Validated Reverse Phase HPLC-UV Method with C18 Kinetex Column to Quantify Nine Commercially Available Veterinary Hormones.

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ABSTRACT

In cattle, assays were developed to monitor hormones levels in plasma, milk, urine and dung by immunoassays and chromatography. Immunoassays offer us to estimate the single hormone levels in an assay whereas chromatographic assays offer us the estimation of multiple hormone levels in an assay. Current work is aimed to validate the developed noninvasive HPLC assay to estimate the fecal hormone metabolites of estradiol, progesterone, cortisone, testosterone along with the commercially available cattle drugs such as as Dinoprost tromethamine, Dexamethasone, Oxytocin, Isofluredone and Clopostenol with a single sample. The developed method successfully proved accuracy, specificity, precision, linearity and range. The developed method can be uses to estimate estradiol, dexamethasone,oxytocin, isoflupredone, clopostenol in nanogram/mL concentrations, dinoprost tromethamine in picogram/mL concentrations and progeterone, hydrocortisone, testosterone can be estimated in microgram/mL concentrations.The validated method can be used for the quantification of hormones to asses breeding cycles, pregnancy status, stress levels and health status.

Keywords: Cattle; HPLC; Steroids; Progesterone; Estrogen; Testosterone; Cortisol; Noninvasive hormone assay.

INTRODUCTION

In cattle, assays were developed to monitor hormones levels in plasma, milk, urine and dung by immunoassays and chromatography. Immunoassays offer us to estimate the single hormone levels in an assay whereas chromatographic assays offer us the estimation of multiple hormone levels in an assay. O R Kling et al., (1982) developed a reverse phase chromatographic method to quantify estrogens, androgens and progestogens by HPLC [1]. Erkoc FU et al., (1989) developed an isocratic chromatographic method to qualify and quantify estrones, testosterone and progesterone [2]. In felids, Brown JL et al., (1994) elucidated the fecal E2 and P4 levels by HPLC and GCMS during reproductive cycle and pregnancy [3]. In elephants, Dehnhard M et al., (2003) predicted parturition by analyzing urine hormone levels by GCMS [4]. Shan Zhao et al., (2004) developed a single sample assay to determine sexual hormones in cosmetics by HPLC [5]. After analyzing fecal estrogen and progesterone levels of pregnant and pseudo pregnant Eurasian lynx, Dehnhard M et al., (2008) hypothesized that chromatographic methods were more suitable to predict pregnancy [6]. Phyllis Wilson (2009) developed and validated a chromatographic method to assess the potency of estrogens and progesterone combination drugs [7]. Xiaofang Wang (2009) developed an isocratic HPLC method to quantify sexual hormones in essential oil [8]. Chengjun Wang (2011) determined naturally occurring estrogens in environmental river and lake water [9]. Na Liu et al., (2015) determined the presence of estrone, estradiol, estriol and 17α-ethynyl estradiol in feces, water samples collected at livestock farms and environmental matrices by HPLC coupled with Fluorescence detector [10]. Yasser Shahbazi et al., (2016) estimated meat estrone levels in cows and buffaloes to distinguish follicular, luteal phases and to evaluate the heating process effect on steroid hormone concentration by HPLC FLD [11]. Xiangning Han et al., (2019) demonstrated a UHPLC-MS method to determine endogenous and exogenous steroid hormones in Antarctic krill [12].

Developed HPLC methods were offer us to monitor hormones levels in animals to assess their health and breeding status. During pregnancy and hormone deficiency, Animals will be treated with commercially available hormones. Dinoprost tromethamine, Dexamethasone, Hydroxyl cortisone, Oxytocin, Isoflupredone and Clopostenol are widely used in cattle for treatment. Dinoprost tromethamine is the widely available prostaglandin used to improve the efficiency of artificial insemination. Dexamethasone is a corticosteroid used to control the inflammatory conditions and for the treatment of primary ketosis in post-partum dairy cows. Hydroxy cortisone and isolupredone are used as anti-inflammatory drugs. Oxytocin is used to induce milk let-down in cattle with proper physical state udder by contracting the smooth muscle cells of the mammary gland. Clopostenol is used to induce parturition and control the breeding pattern in cattle. To test these commercially available hormones, manufacturers use multiple compendial methods which are validated to establish their Limit of detection, Limit of Quantification, Specification, Range, linearity, Precision and accuracy [13-17]. We developed a noninvasive HPLC assay to screen Hormone metabolites in cattle dung which will offer us to evaluate estradiol, progesterone, cortisone, testosterone along with Dinoprost tromethamine, Dexamethasone, Oxytocin, Isoflupredone and Clopostenol. Current work is designed to establish the validity parameters for the assay to quantify above hormones with single analysis.

