Indian Journal of Animal Sciences **93** (1): 39–44, January 2023/Article
https://doi.org/10.56093/ijans.v93i1.117992

Identification of *in vitro* metabolites of boldenone using Camel liver microsomes – A HR-LCMS approach

RAMEES ABDULLA VAZHAT[1, 2✉], N A MOHAMED FAROOK[2], J NALAKATH[1] and PRASEEN O K[1]

Khadir Mohideen College, Bharathidasan University, Adirampattinam, Tamil Nadu 614 701 India

Received: 18 November 2021; Accepted: 9 October 2022

ABSTRACT

Anabolic steroids are widely abused in animal sports to improve their performance. The present study identifies the possible metabolites of boldenone in camel liver. A high-resolution accurate QE mass spectrometer was used to identify the parent boldenone and its metabolites. To investigate the phase 1 biotransformation of boldenone in camel, sodium phosphate buffer and NADPH were used. Chromatographic separation was carried out on a Thermo Hypersil C18 column using acetonitrile and formic acid as mobile phases. The current study, helped in unequivocal detection of six metabolites (Phase 1) for boldenone. The 17b-boldenone is an 3-oxo-Delta (1), Delta(4)-steroid substituted by an oxo group at position 3 and a beta-hydroxy group at position 17. It is prone to oxidation, which results in three hydroxylated metabolites with protonated parent ion of *m/z* 303.1954 ($C_{19}H_{27}O_3$)$^+$. Androsta-1,4-diene-3,17-dione $[M+H]^+$ of *m/z* 285.1848 ($C_{19}H_{25}O_2^+$), hydroxyandrosta-1,4-diene-3,17-dione $[M+H]^+$ of *m/z* 301.1797 ($C_{19}H_{25}O_3^+$) in addition to 17-hydroxy-androsta-1-en-3-one $[M+H]^+$ of *m/z* 289.2162 ($C_{19}H_{29}O_2^+$) were also identified. The structures of the detected metabolites were identified based on their accurate mass, fragmentation pattern, and chromatographic retention time. In this research, camel liver was successfully used for *in vitro* experiments as an alternative to liver microsomes.

Keywords: Anabolic steroids, Biotransformation, Boldenone, Camel liver, *In vitro* metabolism, HRMS

Anabolic-androgenic steroids (AAS) are synthetic derivatives of testosterone, being used by the athletes to enhance the performance and physical appearance (Mottram and George 2000). Anabolic steroids were developed mainly for veterinary use, mostly for the treatment of horses. This androgenic steroid improves growth and is forbidden in meat production and human uses (Cannizzo *et al.* 2007). AAS are metabolized to glucuroindes or sulpho-conjugated compounds in animals or human body and excreted though urine. The *in vitro* and *in vivo* metabolic studies of AAS helps greatly in identifying the metabolites and the parent drugs. It is used to increase the body mass, and enhance strength and endurance in canine, equine, and human athletes (Fred Hartgens *et al*. 2005, Buiarelli *et al.* 2005). The misuse of AAS causes health effects such as liver/kidney dysfunction, tendon damage, and disturbance of endocrine and immune functions (Mayada *et al.* 2015).

Boldenone (androsta-1,4-dien-17a-ol-3-one or 17a-boldenone/androsta-1,4-dien-17b-ol-3-one or 17b-boldenone), is an anabolic steroid with low androgenic activity (Verheyden *et al.* 2010). Boldenone is the dione form of boldione or androsta-1,4-diene-3,17-dione (ADD). It is a derivative of testosterone and is obtained from the dehydrogenisation of testosterone (M. Van Puymbroeck *et al*. 1998). Boldenone is illegally used by the athletes and racehorses to improve sports performance (Soma *et al*. 2007). In 2005, 17b-boldenone also called 1-dehydro testosterone, was included in the World Anti-Doping Agency (WADA) list of banned substances and the Federation Equestrian International (*www.fei.org).* The International Agency for Research on Cancer (IARC) has classified boldenone as a probable human carcinogen like the other androgenic steroids. It's index of carcinogenicity is higher than that of other AAS, such as testosterone, clostebol, nandrolone, and stanozolol (IARC 1987), (De Brabander *et al*. 2004).

Different metabolic studies of boldenone on its phase 1 and phase 2 metabolism in man and animals have been reported (Schanxer and Domke 1993, Balcells *et al*. 2015) a liquid chromatography-tandem mass spectrometry (LC-MS/MS. 17a-boldenone, androsta-1,4-diene-3,17-dione (ADD) and hydroxylated metabolites were identified in animal urine after the administration of 17b-boldenone (Mark Van Puymbroeck *et al*. 1998). ADD is detected as a metabolite and as a precursor of 17b-boldenone due to the enzymatic biotransformation of 17b-boldenone to ADD (Nielen *et al*. 2004).

