Drug Testing and Analysis

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Gel Electrophoretic Detection of Black Market ACE-031

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ABSTRACT

The usage of ACE-031 (Ramatercept), a dimeric fusion protein consisting of a human activin receptor IIB (ACVR2B) fragment linked to an Fc-part of human IgG1, is banned according to chapter S4.3 of the "WADA 2024 List of Prohibited Substances and Methods" due to its potential performance enhancing properties. While ACE-031 has not yet been pharmaceutically approved, it is sold as research chemical on the "black market" (BM). The article presents a study on BM ACE-031 products and its detection by gel-electrophoresis and Western blotting. Of 14 tested products, only 12 contained an ACVR2B-immunoreactive protein. Electrophoretic separation by SDS-PAGE also showed that the 12 ACVR2B-products contained many other proteins in addition to the main compound (ca. 58.4 kDa). Further analyses by mass spectrometry and immunoblotting revealed that the 12 products contained the full-length human activin receptor IIB instead of ACE-031. The absence of an Fc-fusion protein was further confirmed by treatment with IdeS protease, which was unable to cleave the BM products. In addition, it was demonstrated that the protocol we developed to detect luspatercept (another ACVR2B-Fc fusion protein) in human serum could also be successfully applied for the detection of BM ACE-031. Because administering black market products to human subjects was not ethically justifiable, a study was conducted with rats. In rat serum, BM ACE-031 was detectable up to 48 h post administration. However, due to the relatively high dose applied (10 mg/kg body weight) and possible differences in metabolism, the detection window may be different in humans.

1 | Introduction

ACE-031 (Ramatercept) is a soluble homodimeric fusion protein (ActRIIB-IgG1Fc, 343 amino acids) with three N-glycosylation sites [1], which binds (i.e. "traps" and thus blocks) activin receptor type II B (ActRIIB) ligands including myostatin [2]. Myostatin-inhibition leads to an increase in muscle mass and loss of fat. ACE-031 was primarily developed to treat myopathies in humans (e.g., Duchenne muscular dystrophy) [3]. In healthy volunteers, an increase in total body lean mass and thigh muscle volume was observed after administration of ACE-031 [4]. Despite promising results in clinical phase I and II studies without serious or severe adverse events, the development of ACE-031 was terminated in Phase II due to potential safety reasons (e.g., nose bleeding, small dilated blood vessels near the surface of the skin) [3]. Typical doses of ACE-031 were between 0.02–3 mg/kg body weight (BW) for humans and 1–10 mg/kg for mice [3–7].

The main characteristics of the primary structure are shown in Figure 1.

Due to its potentially performance enhancing properties extrapolated from the effects on muscles, ACE-031 is prohibited

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GRGEAETREC IYYNANWELE RTNQSGLERC EGEQDKRLHC YASWRNSSGT  50
IELVKKGCWL DDFNCYDRQE CVATEENPQV YFCCCEGNFC NERFTHLPEA 100
GGPEVTYEPP PTAPTGGGTH TCPPCPAPEL LGGPSVFLFP PKPKDTLMIS 150
RTPEVTCVVV DVSHEDPEVK FNWYVDGVEV HNAKTKPREE QYNSTYRVVS 200
VLTVLHQDWL NGKEYKCKVS NKALPVPIEK TISKAKGQPR EPQVYTLPPS 250
REEMTKNQVS LTCLVKGFYP SDIAVEWESN GQPENNYKTT PPVLDSDGSF 300
FLYSKLTVDK SRWQQGNVFS CSVMHEALHN HYTQKSLSLS PGK        343
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FIGURE 1 | Characteristics of the amino acid sequence of ACE-031 [1]. The ACVR2B fragment (aa 1–115) is marked in red, the triglcyl linker in green (aa 116–118), and the human IgG1 Fc-fragment in blue (aa 119–343). The seven intra-chain disulfide bridges are between cysteins 10–40, 30–58, 65–84, 71–83, 85–90, 157–217, and 263–321 (underlined). Two interchain disulfide bridges at 122 and 125 link two monomers together to a homodimer (bold and underlined). The asparagines of the three N-glycosylation sites are marked in bold.

according to the WADA list 2024 (chapter S4 "Hormone and metabolic modulators," sub-chapter S4.3) [8]. So far, no approved ACE-031 pharmaceuticals are available. Nevertheless, ACE-031 is sold via internet by many black market (BM) companies for "research purposes" targeting bodybuilders and other athletes.

Aims of the project were (1) the biochemical and mass spectrometric characterization of ACE-031 products sold on the BM, (2) an investigation of the electrophoretic detectability in spiked human serum samples, and (3) an administration study with rats because application of BM ACE-031 to human test persons was ethically not justifiable.