MATERIALS AND METHODS

Sample Preparation:

Commercially available veterinary hormones estradiol (Zydus Healthcare Ltd, B.No: S903005), progesterone (Akums Drugs and Pharmaceuticals Ltd, B.No:FGY0015), hydrocortisone (Alkem Lboratories Ltd, B.No:HSP02B), testosterone (Zydus Healthcare Ltd, B.No: G004696), Dinoprost tromethamine (Vvaan Lifesciences Pvt Ltd. B.No: SJ20-67), Dexamethasone (Zydus Animal Health and investments Ltd. B.No: G/28A/2755-A), Oxytocin (Laborate Pharmaceuticals India Ltd. B.No: LLNI-004), Isoflupredone (Zydus Animal Health and investments Ltd. B.No: ISIR2128) and Clopostenol (Montage Laboratoried Pvt Ltd. B.No: MT-239) were stored at 2-8°C till analysis. Samples were diluted with 90% methanol (Methanol: Qualigens, Milli Q water) to get the required concentrations.

HPLC analysis:

HPLC analysis was performed with Waters alliance e2695 HPLC system with 2998 PDA detector and operate with Empower 3 Software. Diluted samples were further diluted to 2fold with Milli Q water and filtered with 0.2μ syringe filters (Millipore) and analysed with the tabulated method conditions (Table 1) to check specificity, accuracy, precision, linearity, range, LOD and LOQ of the method.

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 hormones.

All the samples diluted to 2-fold with water were injected in HPLC and analysed to find out the RT of the compounds for specificity parameter and the samples were diluted, mixed together to get the tabulated concentrations (Table 2) for accuracy, linearity, LOD, LOQ, range and robustness of the method. Each parameter of the method was performed six times to conclude the result.

Analyte	Concentrations range
Estradiol	100, 200,500,1000,5000,10000 ng/mL
Progesterone	0.1,1,2.5,5,7.5,10 µg/mL
Hydrocortisone	2.5,25,50,100,200,250 µg/mL
Testosterone	3.75, 37.5, 75,150,250,375 µg/mL
Dinoprost tromethamine	100,200,400,1000,10000,100000 pg/mL
Dexamethasone	33, 66,330,660,3300,6600 ng/mL
Oxytocin	6.66, 16.66, 83.3, 166.6, 833,1666 ng/mL
Isoflupredone	4,8,40,400,4000 ng/mL
Clopostenol	10,25,125,250,1250,2500 ng/mL

