Evaluation of Extraction Methods for Progesterone Metabolite Determination in Buffalo Feces by Immunoassay

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A B S T R A C T

The present study was planned to develop the best fecal extraction method for progesterone metabolite assay which is based on the concentration of fecal progesterone metabolite (FPM) (5α-pregnan-3α-ol-20-one), obtained in a dried fecal sample of buffaloes. Four clinically healthy female buffaloes of the same age group 4 to 7 years were used for the collection of fecal samples maintained under isomanagerial conditions with an intensive system at the LPM Section of the IVRI institute. The extraction of FPM (5α-pregnan-3α-ol-20-one) was done by various methods using organic solvents like methanol, diethyl ether, ethanol, and 90% methanol and found that extraction with 90% methanol the concentration of FPM (5α-pregnan-3α-ol-20-one) (231.98ng/g), is comparatively higher than other methods and best for a dried fecal sample of buffaloes. In conclusion, the above results suggest that FPM (5α-pregnan-3α-ol-20-one) value using 90% methanol as a solvent in the dried fecal sample assay having high value as compared to other solvents extraction methods like methanol, absolute ethanol, and diethyl ether, and we reported best for a dried fecal sample of buffaloes and preserved for further estimation.

Keywords
Extraction, Feces, Progesterone, Metabolite

Introduction

India possesses a rapidly increasing animal husbandry sector and moves to attain self-sufficiency in the production of livestock products (Dhama et al., 2014). Animal husbandry is a major contributor to the Indian economy and overall contribution is 28-32% in agricultural GDP and 4 to 6% of the national GDP. India possesses the largest buffalo population (109.85 million) in the world (Chand et al., 2015, 20th Livestock census, 2019). The use of steroid hormone concentration measured in feces as a non-invasive method for detecting reproductive status has been used in various research fields such as wildlife endocrinology, animal welfare, ecology, and reproduction (Dantzer et al., 2013; Schwarzenberger and Brown, 2013). Metabolism and excretion of steroids differ widely between species and even between sexes (Touma et al., 2003; Palme et al., 2005) and a mixture of several metabolites with different structures and
polarities. Such non-invasive methods therefore need to be extensively validated for each species (Touma and Palme, 2005). Proper extraction technique is necessary to choose for the steroid metabolites present in the faecal matrix are of varying polarity and composition. Studies of fecal steroid hormones were first conducted mainly on their metabolism and excretion (Adlecreutz et al., 1979), further studies were done regarding the puberty, estrous cycle, pregnancy, abortion, reproductive behavior, seasonality, and the monitoring of treatment therapies in the zoo and domestic animals (Schwarzenberger et al., 1996). The presence of quantified amounts of reproductive steroids was present in cattle feces (Desaulniers et al., 1989; Larter et al., 1994; Isobe et al., 2005) and other animals (Shaw et al., 1995).

The steroid hormone metabolite quantified in faeces, commonly used in wildlife studies, as a non-invasive, non-stressing, economical, and animal-saving technique that permits longitudinal studies by allowing frequent sampling of the same individual (Brown et al., 1994; Graham et al., 1995). Thus, developing enzyme immunoassays (EIAs) against Progesterone metabolites would be more accurate and reliable for non-invasive reproductive monitoring method using fecal samples (Brown et al., 1994; Umapathy et al., 2013; Mithileshwari et al., 2016; Budithi et al., 2016, Marozzi et al., 2019, Amaral et al., 2019). In various studies, the organic solvent was used by the various workers for the extraction of steroid hormone metabolites from feces. Steroids are commonly extracted from feces using ethanol in Goat (Capezzuto et al., 2008), Felid (Brown et al., 1994), Asiatic lion (Umapathy et al., 2007), Scimitar-horned oryx (Morrow and Monfort, 1998), Baboons (Waser et al., 1993). Methanol was used for extraction of fecal steroids in Felid (Graham et al., 1995), Exotic cat (Putranto et al., 2006), White rhinoceros (Schwarzenberger and Walzer, 1995), Black rhinoceros (Schwarzenberger et al., 1993a), Cattle (Schwarzenberger et al. 1996a, 1996b, 1996c; Isobe et al., 2005), Ruminant (Kornmatitsu et al., 2007; Larter et al., 1994), Buffalo (Hattab et al., 2000; Palme et al., 1997; Arunji, 2008; Ashok, 2011), Bengal tiger (Putranto et al., 2006), Goat (Airin et al., 2020). The fecal samples were extracted with diethyl ether in Cattle (Mostl et al., 1984; Hirata and Mori, 1995), Caribou (Desaulnier et al., 1989; Messier et al., 1990), Sow (Choi et al., 1987), Giant ant-eater (Mostl et al., 1984). Progesterone metabolites in feces were extracted using phosphate buffer solution by Hulten et al., (1995) in gilts and by Petroleum ether in Rabbit by Komdorfer et al., (1998). The present study investigated the best fecal extraction method for progesterone metabolite assay based on the concentration of fecal progesterone metabolite (5α-pregnan-3α-ol-20-one) obtained in a dried fecal sample of buffaloes.