2 | Experimental Section

2.1 | Materials

2.1.1 | ACE-031 BM Products

BM ACE-031 specimens were obtained from suppliers, who specialized in selling performance enhancing products via internet (e.g., peptides, steroids). However, all of them were clearly marked as "not for human consumption" or "for research purposes only." In total, 14 different products were investigated. They were bought in UK, Europe, China, and USA. In this article, all products are kept anonymous ("BM-01" to "BM-14") to avoid providing brand-specific information to fraudulent athletes.

Reblozyl (luspatercept-aamt) was obtained from Celgene Europe (Utrecht, Netherlands). It was used as reference for an ACVR2B-Fc fusion protein because ACE-031 was not yet available as pharmaceutical.

2.1.2 | Samples

Serum and urine (human, rat) were from Sigma-Aldrich (St. Louis, MO), Innovative Research (Novi, MI), and BioIVT (West Sussex, UK), respectively, and were used for method development. The methods were then used for analyzing the samples of the animal study.

2.1.3 | Liver Microsomes

Human and rat liver microsomes were purchased from Sigma-Aldrich and were stored at −80°C until usage. NADPH was also from Sigma-Aldrich.

2.1.4 | Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Lithium dodecyl sulfate (LDS) sample buffer (4×), 4-morpholinepropanesulfonic acid (MOPS) running buffer (20x), protein standards (Mark12, SeeBlue Plus2), NuPAGE BisTris precast gels (10% T, *Midi*, 1.0 mm/20 wells), and the electrophoresis chamber (XCell4 SureLock Midi Cell) were obtained from Thermo Fisher Scientific (Waltham, MA). DL-Dithiothreitol (DTT) was from Sigma-Aldrich. The heating block (ThermoMixer C) and power supply (EPS 3501 XL) were from Eppendorf (Hamburg, Germany) and GE Healthcare (Uppsala, Sweden), respectively.

2.1.5 | Coomassie Staining of SDS-PAGE Gels

Gels were stained with Coomassie Brilliant Blue R-250 from Serva (Heidelberg, Germany). Glacial acetic acid (p.a.) and methanol (LiChrosolv, gradient grade) were bought from Merck (Darmstadt, Germany).

2.1.6 | Immunoaffinity Purification

Magnetic beads (Dynabeads M-280 Sheep Anti-Rabbit IgG) were from Invitrogen (Thermo Fisher Scientific; Vilnius, LT), and the chemicals for preparing the IP buffer (25 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 5% glycerol, 0.1% NaN_3, pH 7.4) were from Sigma-Aldrich. Spinfilters for microfiltration (Corning-Costar-Spin-X-filters, 0.22 µm) and ultrafiltration (Amicon Ultra-15 and Ultra-0.5 mL; nominal molecular weight limit [NMWL] 30 kDa) were obtained from Sigma-Aldrich and Millipore (Billerica, MA), respectively. Low protein binding tubes were from Thermo Fisher Scientific (Rockford, IL) and Eppendorf. For immunoprecipitating BM ACE-031 from human and rat samples, a polyclonal anti-ACVR2B antibody from

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Adipogen Life Sciences (San Diego, CA) was used. Protease inhibitor tablets (cOmplete) and the rotator (Mini Labroller) were from Roche (Mannheim, Germany) and Labnet (Edison, NJ). Centrifuges were from Thermo Scientific (Massachusetts, USA; Heraeus Multifuge X3R) and Eppendorf (Centrifuge 5424 R).

2.1.7 | Western Blotting of SDS-PAGE Gels

The PVDF-membrane (Immobilon-P), low-fat dry milk (LFM), and phosphate-buffered saline (PBS) were bought from Millipore (Billerica, MA), Régilait (Saint-Martin-Belle-Roche, France), and Medicago (Uppsala, Sweden), respectively. Gels were blotted with a semidry blotter (Trans-Blot SD) and extra thick blotting paper from BioRad (Hercules, CA). The primary antibodies (custom-biotinylated human activin RIIB antibody clone 60,408 [MAB3392], biotinylated polyclonal human activin RIIB antibody [BAF339], anti-His-tag antibody clone AD1.1.10 [MAB050], biotinylated anti-follistatin antibody [BAF669]) were from R&D Systems (Minneapolis, MN) and the HRP-labelled goat anti-mouse IgG (H + L) highly cross-adsorbed secondary antibody from Thermo Fisher Scientific (31432). The streptavidin horseradish peroxidase (HRP) conjugate was from Roche (Mannheim, Germany). Blots were automatically processed with a BlotCycler from Precision Biosystems (Mansfield, MA) and developed with a substrate from Thermo Fisher Scientific (SuperSignal West Atto). For image acquisition and analysis, a LAS-4000 CCD-camera from Fujifilm (Tokyo, Japan) and GASepo software version 2.3 (Seibersdorf, Austria) was used [9].

