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RESEARCH ARTICLE

ANALYSIS OF PHARMACEUTICAL DRUG PRESERVATIVES IN CATTLE DUNG BY GC-MS: AN ASSESSMENT TOOLTO ESTIMATE VETERINARY MEDICINE LOAD ON CATTLE

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Abstract

In villages farmers often use synthetic hormones and antibiotics without suggestion of medical practitioner. Government restricted the usage of synthetic hormones without prescription of medical practitioner but farmers use these hormones for enhanced production of milk in cows and buffaloes. As of now there is no scientific approach for the detection of usage of synthetic hormones in animals. HPLC, GC, LCMS, GCMS and ELISA are the widely using methods to detect or quantify hormones. Unfortunately these methods cannot give us a clue regarding the origin of hormone. As synthetic hormones have preservatives, the administrated hormones will be utilised by animals and the preservatives will be excreted by animal through dung and urine.Preservatives are the chemicals that are used in drug products to prevent decomposition by microbial growth or by undesirable chemical changes and detection of these preservatives may give us a clue on usage of synthetic hormones. Our GCMS analysis of HPLC fractions proved that these preservatives are excreting in dung and our analysis found Benzoic acid, Benzothiazole, Benzoyl-2-ethoxymethyl benzene, Benzyl Benzoate, Glycerol, Levulonic acid, Phenazinol, Phenol, Propanoic acid and Succinic acid in Dung extracts. Our study provided a clue to investigate synthetic hormones by assessing preservatives using dung and it can be further investigated to evaluate the level of synthetic hormone and medicine load on cattle and their impact on human health.

Introduction:-

Drug Product is a finished dosage form that contains a single or multiple active pharmaceutical ingredients or drug substances along with the excipients or inactive ingredients. Drug substance or active pharmaceutical ingredient is any substance or mixture of substances that are used in the manufacturing of a drug product which are having significant effect in the diagnosis, mitigation, cure, prevention and treatment of disease to affect the structure or function of the body. Inactive ingredients or excipients are the substances that are formulated along with the active ingredient of a medication which are intended for the purpose of long-term storage or stabilization of the active substance orenhanced activity of active substance or for improved physicochemical activity of active substances [1]. Excipients may include fillers, wetting agents, extenders, emulsifiers, solvents, diluents, flavors, adjuvants, anti-adherents, colouring agents, binders, absorption enhancers, sustained release matrices, coatings, disintegrants,glidants, sorbents, sweeteners and preservatives [2, 3].Preservatives are the chemicals that are used in

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drug products to prevent decomposition by microbial growth or by undesirable chemical changes [4,5].Antioxidants such as vitamin A, Vitamin C, amino acids such as cysteine, methionine, mild acids such as citric acid, sodium citrate, benzoic acid, benzoates and synthetic chemicals such as paraben, methyl paraben and propyl paraben are widely using as preservatives in pharmaceutical formulations. In veterinary medicine benzyl alcohol,chlorbutol,benzyl benzoate, casteroil,arachis oil benzoic acid, benzoate,methyl paraben, propyl paraben and succinate are widely using as preservatives. Veterinary practitioneroften uses antibiotics to treat cows and buffaloes to control infections and depending on requirement they suggest synthetic hormones to animals as part of treatment. In villages farmers often use synthetic hormones and antibiotics without suggestion of medical practitioner. Government restricted the usage of synthetic hormones without prescription of medical practitioner but farmers use these hormones for enhanced production of milk in cows and buffaloes. Generally the hormones and antibiotics that are administrated to animals will be utilised by animal and the injected drug substance may penetrated to milk. Consumption of such milk will impact the human health. In Most of the cases usage of gender specific hormones such as progesterone, estrogen and oxytocin will enter the human food chain and impact the human health. Due to the usage of these synthetic hormones in cattle, prematurity of women and enhanced levels of female sex hormones in males are observing frequently, it will be a serious concern if we cannot control the usage of synthetic hormones in cattle. Frequent usage of antibiotics, anti-fungal and anti-viral drugs in cattle also impacts the human health if they enter into human food chain. As of now there is no scientific approach for the detection of usage of synthetic hormones in animals. HPLC,GC,LCMS, GCMS and ELISA are the widely using methods to detect or quantify hormones [6,7]. Unfortunately these methods cannot give us a clue regarding the origin of hormone. As synthetic hormones have preservatives, the administrated hormones will be utilised by animals and the preservatives will be excreted by animal through dung and urine. By considering this fact we worked to detect the preservatives in cattle dung which may give us the information about the pharmaceutical drugs load on animals.