Biotransformation is a part of the metabolic process that

Present address: [1]Camel Forensic Laboratory, Central Veterinary Research Laboratory, PO Box 597, Dubai, United Arab Emirates. [2]Postgraduate and Research Department of Chemistry, Khadir Mohideen College, Bharathidasan University, Adirampattinam, Tamil Nadu. ✉Corresponding author email: aramees@cfldubai.ae

takes place mainly in the liver. Various fractions of the liver can be used for *in vitro* experiments, including slides of fresh liver microsomes, liver cytosol, or S9 fractions. The pathways of biotransformation are divided into phase 1 (reduction, oxidation, and hydrolysis) and phase 2 reactions (conjugation). Phase 1 reactions are mainly catalyzed by the cytochrome P450 system (Brandon *et al*. 2003). In this study, camel liver is used instead of liver microsomes. The present study aims to determine the possible phase 1 metabolites of boldenone in camel liver and identify the metabolites using LC-MS/MS analysis.

Detection of boldenone and its metabolites in biological matrices is usually performed by GC-MS or GC-MS/MS with electron impact ionization (EI) (Popot *et al.* 2003). Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is a sensitive alternative to GC-MS (Buiarelli *et al.* 2005).

MATERIALS AND METHODS

Boldenone and nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Sigma USA. All organic solvents were either HPLC grade or LC-MS grade (Fisher Scientific). Deionized water was generated from an in-house water purification system (PURELAB Flex ELGA). Working solutions were prepared by appropriate dilution in MeOH or acetonitrile (LiChrosolv hyper grade for LC-MS≥99.9%), methanol (LiChrosolv, gradient grade, for LC,≥99.9%), methyl t-butyl ether (Fisher Chemical, HPLC grade,≥99%), formic acid (reagent grade, ≥ 99%), were supplied by Merck KGaA (Darmstadt, Germany).

Camel's liver: Fresh camel liver obtained from a veterinary hospital was cut into a 1 cm thick cube and stored immediately at -80°C. When required, the frozen camel liver pieces were transferred to 1 mL of the plain incubation reagent at 4°C.

In vitro *assay for metabolic reactions: phase 1*: To characterize the *in vitro* metabolic pathway of boldenone, the incubation mixtures containing 50 µL NADPH (10 mM in phosphate buffer), 10 µL of boldenone (1 mM) and 440 µL of phosphate buffer (100 mM, *p*H 7.4) were taken in an Eppendorf tube. The mixture was then vortexed and pre-incubated for 3 min at 37°C in a shaking water bath. Camel liver (1 cm cube) was added and the incubation was continued for 60 min with constant gentle shaking.

The incubation reaction was stopped by the addition of 200 µL of ice-cold methanol. Samples without NADPH were incubated to check for non-enzymatic reactions. All the incubations were performed in duplicates. A substrate blank, a co-factor blank and an enzyme blank along with the test drug were also performed in parallel (Philip *et al.* 2021).

Extraction: The mixture was mixed with 1 mL of methyl tert-butyl ether (MTBE) and 10 µL (1 µg/mL) of the IS stock solution in a 1.5 mL Eppendorf vial. Then, the sample was vortexed for 1 min and then centrifuged at 15,000 rpm for 15 min. The mixture was extracted twice and the organic phase was evaporated to dryness. The dried residue was reconstituted with 50 µL of the mobile phase for LC/ESI-MS/MS analysis.

Liquid chromatography: Boldenone and their *in vitro* metabolites were detected and characterized by liquid chromatography on a Dionex UltiMate 3000 UHPLC+ system. Chromatographic separation was performed using a reverse-phase Thermo Hypersil C18 (100 × 2.1 mm ID, 5 µm particle size) column. The acidic mobile phase consisted of 0.1% formic acid in water (mobile phase A) and acetonitrile (mobile phase B). The chromatographic starting conditions were 100:0, 0.1% formic acid (aqueous): acetonitrile with a 0.3 mL/min flow rate (Table 1).

Table 1. Liquid chromatographic gradient elution mode

Step	Time (min)	A (0.1% Formic acid)	B (Acetonitrile)
0	Initial	100.0	0.0
1	4.00	5.0	95.0
2	8.00	5.0	95.0
3	8.01	100.0	0.0
4	12.00	100.0	0.0

Mass spectrometry: The mass spectrometric analysis was carried out on a QExactive high-resolution accurate mass spectrometer (Thermo Scientific, USA) operated in dual ion mode and the *S*-lens values were set to 50. Analysis was carried out on dual Ion in Full scan AIF mode at 70,000 resolution. The MS data were acquired with a mass resolution of 70,000 over the mass range *m/z* 50-750. The capillary temperature was set to 320°C and the capillary voltage was ±4 kV. The sheath and auxiliary gas were set to 45 and 10 units respectively. The precursor ion detection was carried out using a full scan MS experiment whereas the product ion detection was achieved using a data-independent acquisition (DIA) experiment. The window for DIA was selected as ±1.5 min from the retention time.