Materials and Methods

The present study was conducted at the Livestock Production Management (LPM) farm and the facilities of Physiology and Climatology Division and Nuclear Research Laboratory (NRL), ICAR- Indian Veterinary Research Institute, Bareilly (UP) were utilized.

Four clinically healthy female buffaloes of the same age group 4 to 7 years were used for the collection of fecal samples maintained at the LPM Section of the institute and were maintained under isomanagerial conditions with an intensive system and housed in a well-ventilated brick cemented house with a non-slippery floor and offered adlibitum access to standard ration having green and dry fodder along with concentrate and good quality drinking water.
Screening of suitable extraction method

Approximately 10-15 g fecal samples were collected directly from the rectum in interlocking polythene bags and kept in a hot air oven at 40-50°C till the sample becomes completely dry. The dry samples were further grounded to powder by using pestle and mortar and stored at -20°C until analysis. The samples were further processed for the extraction of P4 metabolites by using organic solvents like methanol, diethyl ether, ethanol, and 90% methanol to select the best among four methods as below. The extraction of steroids in the fecal samples was carried out according to modified methods described by Desaulniers et al., (1989); Thompson et al., (1998); Isobe et al., (2005); Arunji, (2008) and Ashok, (2011).

In Method 1 absolute methanol was used. In this 0.3 g, fecal sample and 5 ml methanol were taken in a tarden centrifuged tube and vortexed in the spinx vortex mixture for 10 minutes than Centrifuged in non-refrigerated Centrifuge (Remi, India) at 3000 rpm for 10 minutes. After that supernatant collected in collecting vial and kept in hot air oven (Yorco instrument, Bombay) overnight for drying at 40-50°C. After drying reconstituted in the appropriate buffer (ELISA buffer- PBS, 0.01% Thiomersal, 0.1% BSA, pH-7.2) and vortexed for 1 minute and preserved in the deep freezer until assay. In the other methods, despite methanol, absolute ethanol, diethyl ether, and 90% methanol were used remaining procedure same as the methanol method. Method 4 using 90% methanol with dry samples selected as the best method for progesterone metabolite assay based on the concentration of metabolite obtained. Hence, all the samples were extracted in 90% methanol and preserved for further estimation. The extraction efficiency of fecal steroids was estimated by adding a known amount of 3H-labeled progesterone tracer in fecal samples before extraction. The extraction efficiency was calculated as a percentage of the amount of labeled hormones observed relative to the amount expected. The extraction efficiency was found to be 85%. The samples were diluted 1:160 with ELISA buffer for progesterone metabolite assay.

Imunoassay procedure

Measurement of FPM (5α-pregnan-3α-ol-20-one) was performed by Immunoassay procedures which were carried out according to standard methods. The Inter-assay and Intra-assay coefficient of variation of Imunoassay was <6.5%, <9.0%, respectively. The recovery of spiked 5α-pregnan-3α-ol-20-one was found to be 95% in fecal extracts analyzed by ELISA. The 5α-pregnan-3α-ol-20-one assay sensitivity was 0.1ng/ml.

