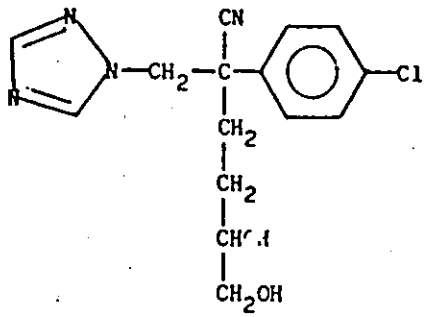
Table of Contents

Subject: RH-0294 Residue Analytical Method in Milk

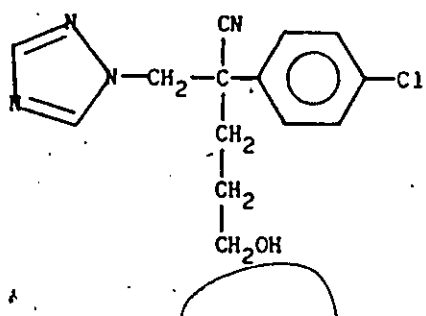
1. Introduction

This report details the RH-0294 residue analytical method in milk. RH-0294 is the major milk metabolite of RH-3866. RH-0294 is oxidatively cleaved to α-(4-chlorophenyl)-α-(2-formylethyl)-1H-1,2,4-triazole-1-propane nitrile in crude milk using potassium periodate. The milk is then centrifuged and the supernatant is collected. The aldehyde is then reduced to α-(4-chlorophenyl)-α-(3-hydroxypropyl)-1H-1,2,4-triazole-1-propane nitrile by addition of sodium borohydride to the supernatant. The product is then purified by Bio-Sil column chromatography. Quantitation is performed by gas liquid chromatography on a Megabore DB-17 column with electron capture detection.

2. Experimental Compounds



RH-0294



RH-66,647

3. Chemicals

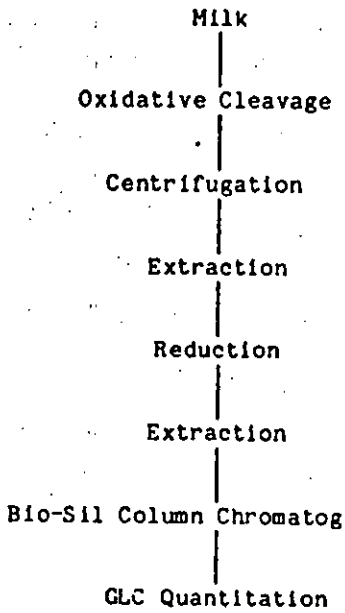
Item	Grade	Source
Acetone	Pesticide	Baker
Bio-Sil A, Mesh 100-200	Reagent	Bio-Rad
Cotton	Nonsterile	Fisher
Ethyl Alcohol/200 proof	Reagent	Pharmco
Ethyl Acetate	Pesticide	Baker
Methanol	Pesticide	Baker
P-10 Gas	HP	Air Products
Potassium Periodate	Reagent	Aldrich
Sodium Borohydride	Reagent	Aldrich
Sodium Sulfate, Anhydrous Granular	Reagent	Mallinckrodt
Toluene	Pesticide	Baker
Water	HPLC	Milli-Q Purification System

4. Equipment

Centrifuge:	Beckman, AccuSpin FR
Centrifuge Tubes:	Nalgene-500 mL, Nalge Co.
Columns:	Glass 250 mm length, 10.5 mm I.D., Teflon stopcock, 200 ml reservoir, Kontes
	GLC Megabore DB-17, 30 m length, J. and W. Scientific
Flasks:	Erlenmeyer, 200 mL, Arthur H. Thomas Co.
	Round bottom, 250 mL, 100 mL, Arthur H. Thomas Co.
Funnels:	Powder, 10 cm, Arthur H. Thomas Co.
	Separatory, 250 mL, Arthur H. Thomas Co.
Gas Chromatograph:	HP-5890, Hewlett-Packard
Graduated Cylinder:	10 mL, 100 mL, Arthur H. Thomas Co.
Pipets:	Glass, disposable, 10 mL, 5 mL, 1 mL, Arthur H. Thomas Co.
	Glass, Pasteur, Fisher Scientific
Rotary Evaporator:	Buchi Rotovapor R, Brinkmann

5 Method

5.1 Flow Chart



5.2 Oxidative Cleavage - Centrifugation

In a 200 mL centrifuge tube, add 10.0 g of milk and 6.0 g of potassium periodate. Shake well and let stand for 10 minutes. Add 10 mLs of ethyl alcohol, shake the sample, and then centrifuge for five minutes at 3000 RPM. Pour the supernatant into a 125 mL separatory funnel. Add 10 mLs of water to the precipitate in the centrifuge tube. Shake well, then centrifuge for five minutes at 3000 RPM. Combine the second supernatant in the separatory funnel containing the initial supernatant. Add 40 mLs of ethyl acetate. Partition and collect the ethyl acetate layer (upper phase) in a 250 mL round bottom flask. Evaporate to dryness by rotary evaporator at 50°C under diminished pressure.