2.1.8 | Tryptic In-Gel Digestion

Iodoacetamide and ammonium hydrogen carbonate were bought from Sigma-Aldrich. Water, formic acid, and acetonitrile (all LC–MS grade) were from Biosolve (Valkenswaard, Netherlands) and Fisher Scientific (Loughborough, UK), respectively. Sequencing Grade Modified Trypsin was obtained from Promega (Madison, WI) and the microfilters (PVDF, 0.22 µm) from Millipore. The vacuum concentrator was from Christ Gefriertrockungsanlagen GmbH (Osterode, Germany).

2.1.9 | Liquid Chromatography High-Resolution Mass Spectrometry (LC-HRMS)

A Vanquish Neo UHPLC-System was coupled to an Orbitrap Exploris 480 mass spectrometer, both from Thermo Fisher Scientific (Germering and Bremen, Germany). The trapping and analytical columns were from Waters (Milford, MA) and Agilent (Woburn, MA), respectively (for further details, see Section 2.2). Mass spectra were processed with the Proteome Discoverer software 1.3 (Thermo Fisher Scientific; San José, CA).

2.2 | Methods

2.2.1 | Animal Study

Male and female Lewis rats (age 8–10 weeks, total number $n = 30$) were purchased from a registered laboratory animal supplier (Janvier Labs, France) and held conventionally or in individually ventilated cages (IVC) of suitable size (Type III/Type IV) either in groups or single-caged. Animals received a standard laboratory rodent diet and water ad libitum. Routine animal examination was performed on daily basis. After 1 week of acclimatization, all animals were treated with a single subcutaneous injection of BM ACE-031 (10 mg/kg body weight; diluted in PBS) [6, 7]. Then, the animals were placed back into their cages. After 24 (Group 1), 48 (Group 2), and 168 h (Group 3), animals (five

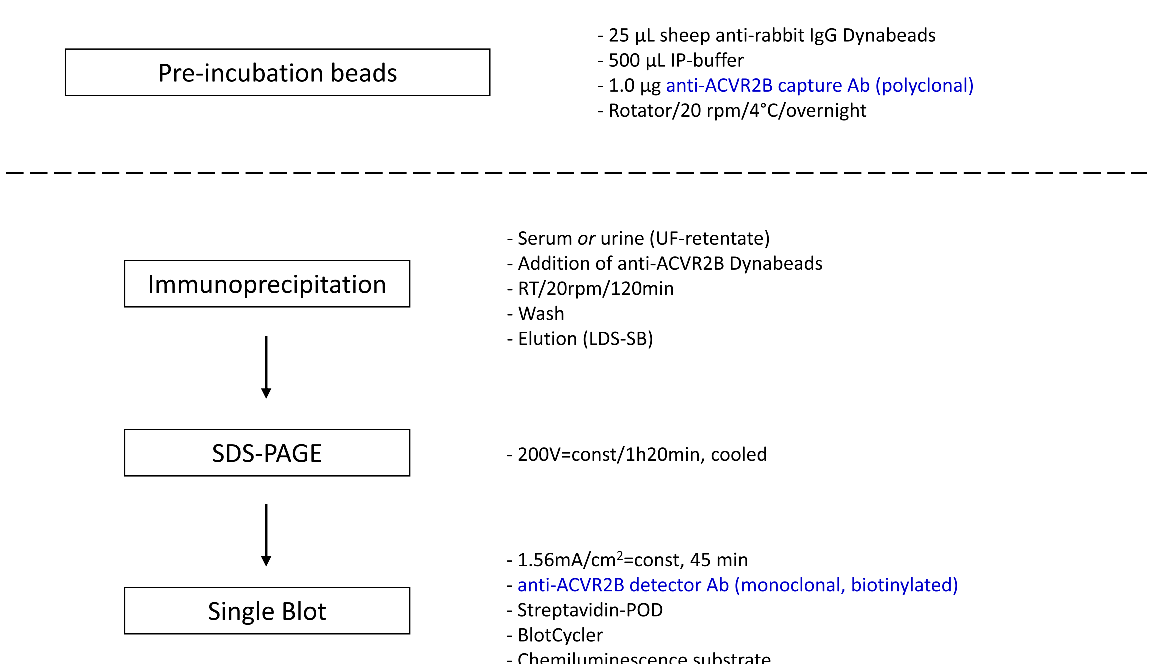

FIGURE 2 | Flow scheme for the detection of black market ACE-031 in serum and urine. Key elements are the IP step employing a polyclonal anti-ACVR2B antibody as well as immunoblotting with a monoclonal anti-ACVR2B antibody after electrophoretic separation.

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male, five female rats for each study group) were anesthetized using isoflurane in medical air and a terminal blood sample (4–5 mL) was drawn via puncture of the retro-orbital sinus vein. Blood was collected in BD Vacutainer SST II Advance tubes and processed as described by the manufacturer. After centrifugation, serum was transferred into test tubes. Following blood sampling, animals were euthanized using pentobarbital sodium and urine was collected. Serum and urine samples were stored at −80°C until further analysis. The study was approved by the Austrian Federal Ministry of Education, Science and Research (file number 2022-0.457.757).