Data analysis: The data were acquired and subsequently analyzed with Thermo XCalibur software. The precursor ion's exact mass in the full scan is considered for the identification, whereas a minimum of three fragment ions in DIA mode were taken for the confirmation.

RESULTS AND DISCUSSION

In vitro studies-phase 1: The LC-MS/MS experiments were performed for the analysis of boldenone and its metabolites. Published reports show seven *in vitro* metabolites after incubation with cattle liver microsomes (Merlanti *et al*. 2007). In the present study, an additional *in vitro* metabolite, namely M6 with protonated molecular ion *m/z* 289.2162 probably 17-Hydroxy-5-Androst-1-en-3-one was also obtained using camel liver. The metabolic conversions of boldenone in camel liver yielded six phase 1 metabolites (M1-M6). The possible metabolites of boldenone identified in camel liver, the retention time of metabolite, mass error, and most predominant fragment ions are listed in Table 2. The metabolites were detected and characterized by positive ionization mode using an LC-MS/MS QExactive high-resolution mass spectrometer.

The precursor ion detection was carried out through a full scan MS experiment and the fragments were confirmed by the MS^2 experiment by positive DIA acquisition mode.

The liver is the main organ responsible for a wide variety of metabolic reactions. The LC-MS/MS analysis of the incubation extracts of boldenone with camel liver and NADPH generating system confirmed six metabolites. Hydroxylation is major biotransformation of several drugs in camels (Al Katheeri *et al.* 2006). In LC-MS/MS analysis, boldenone and its metabolites produced a common selective fragment ion of *m/z* 121.06. Boldenone gives a protonated parent ion $[M+H]^+$ of *m/z* 287.2006 ($C_{19}H_{27}O_2^+$) and eluted at retention of 5.4 min (Fig. 1). At normalized collisional energy (20 eV) boldenone generated the most predominant fragment ions of *m/z* 135.12 (-152Da, $-C_9H_{12}O_2$) also the common fragment ions of *m/z* 121.06 (-166Da, $-C_{11}H_{18}O$). The probable loss of water molecule (-18Da) from the protonated parent *m/z* 287.2 precursors form the fragmented ion *m/z* 269.19 ($C_{19}H_{25}O^+$). The possible structures of fragment ions of boldenone in MS^2 experiments are detailed in Fig. 2A.

Hydroxylated metabolites [M1-M3]: Hydroxylation is the most favoured metabolic pathway of boldenone and other steroids. Three polar hydroxylated metabolites were reported in cattle liver (Merlanti *et al.* 2007). In this study, three mono-hydroxy boldenone *m/z* 302.4079 ($C_{19}H_{26}O_3$) metabolites M1, M2, and M3 were probably formed by oxygenation (monohydroxylation) with a mass increment of 16Da. These polar hydroxylated metabolites M1, M2, and M3 have produced a protonated parent molecular ion $[M+H]^+$ of *m/z* 303.1954 $(C_{19}H_{27}O_3)^+$ and eluted at different retention times of 4.3, 4.6, and 4.8 min (Fig. 4) in order with the position of the hydroxyl group attached on boldenone. The predicted monohydroxylated metabolites are M1 (6a-hydroxy-17b-boldenone), M2 (6b-hydroxy-17b-boldenone) and M3 (6b-hydroxy-17a-boldenone). The proposed biotransformation pathway and the metabolic conversions of 17b-boldenone to its hydroxylated metabolites in camel liver are listed in Fig. 3.

In LC-MS/MS analysis, the probable loss of water (-18 Da) from the mono-hydroxy metabolite yield fragmention of *m/z* 285.18 ($C_{19}H_{25}O_2^+$) and all the other mono-hydroxy metabolite gives the most abundant common fragmented ions of *m/z* 121.07 (-182.13Da, $-C_{11}H_{18}O_2$). The second loss of water molecule results in the fragment ions of *m/z* 267.17 (-36.02Da, $-H_40_2$), and the other ions displayed are *m/z* 133.10 (-170.09Da, $-C_{10}H_{18}O_2$) and 147.12 (-156.09Da, $-C_9H_{15}O_2$). The possible structures of fragment ions of hydroxy boldenone in MS^2 experiments are detailed in Fig. 2B. In LC-MS/MS analysis, *in vitro* studies in phase 1 metabolism of boldenone using camel liver did not detect any dihydroxylated boldenone metabolites.