Materials and Methods:-

Sample collection:

Dung samples were collected carefully from Cows from local dairy farms located at Hyderabad in 4 × 6 inches zip lock bags. Collected samples were placed in Thermocol box with ice packsand stored at -20°C in laboratory till the extraction of metabolites [6].

Extraction of Metabolites:

Prior to the extraction of metabolites, all samples are allowed to reach room temperature. 1±0.05g of dung sample was weighed in 15ml falcon tubes and added with 5 ml of 90% methanol (Methanol: Qualigens, Milli Q water). Samples were vortexed to mix well for 30 seconds by using vortexer and centrifuged at 1500g for 5 minutes. Pelletwas discarded and supernatants were collected in 5ml cryogenic vials (CORNING®). Vilas were sealed with para film and stored at -20°C till further processing [6].

HPLC analysis:

Particles present in extracted metabolite solution were centrifuged to settle down and supernatant was diluted to 2fold with Milli Q water filtered through 0.2µ syringe filters (Millipore). HPLC analysis was performed with Waters alliance e2695 HPLC system equipped with 2998 PDA detectors and Empower 3 Software with the following method conditions [6].

HPLC Method	
Run time	20 minutes
Mobile phase	Acetonitrile and Milli Q water
Gradient programme	Programme starts with 30% acetonitrile, reaches to 50% by 3rd minute and reaches to 100% by 8th minute. 100% acetonitrile continue till 14th minute, reaches to 30% by 18th minute and continue till 20th minute.
Flow rate	1mL per Minute
Sample injection volume	100µL
Sampler temperature	15±5°C
Column	Phenomenex C18 Kinetex 2.6µm 100A° (150*4.6mm)
Column temperature	35±5°C
Detector wavelength	194nm, 214nm, 240nm, 254nm, 280nm and 310nm.

Table 1:- HPLC programme parameters to analyse fecal metabolites.

Fractions collection:
HPLC eluted components were collected at detector outlet as manual fractions in glass vials. Eluted fractions were completely evaporated in an oven at 100°C. Fractions were dissolved in methanol and stored at -20°C till further analysis [7].

Analysis of components by GC-MS:
Silylation Procedure: During sylilation procedure, methanol was evaporated to dryness using nitrogen gas. To the residue, 100µL Acetonitrile and 100µL BSTFA (silylating reagent) were added and heated at 60°C for 1 hour. Samples were submitted to IICT (CSIR-IICT, Hyderabad, India) for further analysis.

GC-MS-MS analysis procedure at IICT: Sample was evaporated and dissolved in 0.5 mL acetonitrile at the time of GC-MS analysis. Analysis was performed in Agilent 6890 Gas chromatography equipped with HP-5MS capillary column (30m length, 250µm internal diameter and 0.25µm film thickness) and 5973N mass selective detector installed with chemstation software (Agilent Technologies, Palo Alto, CA, USA). 1µL sample was injected and analysed with the following method parameters and results were compared in NIST library to identify compounds [7].

Run time	30 minutes
Column initial temperature	50°C with 2 minutes hold-up time
Column final temperature	280°C with 5 minutes hold-up time
Temperature ramp	10°C/minute
Carrier gas flow	Helium gas at 1.2mL flow rate/minute constant flow
Inlet temperature	250°C
GC-MS interface temperature	280°C
Sample injection mode	Split mode with 10:1 split ratio
EI source temperature	230°C
Quadrapole analyser temperature	150°C
MS scan range	m/z 29 to 600

Table 2:- GC-MS programme parameters to analyse sample fractions.

Results and Discussion:-

Hormones are the chemical precursors of the animals that are responsible for major biological process. Hormones produced by animals will be consumed by body and metabolised after their role in body. Metabolised hormones are excreted by dung and urine. Recent research proved that hormones will penetrate into milk in cattle. Consumption of such milk is causing unusual health problems in mankind. So it is very important to monitor the usage of veterinary hormones in the treatment of cattle because by excess administration, hormones will enter into the milk and impact our health. Even Government banned the usage of artificial hormones without proper prescription from veterinary doctor but in ground reality it is not effectively monitored because of lack of identification between naturally produced and synthetic hormones. Preservatives are the additives to drug substances to formulate drug products to avoid degradation and alteration of drug substances. In recent days most of the veterinary medicine is formulated with preservatives especially veterinary hormones. Detection of these preservatives may give us the information of usage of synthetic hormones and it may be useful for us to assess the usage of medicines load on cattle. Our GCMS analysis of HPLC fractions proved that these preservatives are excreting in dung and our analysis found Benzoic acid, Benzothiazole, Benzoyl-2-ethoxymethyl benzene, Benzyl Benzoate, Glycerol, Levulonic acid, Phenazinol, Phenol, Propanoic acid and Succinic acid in Dung extracts.

