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DETECTION AND QUANTIFICATION OF RIBOFLAVIN IN SOME VETERINARY FORMULATIONS USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

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Abstract

Vitamin concentrations in many drugs are impractical, leading to manifestations of vitamin deficiency in animals following recommended dosing. This study was conducted to detect and quantify riboflavin (VB2) in veterinary formulations by high performance liquid chromatography (HPLC). Analysis was performed using analytical HPLC reverse phase chromatography (Agilent 1260 Quadruple Pump Infinite Chamber) with a Zorbax ODS column (5 µ, C18, 4.6 × 250 mm ID), operated at 30 °C. The mobile phase consisted of 5% acetonitrile as solvent (A) and 95% water and 0.05 M formic acid as solvent (B) at a flow rate of 1.0 ml/min and automatic sample injection at 10.0 µl for 12 minutes. With UV detection at 280 nm, riboflavin is present in the samples at varying concentrations. The identification of compounds was performed by comparing their retention and UV spectra with vitamin standards. The results showed that VB2 (riboflavin) ranged from 1 – 11.9 mg/g. The vitamin concentrations in the veterinary formulation used in this study were higher than the actual concentration written on the leaflet. The recommended daily dose (RDA) by WHO and FAO for veterinary drugs for Cow, goats and Sheeps is 1.3 mg / day. The result shows that the riboflavin concentration (VB2L) written on the leaflet is different from the actual concentration (VB2A) present in the capsule and most of the veterinary drugs are higher than the RDA. There is need for quality control to make sure these concentrations written on the leaflet are the actual concentration present in the veterinary formulation.

Keywords: Veterinary Formulation, Vitamins, RDA, HPLC, Riboflavin

INTRODUCTION

Riboflavin (Vitamin B2) is an essential nutrient not just for humans, but also for animals. It plays a pivotal role in energy production, fat metabolism, and cellular growth and function in animals (Suwannasom *et al.*, 2020*)*.

In animals, riboflavin is crucial for energy production, being a precursor of the coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) which participate in various metabolic pathways (Squires, 2013). National Research Council (2012) states that "Riboflavin needs in livestock and poultry vary, but they're generally obtained from foodstuffs like grains, greens, and protein sources. Depending on the type of animal and its life stage, the riboflavin requirement will differ." Riboflavin deficiency in animals leads to symptoms such as growth retardation, ataxia, skin lesions, hair loss, and reproductive problems (Tallmadge and Miller, 2019). Aquatic Animals e.g Fish require riboflavin for growth and maintenance likewise deficiency in fish diets can result in poor growth, eye cataracts, and even increased mortality as reported by the National Research Council (2011).

Riboflavin is essential for reproduction in animals. Deficiencies can lead to embryonic mortality and reduced fertility (Ma *et al*., 2016). With the rise of the livestock industry, there's been increased interest in producing riboflavin through fermentation for animal feed supplementation (Abbas and Sibirny, 2011).

Beyond metabolic functions, riboflavin plays a role in maintaining animal health. Recent studies by Lei

and Miller (2013) have shown its potential for enhancing immunity and resistance to certain diseases.

To ensure that these veterinary formulations contain the indicated amounts of vitamins written on the leaflet, there is need for quality control. This research is aimed to detect the presence of the said vitamin and to quantify the multivitamin capsule to ensure that the actual concentration of Riboflavin present in the multivitamin capsule is the actual amount written on the leaflet of the multivitamin capsule pack as well as to identify if these companies are counterfeiting these vitamins. Quality control testing for these vitamins is therefore required.

Materials and Method

Reagents and Apparatus

HPLC and analytical grade reagents, HPLC grade water milli-Q Gradient, acetonitrile HPLC gradient and formic acid used were purchased from Sigma Aldrich. All solutions were prepared with HPLC grade water and glass wares used were carefully cleaned and dried. Vitamins Standard Riboflavin, Ammonium hydroxide solution and Sodium hydroxide pellet.