Androsta-1,4 diene-3,17-dione (ADD) [M4]: In cattle liver, the *in vitro* studies reported the biotransformation of boldenone into androsta-diene-dione and vice versa (Merlanti *et al.* 2007). The biotransformation of boldenone into androsta-1,4-diene-3,17-dione (ADD) was found to be the most abundant *in vitro* metabolite in camel liver. In LC-MS/MS analysis ADD generates a protonated parent ion $[M+H]^+$ of *m/z* 285.1848 ($C_{19}H_{25}O_2^+$) and eluted at a retention time of 5.5 min (Fig. 4B). Fragmentation with collisional energy 30 eV, generated the most predominant fragment ions of *m/z* 121.07 (-164.12Da, $-C_{11}H_{16}O$). The other major fragments observed for this metabolite are *m/z* 151.11 (-134.07Da, $-C_9H_{10}O$), *m/z* 133.10 (-152.087Da, $-C_9H_{12}O_2$) and a probable loss of water molecule (-18Da) from the parent to yield *m/z* 267.17 ($C_{19}H_{23}O^+$). The possible structures of fragment ions of ADD in MS^2 experiments are detailed in figure 3C.

ADD-OH [M5]: The metabolite M5 was probably formed by oxygenation (mono-hydroxylation) of ADD with a mass increase of 16Da (Table 2). In LC-MS/MS analysis the metabolite M5 produced a protonated molecular ion $[M+H]^+$ of *m/z* 301.1797 ($C_{19}H_{25}O_3^+$). The hydroxylated metabolite of ADD, probably 6-hydroxyandrosta-1,4-diene-3,17-dione eluted at a retention time of 4.7 min (Fig. 4C). At normalized collision energy (20 eV) the probable loss of water molecule (-18 Da) from the parent *m/z* 301.17 yields a fragmented ion of 283.17 ($C_{19}H_{23}O_2^+$) and the loss of second water molecule (-36Da, $-H_2O_2$) from ADD-OH results in a product ion of *m/z* 265.16 ($C_{19}H_{21}O^+$). The fragmented ions of *m/z* 121.06 ($-C_{11}H_{16}O_2$, -180.11 Da)

Table 2. Summary of observed Phase 1 metabolite produced after the *in vitro* incubation of boldenone with NADPH generating system in camel liver and most abundant product ions recovered in LC-MS/MS DIA experiment

	Analyte	RT (min)	Elem.comp	Mass (*m/z*) [M+H] theoretical/Observed	Error (ppm)	Fragments
M	Boldenone	5.3	$C_{19}H_{26}O_2$	287.2006/287.2006	0	121.06 ,135.12,269.18
M1	Hydroxy-boldenone	4.3	$C_{19}H_{26}O_3$	303.1955/303.1954	-0.33	121.06, 267.17,133.06
M2	Hydroxy-boldenone	4.6		303.1955/303.1954	-0.33	121.06, 267.17,133.06
M3	Hydroxy-boldenone	4.8		303.1955/303.1954	-0.33	121.06, 267.17,133.06
M4	Androsta-1,4diene-3,17-dione (ADD)	5.5	$C_{19}H_{24}O_2$	285.1849/285.1848	-0.35	121.06,147.06,131.1
M5	Hydroxyandrosta-1,4-diene-3,17-dione (ADD-OH)	4.7	$C_{19}H_{24}O_3$	301.1798/301.1797	-0.33	121.06, 283.16,163.11
M6	Hydroxyandrosta-1-en-3-one	5.8	$C_{19}H_{28}O_2$	289.2162/289.2162	0	121.06, 271.21,187.15