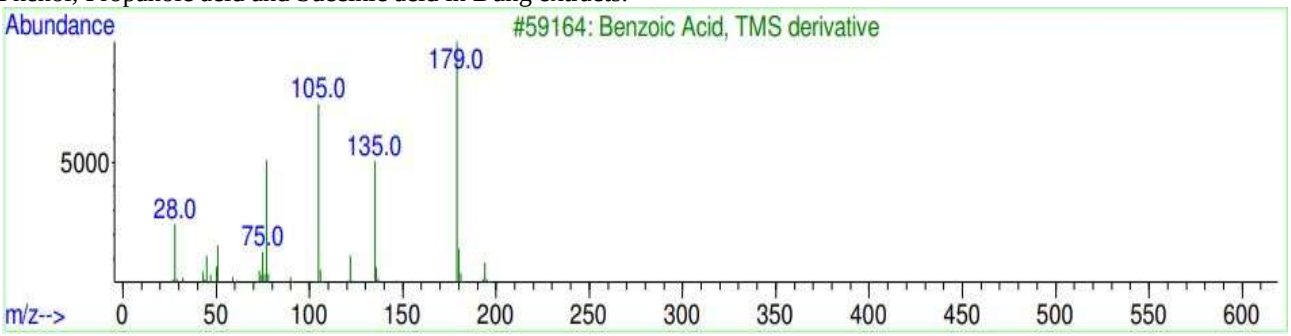

Figure 1:- GC MS Identification of Benzoic Acid in Dung extracts.

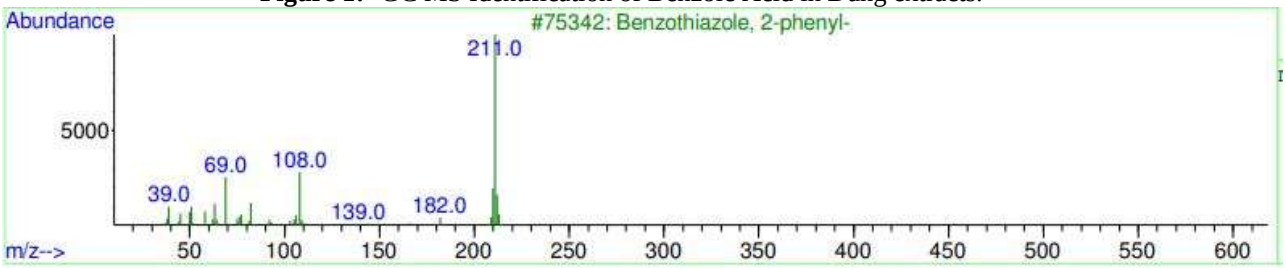

Figure 2:- GC MS Identification of Benzothiazolein Dung extracts.

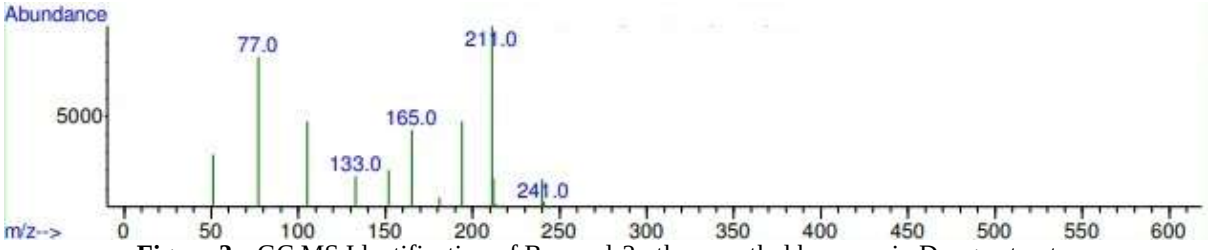

Figure 3:- GC MS Identification of Benzoyl-2-ethoxymethyl benzene in Dung extracts.