All measurements were taken using analytical balance. HPLC (Agilent 1260 quad pump infinity compartment), Ultrasonic bath Branson 5510, Vortex mixer Vortexer S0100A clever scientific, Centrifuge 80-2, Weighing balance mettler Toledo, Spatula, Measuring cylinder, Pestle and mortal, Volumetric flask, Beaker, Vials, glass Vials, Rubber Test-tube, Micropipette, Glass rod and Test tube rack.

Preparation of sample and solution

Brands of veterinary formulations from various pharmaceutical companies were purchased from the Abubakar Rimi market in Kano City. Veterinary drug formulas were coded as follows: INP, ATP, PWV, CMP, VVP, SSP and VEP.

The samples were grinded into a fine powder with a mortar and pestle. Fifty milligrams (50mg) of each sample were dissolved in 10 ml of HPLC grade water and vortexed for 1 min, homogenized samples were then degassed in an ultrasonic bath for 15 min. The mixture was then centrifuged for 5 min at 3000 rpm and then stored at 4°C for later use (Moreno and Salvador, 2000). Sodium hydroxide 0.1 g was weighed and dissolved in 100 ml HPLC grade water in a 100 ml volumetric flask.

Stock Standard Preparation

To prepare VB2 stock standard solution (riboflavin); the riboflavin standard (50 mg) was accurately weighed and dissolved in 10 ml of HPLC grade water in a 100 ml volumetric flask to obtain a solution of 5 mg/ml. Working standard solutions were prepared from stock standards on the day of use by diluting with HPLC grade water from the concentration of 0.005 mg/ml to 0.12 mg/ml. Standard solutions were stored at 4°C and prepared daily.

Chromatographic condition/Injection

The extracted sample that was stored at 4^0C were put in HPLC viles and then arranged in the sample tray which is attached to the HPLC, the samples are automatically injected into the auto sampler injector after setting the chromatographic condition. The chromatographic condition for the Reversed-phase Chromatographic HPLC with Zorbax ODS Column (5 µ, C18, 4.6x250 mm) was used: the mobile phase was acetonitrile 5 % as Solvent (A) and water 95 % and 0.05 % formic acid as Solvent (B). The column was operated at 30°C and the flow rate was 1.0 ml/ min, and the auto injection volume was at 10.00 µl for 12 min.

Detection was performed with UV-DAD detector at 280 nm for riboflavin (Antakli *et al*., 2015).

RESULTS AND DISCUSSION

In this study the riboflavin was identified using retention time and peak area match against those of the vitamin standards, while quantification was performed by means of peak area match against the standard.

The typical chromatograms of pure Standard and UV-Spectrum of VB2 were observed at 280nm for UV-DAD detector as presented in Fig 1. This demonstrated excellent separation of VB2 in 12.0 mins. The value of retention times was 7.1 mins for VB2, and the detection of the riboflavin in respect to the retention time and wavelenght was similar to the work of Borivoj *et al*., (2004) ; Pengfei *et al*., (2012).

Table 1 confirms the present of riboflavin in all the

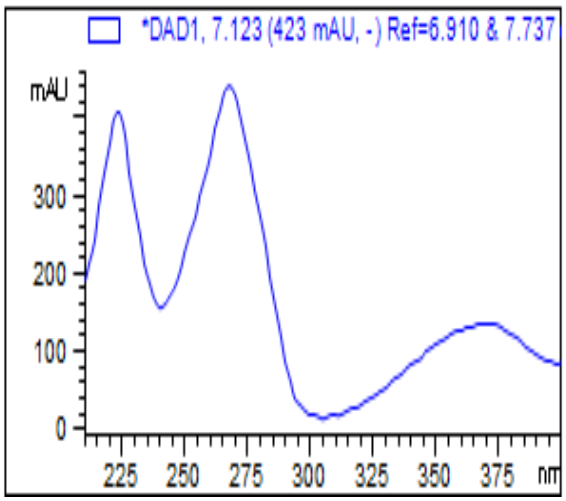
multivitamin capsules, they all absorbed at 280nm with retention time of 7.1 min. This also supported the findings of Hosain *et. al*. (2021) showing similar retention time and separation at 280 nm of Riboflavin.