Statistical Analysis

The data generated in the present study were analyzed using JMP 9.0 software.

Results and Discussion

The present study was designed to develop the best extraction method for progesterone metabolite assay based on the concentration of FPM (5α-pregnan-3α-ol-20-one) obtained in a dried fecal sample of buffaloes. After fecal samples dried on 40-50°C in hot air oven by using variou organic solvents like methanol, diethyl ether, ethanol, and 90% methanol. After extraction samples were analyzed using ELISA and the values are presented in table 1. The average values of all the four samples extracted by the ethanol (80.11ng/g), diethyl ether (95.62ng/g), methanol (191.08ng/g), and 90% methanol (231.98ng/g) in a dried fecal sample. Based on the concentration of fecal progesterone metabolite, it was found that the extraction of
a fecal sample with 90% methanol is comparatively higher than other methods.

In the previous studies extraction of fecal progesterone was performed using methanol as solvent was reported by various authors in different species such as Schwarzenberger et al., (1993a) in Black rhinoceros, (1996b) in cows and Schwarzenberger and Walzer, 1995 in white rhinoceros, Graham et al., (1995) in felids, Putranto et al., (2006) in exotic cats, Ghosal et al., (2010, 2012) in Asian elephants, Yujiang et al., (2019) in porpoise. In Ruminant species such as Larter et al., (1994), Kornmatisuk et al., (2007), Isobe et al., (2005) in cows, Hattab et al., (2000), Arunji, (2008), Ashok, (2011) in buffaloes and Jack et al., (2012) in goats. Extraction of fecal progesterone using 90% methanol in black rhinoceros by Edwards et al., (2014), Chelonians by Umapathy et al., (2015) and by 80% methanol in cattle by Palme et al., (1997), goats by Airin et al., (2020), ewes by Amaral et al., (2019), Tsushima leopard cat by Adachi et al., (2010) as well as in Asian Elephants by Kumar et al., (2014, 2019) and guanacos by Marozzi et al., (2019).

| Sample | Animal No. | Solvents used | Ethanol | Diethyl Ether | Methanol | 90% Methanol |
|--------|------------|---------------|---------|---------------|----------|--------------|
| Dry    | 114        | Ethanol       | 159.64  | 50.40         | 282.00   | 322.81       |
|        | 350        | Ethanol       | 52.99   | 50.40         | 222.71   | 273.77       |
|        | 1038       | Ethanol       | 46.50   | 50.40         | 148.66   | 173.58       |
|        | 1091       | Ethanol       | 61.31   | 50.40         | 110.95   | 157.78       |
| Average|            | Ethanol       | 80.11   | 50.40         | 191.08   | 231.98       |

Table I Faecal progesterone metabolite (5α-pregnan-3α-ol-20-one) concentration (ng/g) (Mean) in buffaloes

This study showed a high FPM (5α-pregnan-3α-ol-20-one) value using 90% methanol as a solvent in the dried fecal sample. There is an average value of 231.98ng/g for FPM (5α-pregnan-3α-ol-20-one) which is higher than other methods. Our studies show the better application of 90% methanol as a solvent in dry fecal samples which is similar to previous reports by Edwards et al., (2014) in black rhinoceros and Umapathy et al., (2015) in Chelonians. So method using 90% methanol as a solvent is best and increase accuracy for determination of FPM (5α-pregnan-3α-ol-20-one) in a dried fecal sample of buffaloes for different investigation.

In Conclusion, the above results suggest that fecal progesterone metabolite (5α-pregnan-3α-ol-20-one) value using 90% methanol as a solvent in the dried fecal sample assay having high value as compared to other solvents extraction methods like methanol, absolute ethanol, and diethyl ether, and we reported best for a dried fecal sample of buffaloes and preserved for further estimation.

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Disclosure statement

No potential conflict of interest was reported by the authors.
Abbreviation

Fecal progesterone metabolite (FPM), Indian Veterinary Research Institute (IVRI), Livestock Production Management (LPM), Nuclear Research Laboratory (NRL), Artificial Insemination (AI), ELISA (Enzyme-Linked Immunosorbent Assay)

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