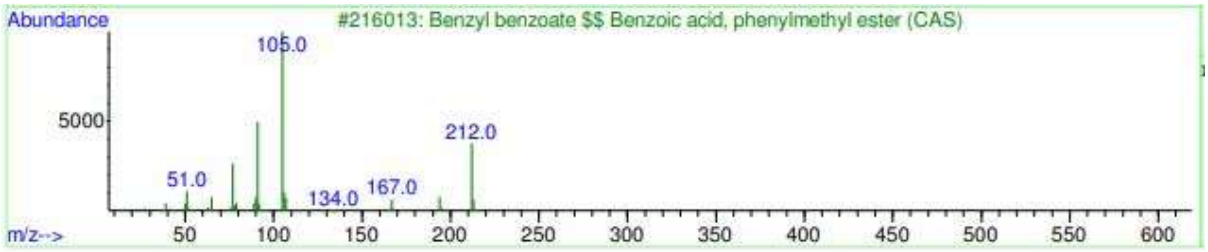

Figure 4:- GC MS Identification of Benzyl Benzoate in Dung extracts.

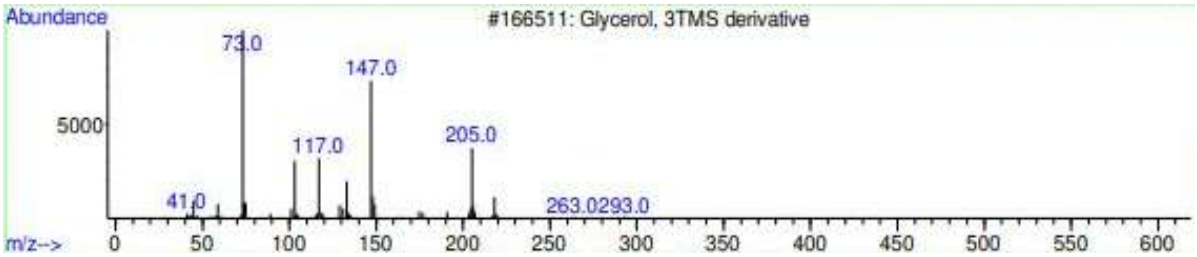

Figure 5:- GC MS Identification of Glycerol in Dung extracts.

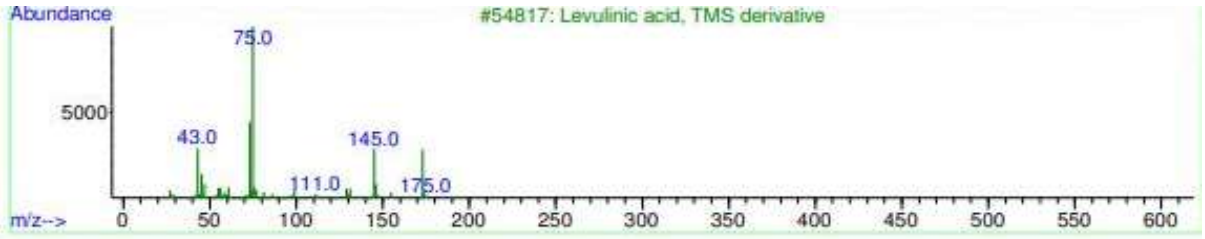

Figure 6:- GC MS Identification of Levulonic acidin Dung extracts.

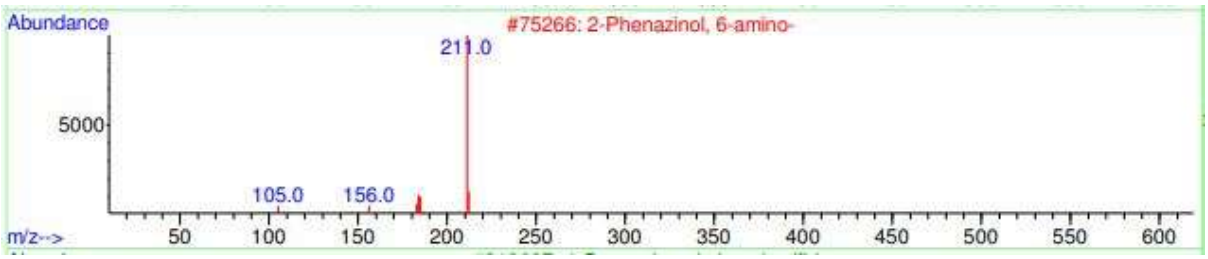

Figure 7:- GC MS Identification of Phenazinolin Dung extracts.