Table 2 shows the statistical analysis of the mean concentration of the actual riboflavin present in the veterinary formulation with p value 0.001. In the veterinary formulation samples analyzed, it can be observed that the sample of veterinary formulation coded (INP) has the highest concentration of VB2A (11.9 ± 0.141 mg/g) among the various samples analyzed. This is followed by sample coded (SP) (8.95±0.014 mg/g) and sample of the veterinary formulation sample coded (CMP) has the least concentration of VB2A (1.0±0.141 mg/g) compared to the other samples analyzed

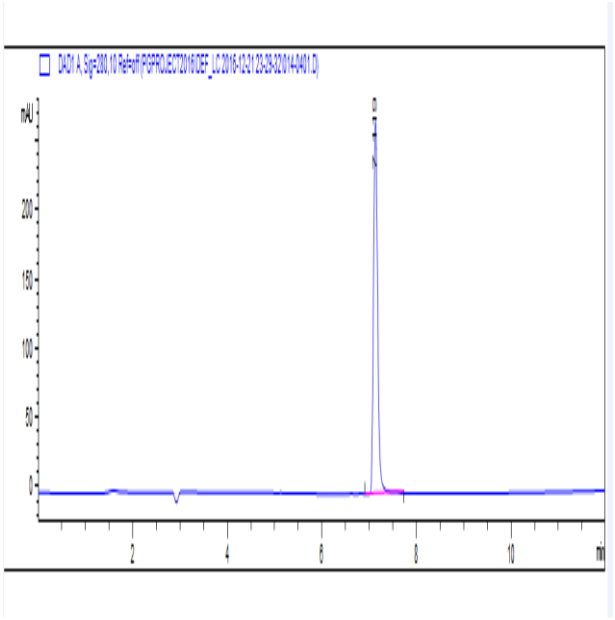
Table 3 shows the results for the actual conc. of Riboflavin and the label concentration of the riboflavin stated on the leaflet of the veterinary formulation pack. There was difference in the concentration of 0.1, 0.05, 0.4, 1.0, 0.3, 0.35, 1.52 respectively between the VB2L and VB2A. This shows that the companies hike the actual amount as compared to the amount on the label.

Table 1: Riboflavin (VB2) Veterinary Samples analyzed showing retention Time of at different concentrations using HPLC

Sample	Concentration (mg/g)	Retention time (min)	Wavelength (nm)	Absorbance (mAu)
IN-P	12	7.1	280	50
AT-P	4	7.1	280	50
VO-P	4.8	7.1	280	50
CM-P	2	7.1	280	50
V-P	2.5	7.1	280	50
S-P	9.3	7.1	280	50
VE-P	4.8	7.1	280	50



(a)



(b)

Fig 1: UV-Spectrum of (a) VB2 (Riboflavin) and (b) VB2 (Riboflavin) Pure Standard Chromatogram

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VO-P	4.8	7.1	280	50
CM-P	2	7.1	280	50
V-P	2.5	7.1	280	50
S-P	9.3	7.1	280	50
VE-P	4.8	7.1	280	50

Table 2: Mean Concentration of VB2 (riboflavin) in veterinary formulation

S/N	SAMPLE CODE	MEAN CONC. ± STD OF VB2A (mg/g)	P-Value	F- Value
1	INP	11.9 ± 0.141		
2	ATP	3.95 ± 0.071		
3	VOP	4.4 ± 0.424		
4	CMP	1 ± 0.141	0.001	194.453
5	VP	1.2 ± 0.283		
6	SP	8.95 ± 0.014		
7	VEP	3.28 ± 0.028		

Riboflavin Actual conc. (VB2A), STD Standard deviation

Table 3: **Sample of various brands of riboflavin supplement with their label and Actual concentration**