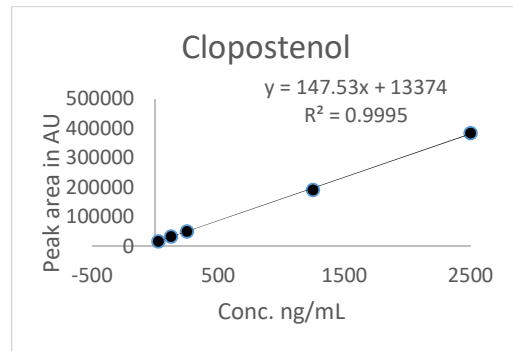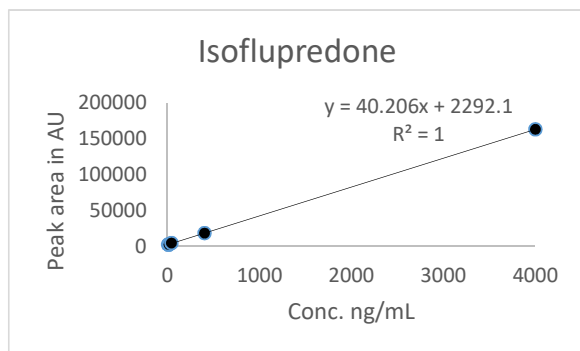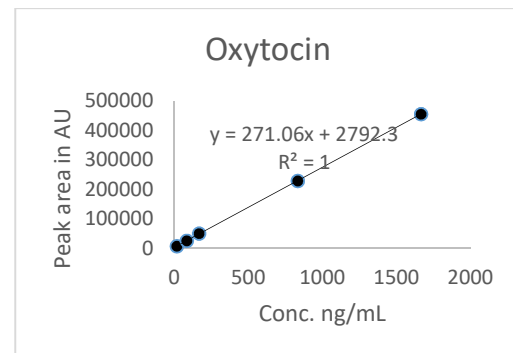Table 2: Concentrations of the samples prepared to verify method validation parameters.
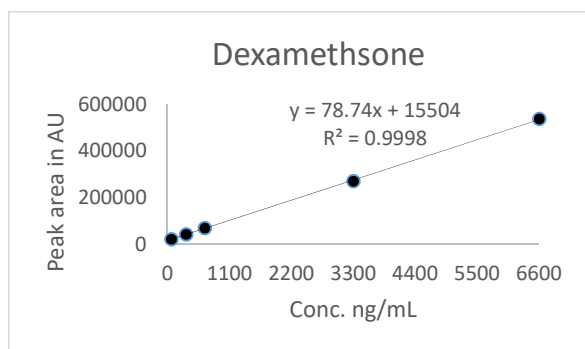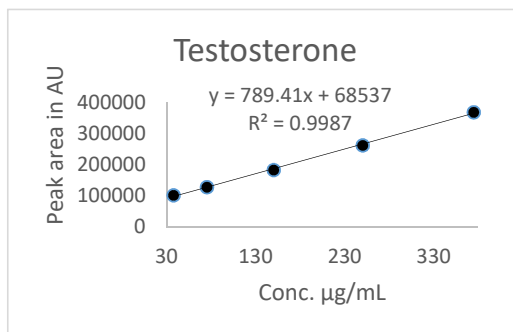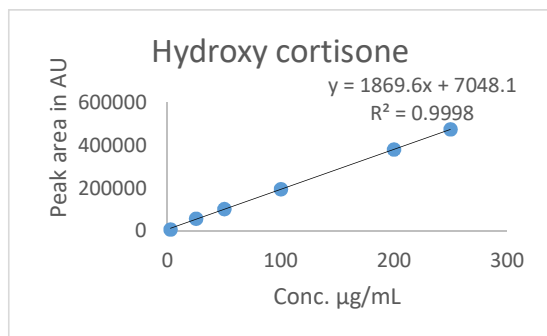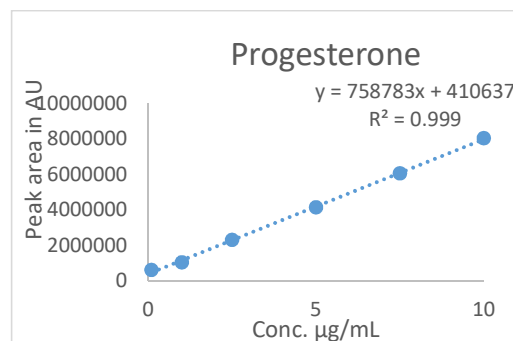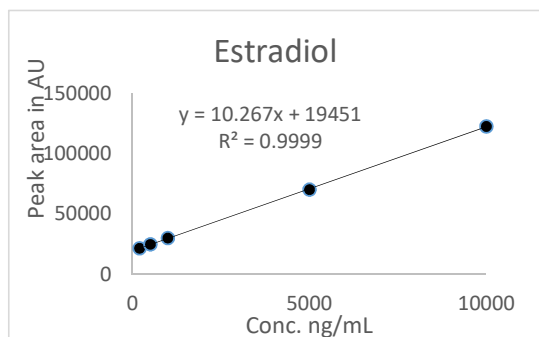
RESULTS AND DISCUSSION

In HPLC methods, compounds will be identified by Retention time (RT) and specificity of the method is the ability of the method to assess the target analyte unequivocally among other components. Initially each compound was analysed in HPLC to find the retention time of the compounds and the retention times of the standards in the standard curve range were compared to evaluate the specificity of the respective compound. Each compound showed specific retention time and the %CV of retention times were calculated to evaluate the variation in retention times of the compounds and the results were tabulated in Table 3.