2.2.2 | In Vitro Metabolism Study

The study followed a published protocol [10]. Briefly, human and rat liver microsomes were slowly thawed on ice. Then, 10 µg of BM ACE-031 (1 µg/µL) were added to 175 µL of phosphate buffer (100 mM, pH 7.4) and 5 µL of microsomes (20 mg/mL). The mixture was preincubated for 5 min in a Thermomixer at 37°C. To initiate the reaction, 10 µL of NADPH (20 mM in phosphate buffer) were added, followed by incubation at 37°C/300 rpm for 5, 60, 120, 300 min, and 24 h. Two controls were also prepared: (1) BM ACE-031 in buffer with NADPH but without microsomes and (2) microsomes plus NADPH in buffer but without BM ACE-031. They were incubated at 37°C/300 rpm for 60 min only. Following incubation, samples were immediately stored at −80°C to terminate the reaction.

2.2.3 | SDS-PAGE

SDS-PAGE was performed as described earlier [11]. Samples were mixed with LDS-sample buffer (1×, 0.1 M DTT) and heated at 95°C/5 min/650 rmp (Thermomixer). Electrophoresis was performed at constant voltage (200 V/75 min) with MOPS running buffer and under cooled conditions. On gels stained with *Coomassie R-250*, ca. 10 µg of each BM product was applied. For Western blots of the BM products, between ca. 2 and 50 ng were investigated, and for the microsome study, 1 µL of each supernatant was used. Samples of the animal study as well as human specimens spiked with ACE-031 were immunoaffinity purified and processed as described below.

2.2.4 | Coomassie R-250 Stain of SDS-PAGE Gels

After electrophoresis, gels were incubated in fixing solution (50% methanol/10% acetic acid, 15 min) followed by staining in 0.025% Coomassie R-250 (40% methanol/10% acetic acid, overnight). Gels were destained with 20% methanol/10% acetic [12].