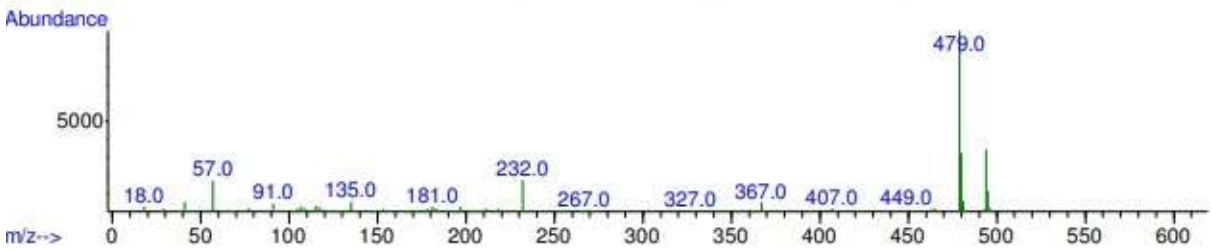

Figure 8:- GC MS Identification of Phenol in Dung extracts.

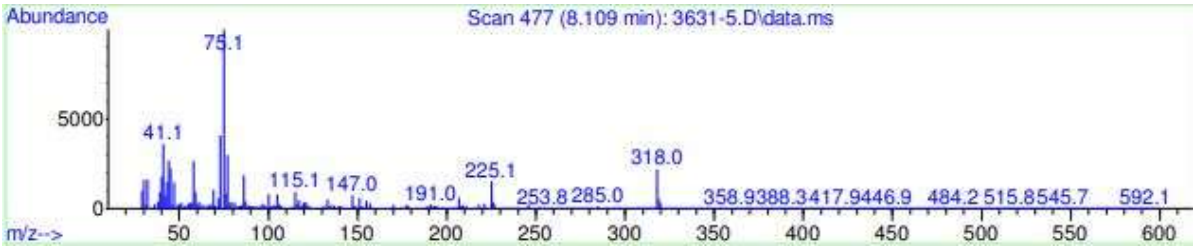

Figure 9:- GC MS Identification of Propanoic acid in Dung extracts.

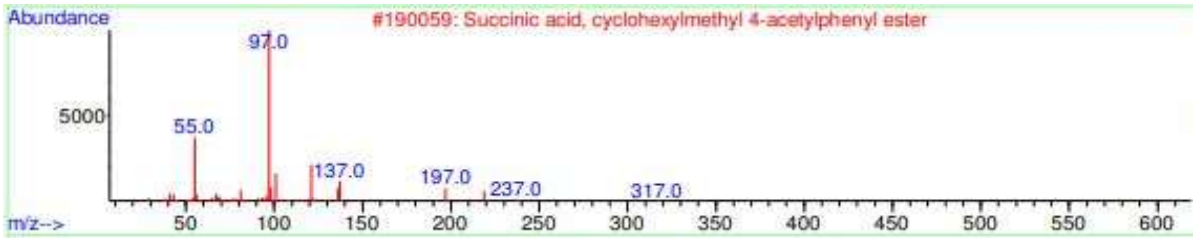

Figure 10:- GC MS Identification of Succinic acidin Dung extracts.

Hydroxyprogesterone, Progesterone, Testosterone, Isoflupredone, Prednsolone and Burselin are the commonly used formulations in cattle and they were formulated with Benzyl alcohol and its derivatives as preservatives. Our GCMS analysis detected Benzoic acid, Benzothiazole, Benzoyl-2-ethoxymethyl benzene and Benzyl Benzoate in dung extracts. Recent research raised safety concerns on the usage of those preservatives and their impact on the animal and human health. As there is a chance of biomagnfication in food cycle and these preservative may enter in our food cycle through milk and they impact our health [8]. So the usage of medicines and hormones need to be monitored carefully in cattle.

Conclusion:-

Cattle play a crucial role in human diet and the cattle milk play a crucial role on our health. Usage of synthetic hormones in cattle may lead to the milk with unwanted hormones and its consumption will impact the human health. As of now very limited information is available on the identification of natural hormones and synthetic hormones. Our study focussed to understand the feasibility of preservative assessment by non-invasive methodsby assessing dung samples. Our study provided a clue to investigate synthetic hormones by assessing preservatives using dung

samples collected from cattle and it can be further investigated to evaluate the level of synthetic hormone and medicine load on cattle and their impact on human health.

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Declaration of interests:

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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