Analyte	Retention time observed	%CV of Retention time
Dinoprost tromethamine	2.547-2.562	0.153
Dexamethasone	3.065-3.133	0.605
Hydrocortisone	3.360-3.374	0.109
Oxytocin	4.414-4.439	0.153
Isoflupredone	4.713-4.744	0.165
Clopostenol	4.941-4.940	0.153
Progesterone	7.369-7.395	0.093
Estradiol	8.983-9.006	0.073
Testosterone	10.340-10.364	0.062

Table 3: Retention time of the compounds to evaluate Identity and Specificity of the compounds.

Linearity of the method is the ability to obtain test results proportional to the concentration of analytes. R^2 value is the measure of fitness in linearity model and ≥ 0.95 value is the acceptable value for linearity. Standard range was prepared according to the concentrations mentioned in table 2 and linearity plots were plotted for standard concentrations against peak areas using MS-Excel. All the analytes showed proportional response of peak areas to concentrations with 0.999 R^2 values.

Estradiol

$y = 10.267x + 19451$
$R^2 = 0.9999$

Progesterone

$y = 758783x + 410637$
$R^2 = 0.999$

Hydroxy cortisone

$y = 1869.6x + 7048.1$
$R^2 = 0.9998$

Testosterone

$y = 789.41x + 68537$
$R^2 = 0.9987$

Dexamethsone

$y = 78.74x + 15504$
$R^2 = 0.9998$

Oxytocin

$y = 271.06x + 2792.3$
$R^2 = 1$

Isoflupredone

$y = 40.206x + 2292.1$
$R^2 = 1$

Clopostenol

$y = 147.53x + 13374$
$R^2 = 0.9995$

Dinoprost tromethamine

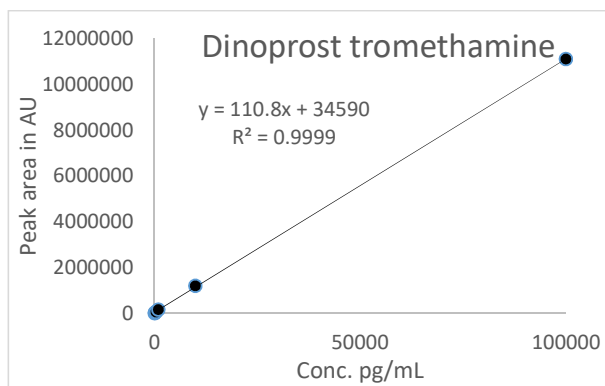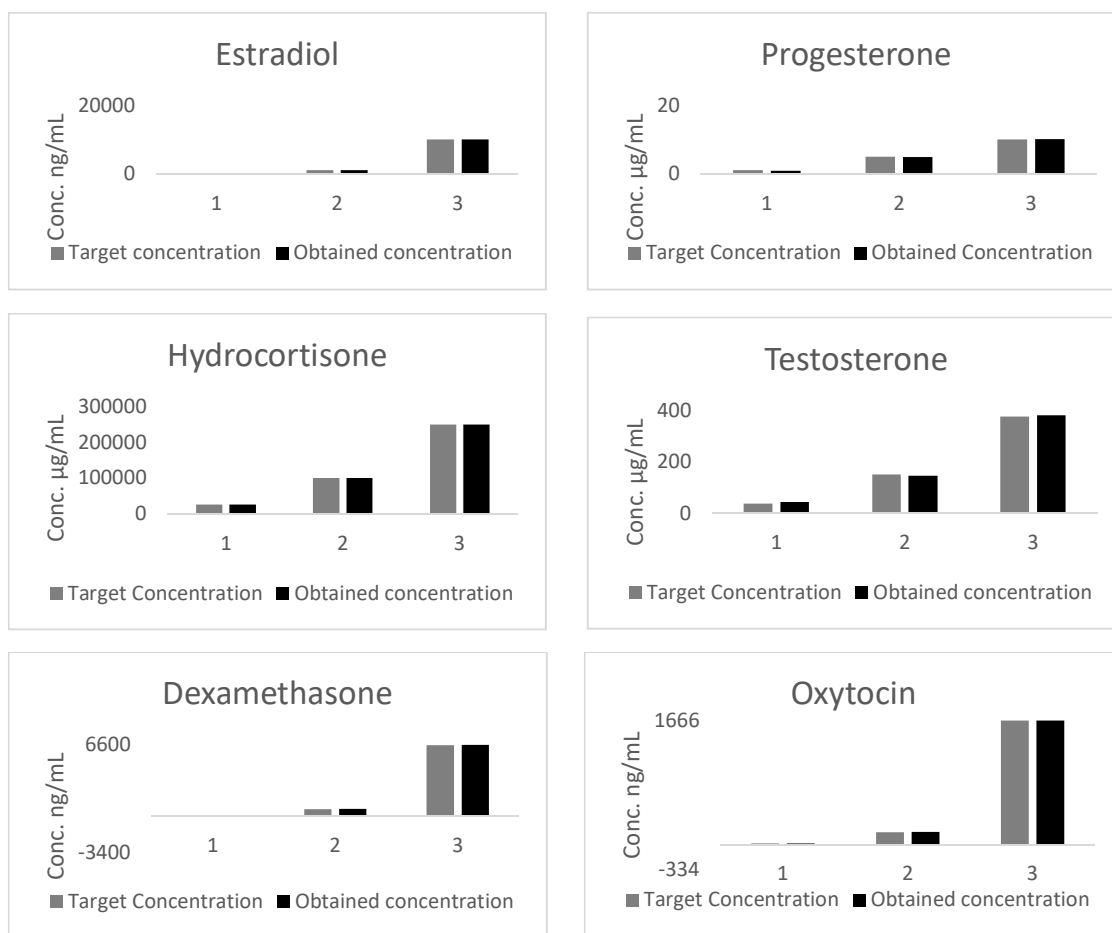$y = 110.8x + 34590$
$R^2 = 0.9999$