S/N	Sample	WT. (mg)	VB2L Conc. (mg)	**VB2A** Conc. (mg)
1.	IN-P	1000	12.00	11.9
2.	AT-P	1000009	4.00	3.95
3.	VO-P	100000	4.80	4.40
4.	CM-P	100000	2.00	1.00
5.	V-P	100000	2.50	1.20
6.	S-P	100000	9.30	8.95
7.	VE-P	100000	4.80	3.28

Weight of Rivofllavin (WT), concentration (Conc.), milligram (mg). Rivoflavin Label (VB2L) Conc. Rivoflavin Actual

Weight of Rivofllavin (WT), concentration (Conc.), milligram (mg). Rivoflavin Label (VB2L) Conc. Rivoflavin Actual (VB2A) Conc

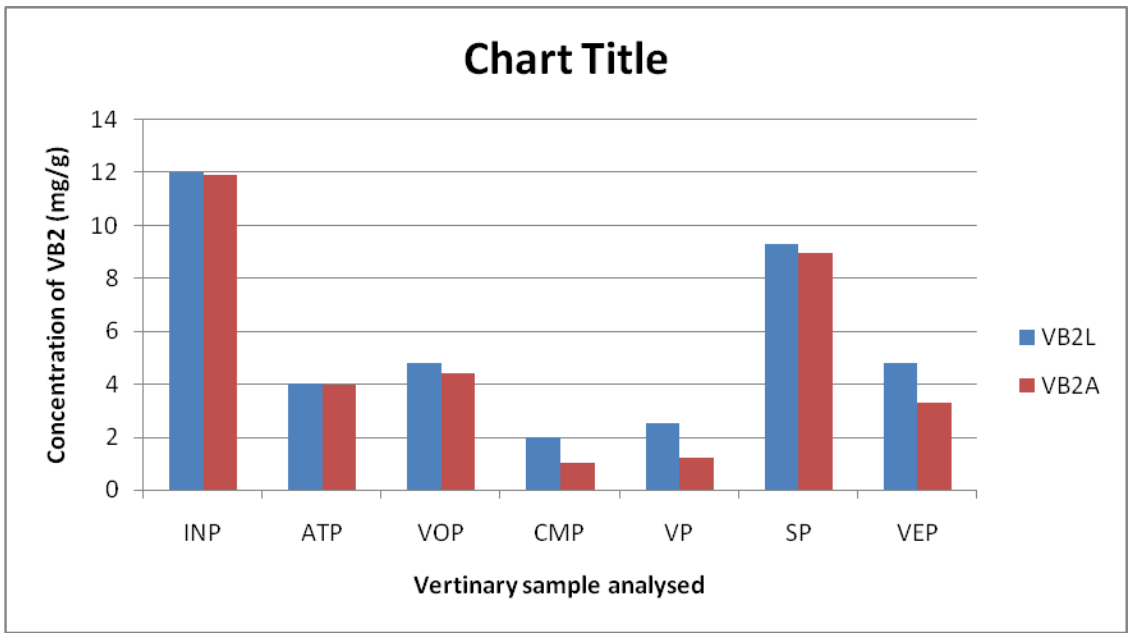

Fig 1: Amount of VB2 (Riboflavin) mg/g in Veterinary formulation

CONCLUSION

The results revealed that VB2 was found in the range of 11.9 ± 0.141 – 0.7 ± 0.14 mg/g. This shows that there was a significant difference between the label concentration and the actual concentration of VB2 veterinary formulations. This calls for proper and adequate quality control test of the pharmaceutical companies producing this formulation. There is need for serious quality control measures to be implemented so as to reduce this problem. Riboflavin plays a pivotal role in energy production, fat metabolism, and cellular growth and function in animals (Suwannasom *et al*, 2020). Riboflavin deficiency in animals leads to symptoms such as growth retardation, ataxia, skin lesions, hair loss, and reproductive problems (Tallmadge and Miller, 2019). Deficiency of this vitamin may lead to reduced feed intake, low performance, dermatitis and skin lesions, retarded embryonic development, retarded growth and nervous disorders like leg paralysis and curled toes in poultry (FEFANA, 2014).

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