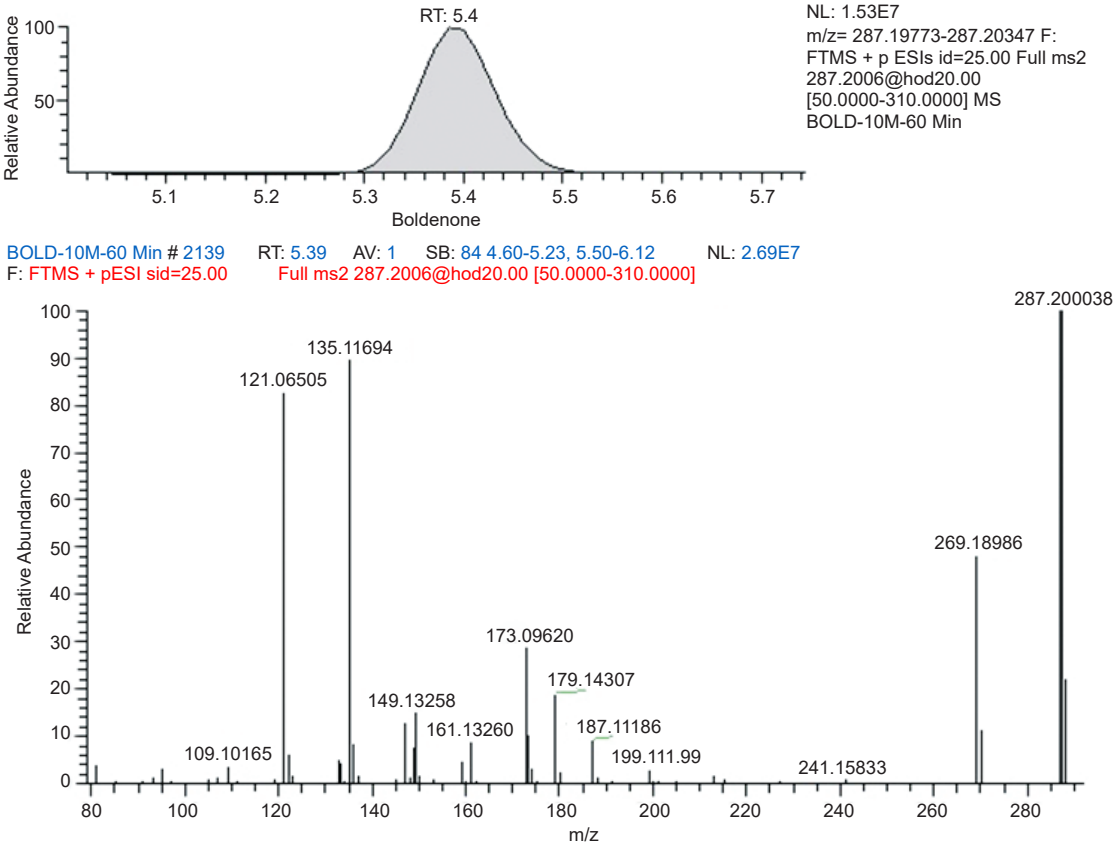

Fig. 1. LC-MS/MS Chromatogram and Electrospray ionization (ESI) product ion mass spectrum of boldenone ($C_{19}H_{27}O_2^+$) in positive ionization mode.

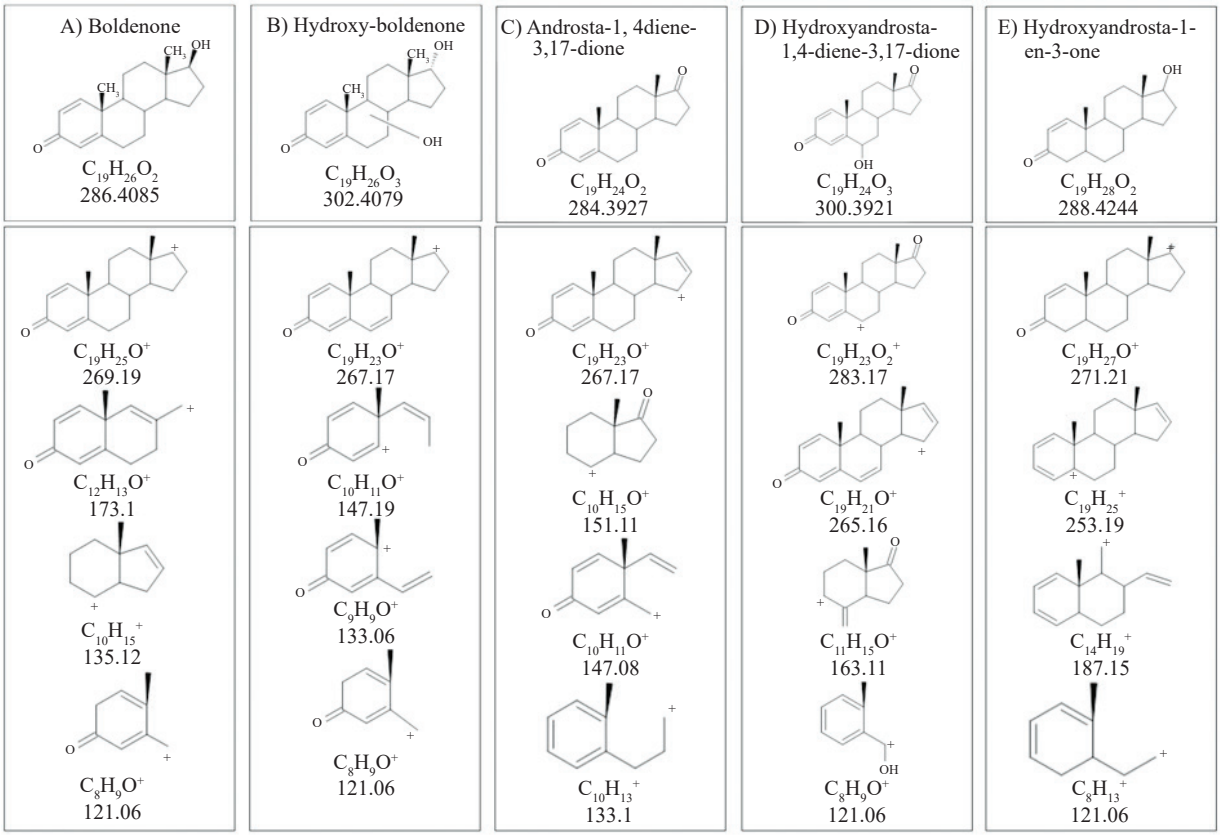

Fig. 2. Proposed chemical structures of fragment ions of boldenone and its phase 1 metabolites under positive ESI conditions.