Figure 1: Linearity Curves of Estradiol, Progesterone, Hydro cortisone, Testosterone, Dexamethasone, Oxytocin, Isoflupredone, Clopostenol and Dinoprost tromethamine.

Accuracy of the method expresses the closeness of agreement between the obtained value and the reference value. The accepted criteria for the accuracy of found value is ± 20% to the conventional true value or an accepted reference value. Accuracy of method was established by spiking the standard with blank solution at lower, higher and middle range of the curve and all the analytes showed recovery within 80-120%.
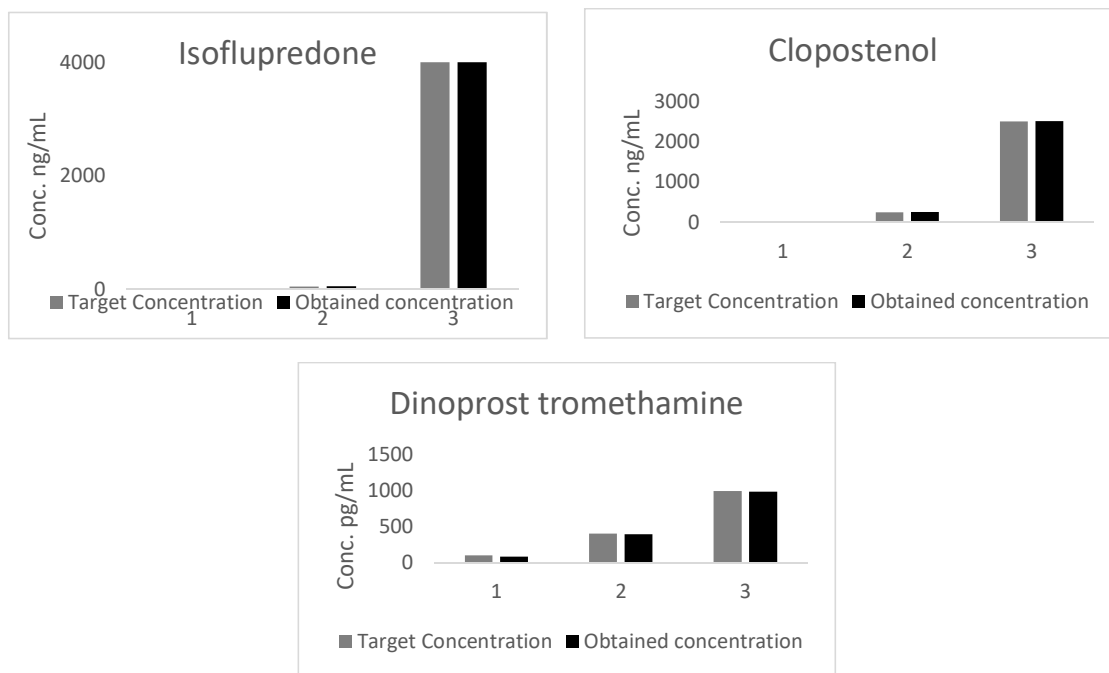
Figure 2: Accuracy of Estradiol, Progesterone, Hydro cortisone, Testosterone, Dexamethasone, Oxytocin, Isofluredone, Clopostenol and Dinoprost tromethamine at low, middle and high ranges of concentration curve.