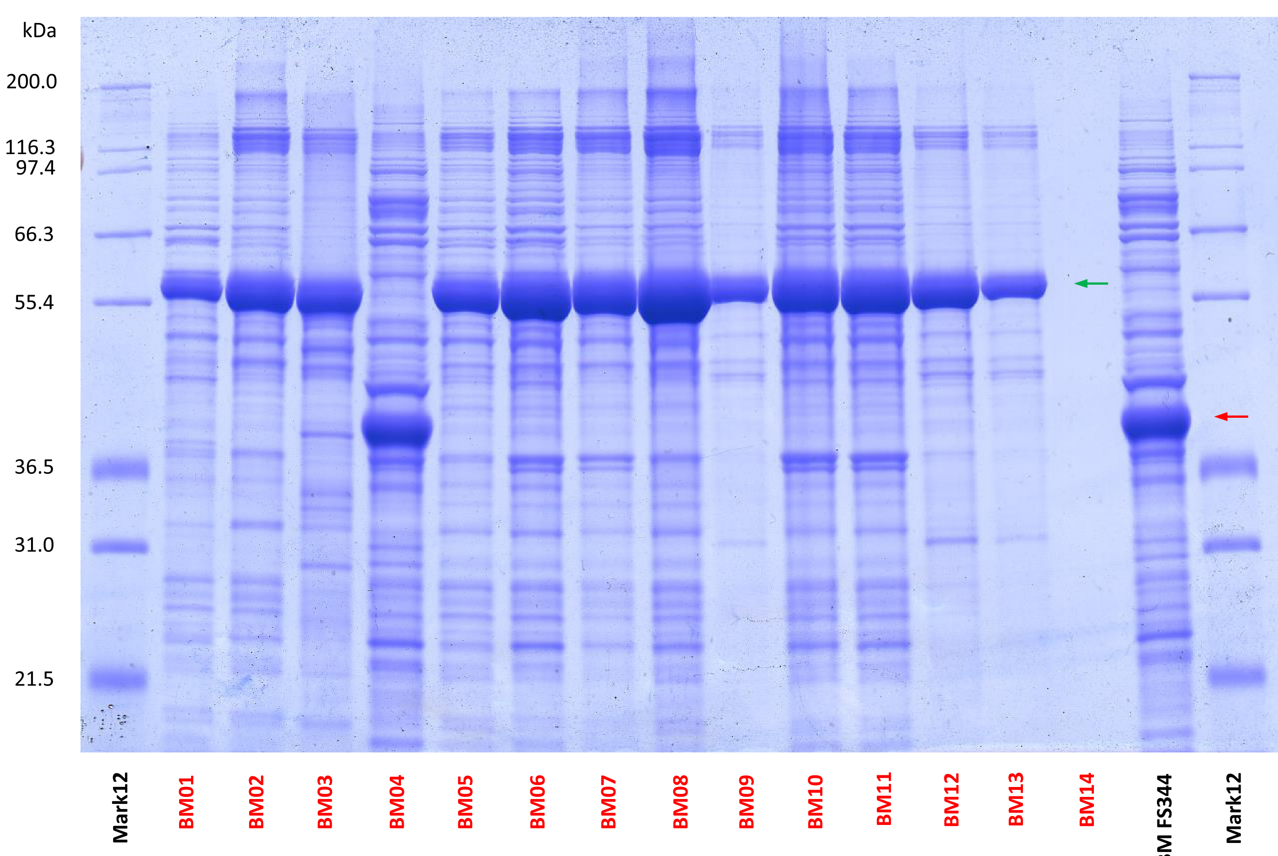

FIGURE 3 | Separation characteristics of 14 ACE-031 black market products (BM01 to BM14) on SDS-PAGE (10% T, MOPS running buffer; ca. 10 µg on gel, Coomassie R-250 stain). Twelve products looked quite identical after electrophoretic separation (the green arrow indicates the main band). Product BM04 did not contain ACE-031 but black market follistatin 344 instead (red arrow; also see Figure 4B). No proteins were found in BM14. Product BM06 was chosen for the administration study. Mark12 is the molecular weight marker.

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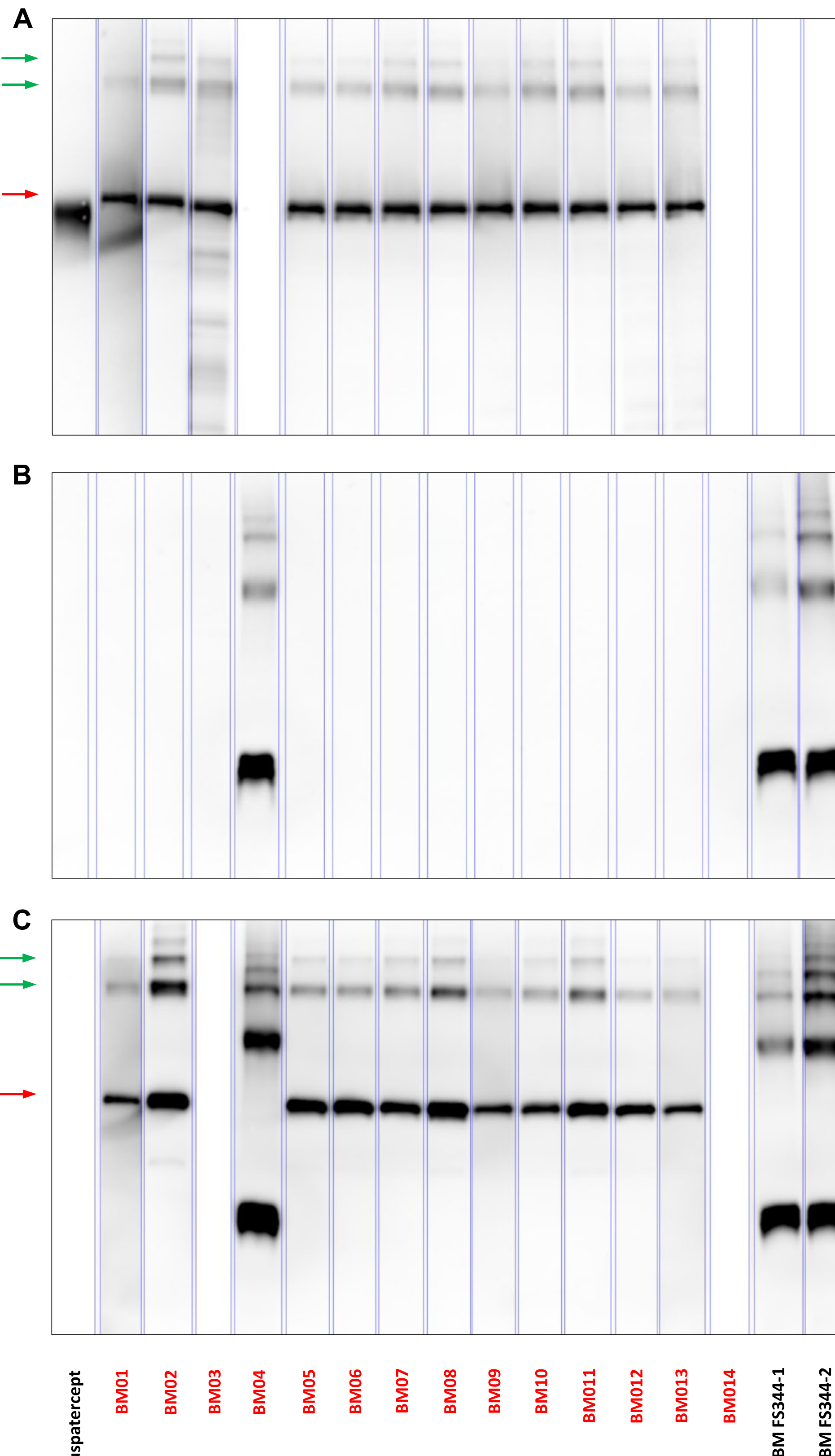