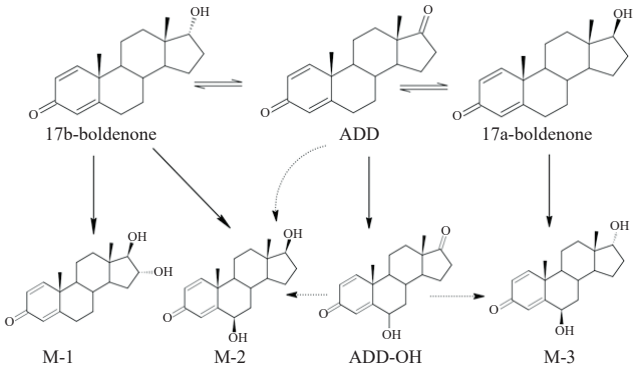

Fig. 3. Biotransformation pathway of boldenone to its hydroxylated metabolite

and 163.11 (-$C_8H_{10}O_2$, -138.07 Da) are also generated in the MS^2 experiment by positive DIA acquisition mode. The possible structures of fragment ions of ADD-OH in MS^2 experiments are detailed in Fig. 2D.

17-Hydroxy-androsta-1-en-3-one [M6]: The metabolite M6 (androsta-1-en-3-one,17-ol) was probably formed by the reduction of the steroid ring of boldenone. In LC-MS/MS analysis this metabolite generates a protonated parent ion $[M+H]^+$ of *m/z* 289.2162 ($C_{19}H_{29}O_2^+$) and eluted at a retention time of 5.8 min (Fig. 4D). In the MS^2 experiment the elimination of water molecule (-18 Da) from the precursor ion yields *m/z* 271.21 ($C_{19}H_{27}O^+$). Like other boldenone metabolites, M6 also shows the fragment ion of *m/z* 121.06 (-168.11Da -$C_{10}H_{16}O_2^+$). At collisional energy 20 eV the metabolite M6 also displayed the fragmented ions of *m/z* 187.14 (-102.17Da, -$C_5H_{10}O_2$) and the loss of the second water molecule resulted in *m/z* 253.19 (-36 Da, -H_4O_2). The possible structures of fragment ions of M6 in MS^2 experiments are detailed in Fig. 2E.

The successful approach using camel liver in lieu of liver microsomes for carrying out *in vitro* experiments for detecting boldenone metabolites is proposed. Results obtained from the present study contributed to elucidating the biotransformation of 17b-boldenone in camel liver. A total of six metabolites for boldenone in phase 1 metabolism were successfully identified, three polar hydroxylated metabolites of boldenone *m/z* 302.4079 ($C_{19}H_{26}O_3$) were identified, androsta-1,4-diene-3,17-dione *m/z* 284.3927 ($C_{19}H_{24}O_2$) was the major metabolite of 17b-boldenone when boldenone is incubated with camel liver. Hydroxy-androsta-1,4-diene-3,17-dione *m/z* 300.3921 ($C_{19}H_{24}O_3$) and hydroxyandrosta-1-en-3-one *m/z* 288.4244 ($C_{19}H_{28}O_2$)