5.3 Reduction

In the 250 mL round bottom containing the sample, add 10 mLs of water, 1.0 mL of methanol and 1.0 g of sodium borohydride. Swirl the flask and let stand for 15 minutes. Pour the flask contents into a 125 mL separatory funnel. Rinse the flask first with 10 mLs of water, followed by 20 mLs of ethyl acetate combining all fractions in the separatory funnel. Shake separatory funnel, pour the water phase (bottom layer) into another 125 mL separatory funnel and collect the ethyl acetate layer by passing it through 20 g of sodium sulfate (anhydrous) held in a 10 cm powder funnel plugged with cotton into a 250 mL round bottom flask. Partition the aqueous phase again with two 20 mL fractions of ethyl acetate combining all fractions in the same 250 mL round bottom. Evaporate the ethyl acetate fraction to dryness by rotary evaporator at 45°C at diminished pressure.

5.4 Bio-Sil Chromatography

Activate the Bio-Sil (100-200 mesh) by heating for 48 hours at 200°C. Remove from the oven and store in a tightly capped jar.

Pack a 25 cm x 10.5 mm I.D. glass column plugged with cotton with 15 mL (packed) of the activated Bio-Sil. Top the column with 1 g of anhydrous granular sodium sulfate.

Redissolve the residue from the ethyl acetate partition, section 5.3, in 20 mL of 5% acetone/toluene. Quantitatively transfer the solution to the column and elute to the top of the sodium sulfate layer. Wash the 250 mL round bottom flask with 20 mLs of 5% acetone/toluene (v/v), add the wash to the column, and elute to the top of the sodium sulfate layer. Add 20 mLs of 50% acetone/toluene (v/v), and elute to the top of the bed. Discard all washes. Elute the product with 60 mL of 90% acetone/toluene (v/v) into a 250 mL round bottom flask. Evaporate the acetone/toluene solution to dryness by rotary evaporator at 45°C under diminished pressure. The sample is ready for GLC analysis.

5.5 GLC Quantitation

5.5.1 Instrument and Conditions

GLC	Hewlett-Packard - 5890
Detector	^{63}Ni ECD
Column	Megabore DB-17, 30 m x 0.53 mm
Carrier Gas	P-10 (10% methane/argon)
Column Flow Rate	8.5 ml/min
Detector Purge Flow Rate	60 ml/min
Injector Temperature	240°C
Detector Temperature	325°C
Initial Temperature	240°C
Initial Hold Time	11 min
Program Rate	10°C/min
Final Temperature	255°C
Final Hold Time	12.50 min
Total Run Time	25 min
Injection Volume	3 ul

5.5.2 Data Reduction

Data was collected and integrated by a Nelson Analytical 4400 series data system consisting of Nelson Analytical Chromatography Software and Hewlett-Packard Model 200 modular computer.

5.5.3 Preparation of Standard Curve

Standard solutions of the final product α-(4-chlorophenyl)-α-(3-hydroxypropyl)-1H-1,2,4-triazole-1-propane nitrile in 8% acetone/toluene (v/v)) were prepared by serial dilution in the concentration range of 0.60 ug/ml - 0.10 ug/ml, to reflect RH-0294. The molecular weight conversion factor is 0.91. Three ul of each standard is injected. The standard curve of peak area vs. concentration is constructed. The standard curve is linear within the concentration range. Standard curves are prepared for each analysis day.

5.5.4 Quantitation

Three ul of the sample, section 5.4, is injected into the GLC. If necessary, the sample is diluted to an appropriate volume to give a response within the standard curve range. The peak area is computed and the concentration is determined by the data reduction system as follows:

Equation 1:

$$\frac{\text{Total Volume (mL)} \times \text{Concentration (ug/mL)} \times 100}{\text{Average Recovery (\%)} \times \text{Sample Weight (g)}} = \text{ppm}$$

5.5.5 Fortification Recovery

Samples are fortified with known amounts of RH-0294. Measure the peak area, determine ug/mL from the standard curve, and calculate percent recovery from equation 2.

Equation 2·

$$\frac{\text{(ug/ml Found) x Final Sample Volume (ml) x 100}}{\text{Fortification (ug)}} = \text{\% recovery}$$

5.5.6 Representative Chromatograms

Figures 1-6 illustrate standard chromatogram and the resulting standard curve. Figures 7-11 illustrate representative chromatograms of control and fortified samples.

5.5.7 Sample Calculations

Table 1 summarizes the data used in this example. For the fortification shown in Figure 8, a peak of area 173 corresponds to a concentration of 0.020 ug/ml from the standard curve. From equation 2·

$$\text{\% recovery} = \frac{\text{(0 020 ug/ml) x 10 ml x 100}}{\text{0.26 ug}} = 79\%$$

5.6 Method Sensitivity and Recovery

Fortifications of 0.013 ppm - 0.51 ppm exhibit 92% ± 13.50%. Table 2 details the recovery data.