FIGURE 4 | Separation characteristics of 14 ACE-031 black market products (BM01 to BM14) on SDS-PAGE (10% T, MOPS running buffer; ca. 50 ng on gel). Immunoblots with anti-ACVR2B antibody BAF339 (A), anti-follistatin antibody BAF669 (B), and anti-His antibody MAB050 (C). BM04 did not contain immunoreactive ACVR2B (A) bust instead BM follistatin 344 (B). All ACE-031 products except BM03 were positive for His-tags (C). Luspatercept (Lane 1) served as positive control for an ACVR2B-Fc fusion protein, and the two black market follistatins (BM FS344-1 and -2) as positive controls for follistatins with His-tags (Lanes 16 and 17). The red arrow indicates the monomer, and the green indicates the dimer and trimer of the black market products.

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2.2.5 | Semidry Western Blotting

Before blotting, SDS-PAGE gels were equilibrated in Bjerrum buffer (48 mM Tris/39 mM glycine/0.375% SDS/5% methanol; 3 × 5 min). For the transfer, one sheet of extra thick blotting paper on each side of the gel and an Immobilon-P membrane was used. Blotting was performed at constant current (1.56 mA/cm^2, 45 min) [13, 14]. Membranes were blocked in 5% low-fat milk in PBS (LFM/PBS) for 60 min at room temperature followed by incubation in primary (biotinylated *MAB3392*: 1 µg/mL, 720 min; *BAF339* and *BAF669*: 0.1 µg/mL, 720 min; *MAB050*: 0.2 µg/mL, 300 min) and secondary antibodies (*31432*:1/100,000, 720 min) or streptavidin-HRP (1/4000, 300 min) diluted in 1% LFM/PBS. All incubations were automatically performed in a BlotCycler overnight (coldroom). Washes between incubations were with PBS (9 × 5 min). Subsequently, membranes were developed with chemiluminescent substrate (SuperSignal West Atto) and images acquired with a LAS-4000 camera.

2.2.6 | Immunoaffinity Purification of Samples Containing BM ACE-031

The protocol was based on our previously published procedure for the purification of luspatercept in human specimens with slight modifications [15].

Briefly, Dynabeads (25 µL) were washed with IP-buffer (3 × 1000 µL) and then coated with 1 µg of anti-ACVR2B antibody in 500 µL IP-buffer (rotator, 20 rpm/coldroom/overnight). Subsequently, beads were washed with IP buffer (1 × 1000 µL), resuspended in 500 µL IP-buffer and then added to 500 µL of microfiltrated (16,000 g/10 min) serum sample. After 120 min on a rotator at room temperature, tubes were put into a magnet (DynaMag-2), and after ca. 2 min, the supernatant was removed. After three washes with 1500 µL IP-buffer and two washes with Tris–HCl buffer (50 mM, pH 7.4), bound proteins were eluted by heating the beads with 18 µL LDS-sample buffer (1×; 0.1 M DTT) at 95°C/5 min/650 rpm. After cooling down, the eluate was loaded onto an SDS-PAGE gel, run at constant voltage, and immunoblotted. The blocked membranes were processed in a BlotCycler with primary and HRP-labelled secondary antibodies or streptavidin-HRP.

For immunoaffinity purification of urine, samples of each animal study group were pooled (because the available amount of urine of each rat was quite low), supplemented with Tris–HCl buffer (3.75 M, pH 7.4) and cOmplete protease inhibitor, then centrifuged (4000 g/15 min) and the supernatant ultrafiltrated (Amicon Ultra-15; 4000 g / 15 min). Then, the retentate was washed two times with 50 mM Tris–HCl buffer (pH 7.4) and further concentrated down to ca. 20 µL with an Amicon Ultra-0.5 mL filter (14,000 g/15 min). After adjusting the volume to 500 µL with IP-buffer (2 mL Eppendorf tube), the retentate was added to the washed anti-ACVR2B antibody-coated Dynabeads. All subsequent steps were the same as for serum samples. Figure 2 summarizes the main steps of the detection protocol.

