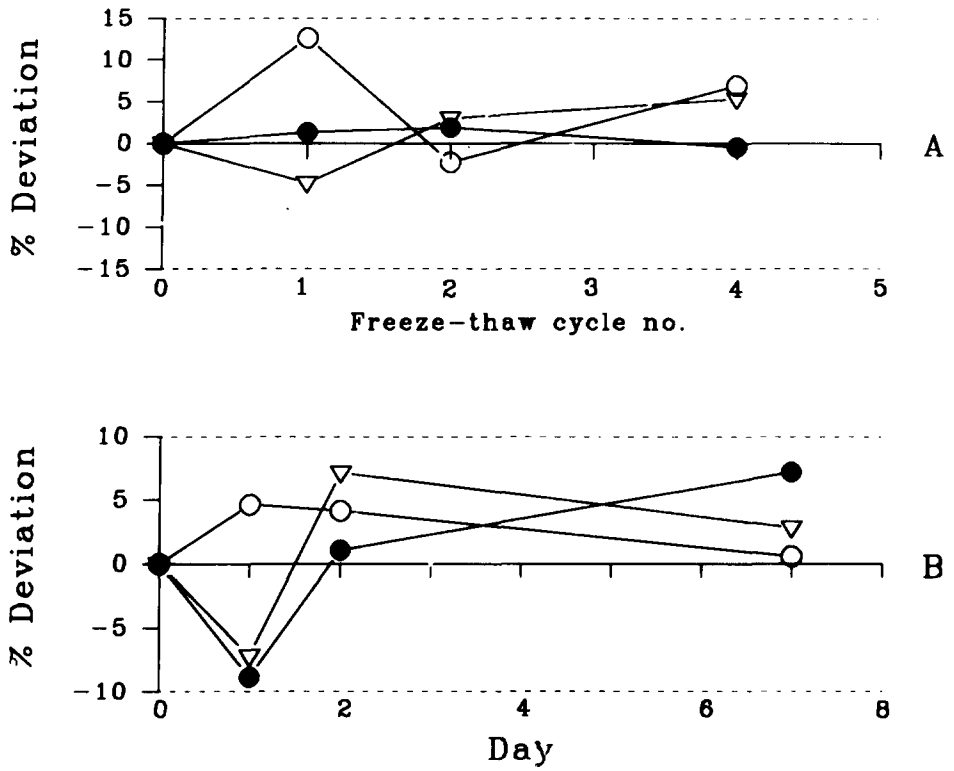

Fig. 3. (A) Effect of f–t cycles of milk and (B) storage of dry residue at −30°C on the stability of 81/470 (○, 10; ●, 100; ▽, 1000 ng ml^{-1}).

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