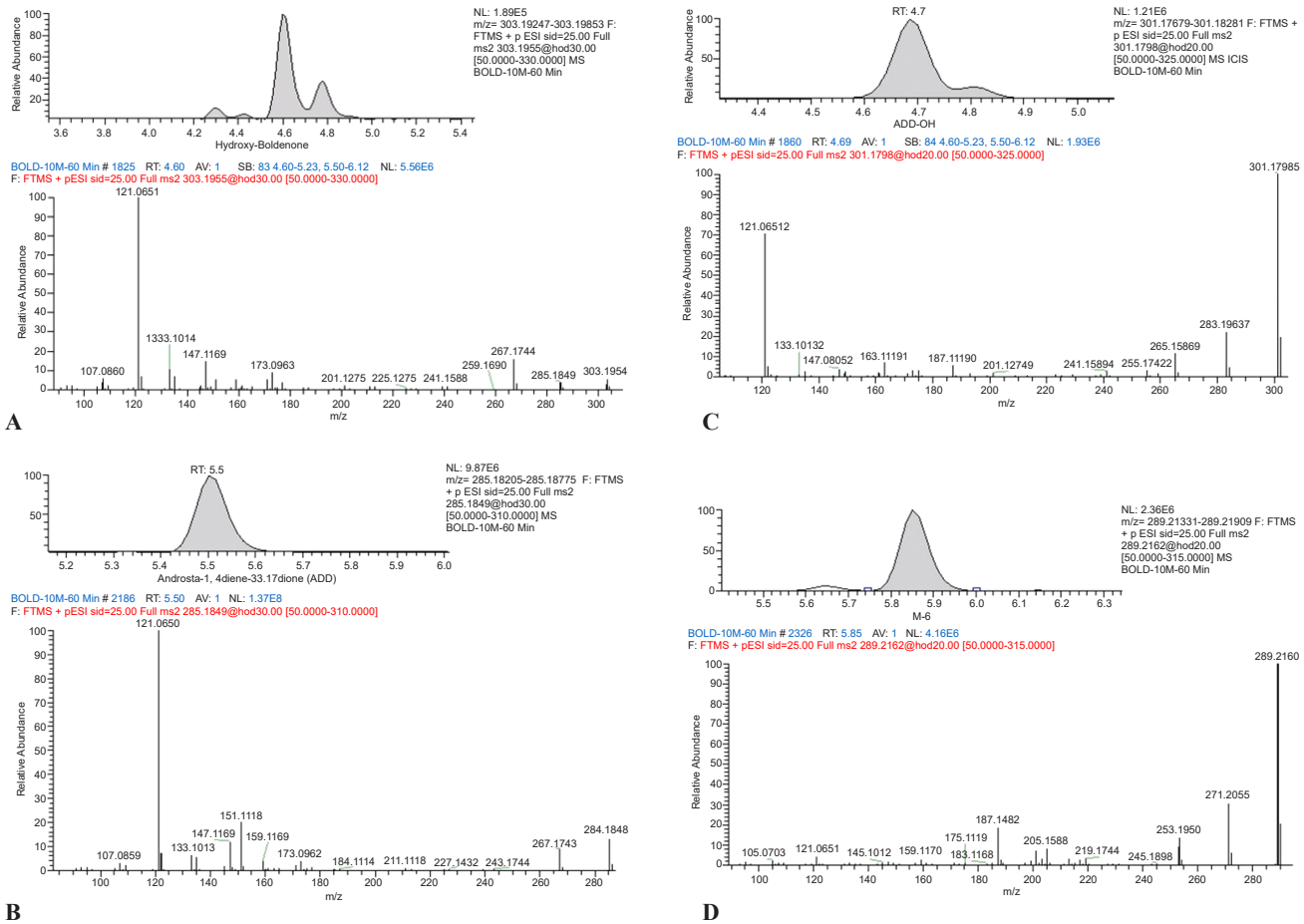

Fig. 4. **A.** LC-MS/MS Chromatogram and ESI product ion mass spectrum of mono-hydroxylated boldenone ($C_{19}H_{27}O_3^+$) in positive ion mode; **B.** LC-MS/MS Chromatogram and ESI product ion mass spectrum of Androsta-1, 4diene-3, 17-dione ($C_{19}H_{25}O_2^+$) metabolite in positive ion mode; **C.** LC-MS/MS Chromatogram and ESI product ion mass spectrum of ADD-OH ($C_{19}H_{25}O_3^+$) metabolite in positive ion mode; **D.** LC-MS/MS Chromatogram and ESI product ion mass spectrum of 17-Hydroxy- androsta-1-en-3-one ($C_{19}H_{29}O_2^+$) metabolite in positive ion mode.

are the other metabolites. The chromatographic separation of polar hydroxylated boldenone was performed using the Thermo Hypersil C18 column with the developed LC-MS/MS method. A possible structure of the fragmented ions of boldenone and its metabolites under ESI conditions were suggested. The metabolites identified help in doping control of boldenone in camel races. Further studies are required to determine the metabolites of boldenone in administrated camel samples.

ACKNOWLEDGEMENTS

The authors are thankful to Dr. Ali Ridha, Director General, (Central Veterinary Research Laboratory) for the valuable suggestions. We acknowledge the support and assistance extended by the staff at Camel Forensic Laboratory in this project. The authors are also grateful to the Postgraduate and Research Department of Chemistry, Khadir Mohideen College, Bharathidasan University, Tiruchirappalli for providing the support.

REFERENCES

Al Katheeri N A, Wasfi I A, Lambert M, Giuliano Albo A and Nebbia, C. 2006. *In vivo* and *in vitro* metabolism of dexamethasone in the camel. *Veterinary Journal* **172**(3): 532–43.

Balcells G, Pozo O J, Esquivel A, Kotronoulas A, Joglar J, Segura J and Ventura R. 2015. Screening for anabolic steroids in sports: Analytical strategy based on the detection of phase I and phase II intact urinary metabolites by liquid chromatography tandem mass spectrometry. *Journal of Chromatography A* **1389**: 65–75.

Brandon E F A, Raap C D, Meijerman I, Beijnen J H and Schellens J H M. 2003. An update on *in vitro* test methods in human hepatic drug biotransformation research: Pros and cons. *Toxicology and Applied Pharmacology* **189**(3): 233–46.

Buiarelli F, Cartoni G P, Coccioli F, Giannetti L, Merolle M, Neri B and Terracciano A. 2005. Detection of boldenone and its major metabolites by liquid chromatography — tandem mass spectrometry in urine samples. *Analytica Chimica Acta* **552**: 116–26.