Range of an analytical procedure is the interval between the lower and upper concentrations of analyte in the sample which shows precision, accuracy and linearity. Precision of an analytical procedure is the expression the closeness between a series of measurements obtained from multiple assays and it will be expressed in terms of variance or standard deviation or coefficient of variation of a series of measurements. Spike sample was analysed six times to check precision and the concentration was derived from the established standard curve. Standard concentrations showed 80-120% recovery was considered to determine the ranges of the assay for above analytes and the assay range of the analytes were mentioned in table 4. Precision was calculated at low, middle and high and ranges of standard curve and represented in Table-5 and Figure-3.

Analyte	Concentrations range
Estradiol	200,500,1000,5000,10000 ng/mL
Progesterone	1000,2500,5000,7500,10000 ng/mL
Hydrocortisone	25,50,100,200,250 μg/mL
Testosterone	37.5, 75,150,250,375 μg/mL
Dinoprost tromethamine	100,200,400,1000 and 1000,10000,100000 pg/mL
Dexamethasone	66,330,660,3300,6600 ng/mL
Oxytocin	16.66, 83.3, 166.6, 833,1666 ng/mL
Isofluredone	8,40,400,4000 ng/mL
Clopostenol	25,125,250,1250,2500 ng/mL

Table 4: Standard curve ranges of analytes.

Analyte	%CV of concentrations at low, middle and high range of standard curve		
	Low	Middle	High
Estradiol	2.24	7.96	1.12
Progesterone	5.00	0.01	6.46
Hydrocortisone	5.99	0.39	0.46

Testosterone	4.11	0.28	1.55
Dinoprost tromethamine	6.31	1.97	1.46
Dexamethasone	4.81	9.09	4.72
Oxytocin	9.50	2.69	4.05
Isoflupredone	6.84	9.66	6.20
Clopostenol	6.91	0.78	1.87