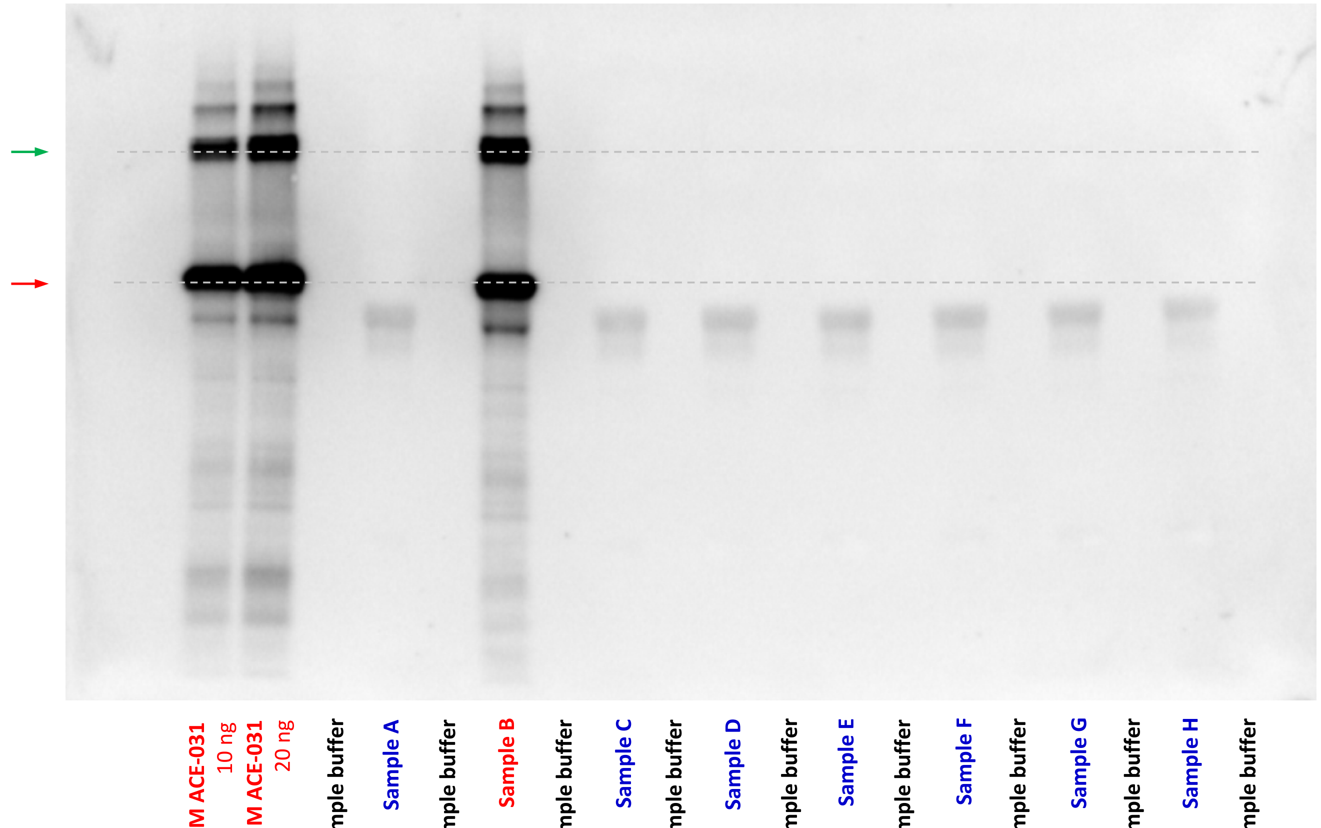

FIGURE 5 | Results of the inter-laboratory myostatin inhibitors' study. Of the eight blind serum samples, the one containing black market ACE-031 (Sample B) was correctly identified. IP with polyclonal ACVR2B antibody coated magnetic beads. Immunoblot after SDS-PAGE with clone 60408 anti-ACVR2B antibody. The red arrow indicates the monomer, and the green indicates the dimer.

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2.2.7 | IdeS Protease Digestion

Digestion of BM ACE-031 products was performed with IdeS protease from Promega as described by the manufacturer with modifications. Briefly, 3 μL of enzyme (50 U/μL) were added in access to BM ACE-031 (ca. 10 μg in 50 mM Tris–HCl buffer, pH 7.4). Reblozyl was used as positive control. Incubations were at 37°C for 60 min.