Cannizzo F T, Zancanaro G, Spada F, Mulasso C and Biolatti B. 2007. Pathology of the testicle and sex accessory glands following the administration of boldenone and boldione as growth promoters in veal calves. *Journal of Veterinary Medical Science* **69**(11): 1109–16.

De Brabander H F, Poelmans S, Schilt R, Stephany R W, Le Bizec B, Draisci R, Sterk S S, van Ginkel L A, Courtheyn D, Van Hoof N, Macrì A and De Wasch K. 2004. Presence and metabolism of the anabolic steroid boldenone in various animal species: A review. *Food Additives and Contaminants* **21**(6): 515–25.

Fred Hartgens, Wouter D. Van Marken Lichtenbelt, Spike Ebbing, Niels Vollaard, Gerard Rietjens and Harm Kuipers. 2001. Androgenic-anabolic steroid—induced body changes in strength athletes. *The Physician and Sportsmedicine* **29**(1): 49–46.

IARC I A for R on C. 1987. Evaluation of carcinogenic risks. Overall evaluations of carcinogenicity: IARC Monographs, 1–42.

Mayada R F, Taghred M S and Haytham A A. 2015. Boldenone-induced apoptotic, structural, and functional alterations in the liver of rabbits. *World Rabbit Science* **23**(1): 39–46.

Merlanti R, Gallina G, Capolongo F, Contiero L, Biancotto G, Dacasto M and Montesissa C. 2007. An *in vitro* study on metabolism of 17β-boldenone and boldione using cattle liver and kidney subcellular fractions. *Analytica Chimica Acta* **586**(1-2 SPEC. ISS.): 177–83.

Nielen M W F, Rutgers P, Van Bennekom E O, Lasaroms J J P and Van Rhijn J A. 2004. Confirmatory analysis of 17β-boldenone, 17α-boldenone and androsta-1,4-diene-3,17-dione in bovine urine, faeces, feed and skin swab samples by liquid chromatography-electrospray ionisation tandem mass spectrometry. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences* **801**(2): 273–83.

Philip M, Karakka Kal A K, Subhahar M B, Karatt T K, Mathew B and Perwad Z. 2022. *In vitro* studies of hypoxia inducible factor-prolyl hydroxylase inhibitors daprodustat, desidustat, and vadadustat for equine doping control. *Drug Testing and Analysis* **14**(2): 317–48.

Philip M, Karakka Kal A K, Subhahar M B, Perwad Z and Karatt T K. 2021. Characterization of equine liver microsome-generated metabolites of growth hormone secretagogue small molecule ibutamoren. *Rapid Communications in Mass Spectrometry* **35**(23).

Philip M, Mathew B, Karatt T K, Perwad Z, Subhahar M B and Karakka Kal A K. 2021. Metabolic studies of hypoxia-inducible factor stabilisers IOX2, IOX3 and IOX4 (*in vitro*) for doping control. *Drug Testing and Analysis* **13**(4): 794–816.

Popot M A, Stojiljkovic N, Garcia P, Bonnaire Y and Tabet J C. 2003. First mass spectrometric detection of boldenone in horse mane samples. *Chromatographia* **57**(3–4): 255–60.

Schanxer W and Domke M. 1993. Metabolism of anabolic steroids in man: synthesis and use of reference substances for identification of anabolic steroid metabolites. *Analytica Chimica Acta* **275**: 23–48.

Soma L R, Uboh C E, Guan F, Mcdonnell S and Pack J. 2007. Pharmacokinetics of boldenone and stanozolol and the results of quantification of anabolic and androgenic steroids in race horses and nonrace horses. *Journal of Veterinary Pharmacology and Therapeutics* **30**(2): 101–08.

Van Puymbroeck M, Kuilman M E M, Maas R F M, Witkamp R F, Leyssens T, Vanderzande D, Gelan J and Raus J. 1998. *In vitro* liver models are important tools to monitor the abuse of anabolic steroids in cattle. *Analyst* **123**(12): 2453–56.

Van Puymbroeck Mark, Kuilman M E M, Maas R F M, Witkamp R F, Leyssens L, Vanderzande D, Gelan J and Raus J. 1998. Identification of some important metabolites of boldenone in urine and feces of cattle by gas chromatography-mass spectrometry. *Analyst* **123**(12): 2681–86.

Verheyden K, Noppe H, Zorn H, Van Immerseel F, Bussche J Vanden, Wille K, Bekaert K, Janssen C R, De Brabander H F and Vanhaecke L. 2010. Endogenous boldenone-formation in cattle: Alternative invertebrate organisms to elucidate the enzymatic pathway and the potential role of edible fungi on cattle's feed. *Journal of Steroid Biochemistry and Molecular Biology* **119**(3–5): 161–70.