Table 5: Analyte concentrations %CV at low, middle and high range of standard.
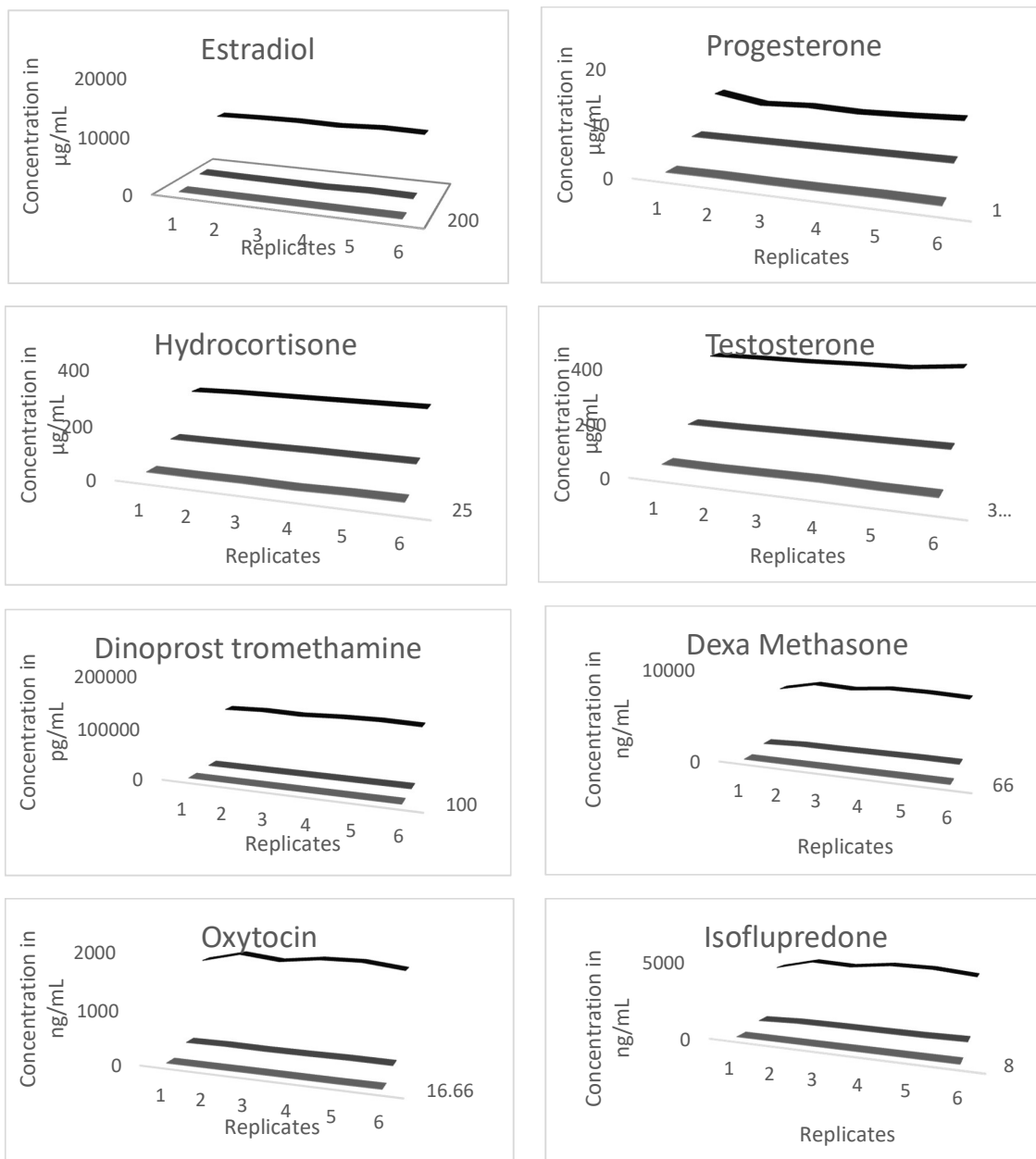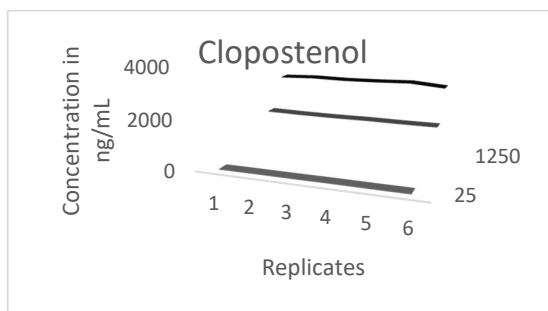
Figure 3: Precision of Estradiol, Progesterone, Hydro cortisone, Testosterone, Dexamethasone, Oxytocin, Isoflupredone, Clopostenol and Dinoprost tromethamine at low, middle and high ranges of concentration curve.

LOD of the assay is the lowest detectable concentration of the analyte in a sample which can be detected but not necessarily quantitated as an exact value and LOQ of the assay is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. LOQ of the assay can be determined by visual evaluation or signal to noise ratio or from the standard deviation of the response and the slope. We evaluated the LOD and LOQ values based on visual evaluation by back calculating recovery of targeted concentrations and the LOD and LOQ values were tabulated in Table 6.

Analyte	LOD	LOQ
Estradiol	40 ng/mL	200 ng/mL
Progesterone	0.1 µg/mL	1 µg/mL
Hydrocortisone	2.5 µg/mL	25 µg/mL
Testosterone	1.5 µg/mL	37.5 µg/mL
Dinoprost tromethamine	50 pg/mL	100 pg/mL
Dexamethasone	6.6 ng/mL	66 ng/mL
Oxytocin	6.6 ng/mL	16.6 ng/mL
Isoflupredone	4 ng/mL	8 ng/mL
Clopostenol	2.5 ng/mL	25 ng/mL

Table 6: LOD and LOQ values of analytes.

CONCLUSION

The developed noninvasive HPLC assay offered us to estimate the fecal hormone metabolites of estradiol, progesterone, cortisone, testosterone along with the commercially available cattle drugs such as as Dinoprost tromethamine, Dexamethasone, Oxytocin, Isoflupredone and Clopostenol with a single sample. The developed method successfully proved accuracy, specificity, precision, linearity, range and established with limit of detection and Quantification concentrations. So the developed method can be used for quantification of hormones to assess breeding cycles, pregnancy status, stress levels and health status.

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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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