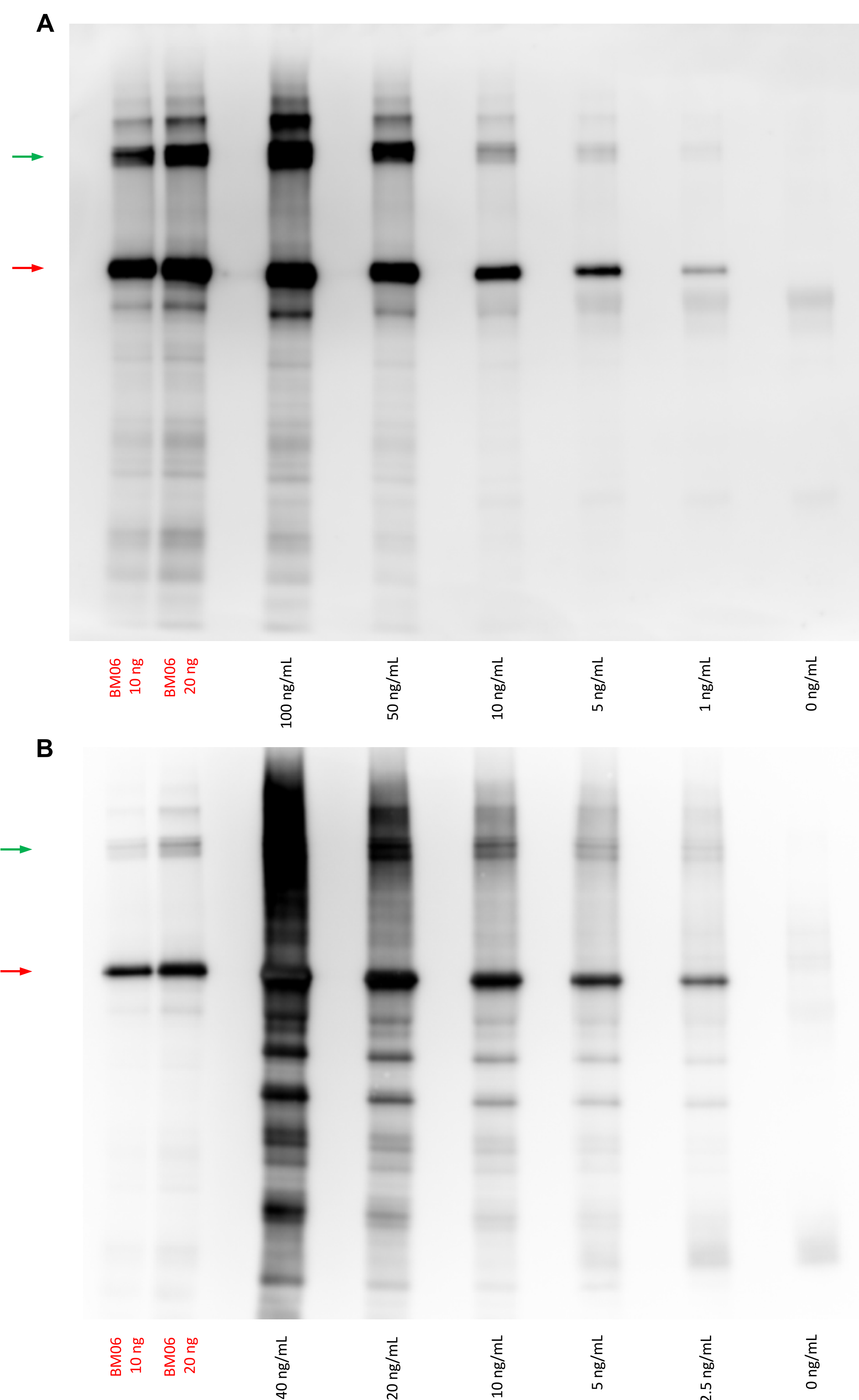

FIGURE 6 | Method verification for the detection of BM ACE-031 in rat serum (A) and rat urine (B). SDS-PAGE and Western blot with clone 60408 anti-ACVR2B antibody after IP with a polyclonal anti-ACVR2B antibody. ACE-031 monomer (red arrow). ACE-031 dimer (green arrow). The negative control (0 ng/mL) shows no interferences in the mass range of the monomer and dimer.

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2.2.8 | Tryptic Digestion

The procedure followed a protocol published by Shevchenko et al. [16]. In brief, Coomassie-stained SDS-PAGE bands of interest were excised from the gel and minced. Subsequently, gel pieces were destained with 50 mM NH_4HCO_3 in 50% acetonitrile (37°C/1000 rpm/2 × 15 min; Thermomixer), followed by reduction of disulfide-bridges in 10 mM DTT/50 mM NH_4HCO_3 (57°C/800 rpm/30 min) and carbamidomethylation (55 mM iodoacetamide/50 mM NH_4HCO_3, 25°C /800 rpm/60 min/dark). Residual reagents were removed by washing the gel pieces with 50 mM NH_4HCO_3/50% acetonitrile (37°C/800 rpm /2 × 15 min) and acetonitrile (37°C /800 rpm/15 min). Finally, trypsin (dissolved in 50 mM NH_4HCO_3) was added and proteins were digested at 37°C in an incubator overnight. The supernatant was collected in a LoBind Eppendorf tube, and remaining peptides were further extracted with 50 mM NH_4HCO_3, 5% formic acid, 50% acetonitrile/5% formic acid, and acetonitrile. To remove small gel particles, the pooled extract was microfiltrated using centrifugal filters, which were pre-washed with 50% acetonitrile/5% formic acid. The filtrate was transferred to a Lobind tube and then dried by vacuum centrifugation (50°C/150 min). Peptides were redissolved in 5% formic acid (37°C/1400 rpm/30 min) and stored at −30°C until analysis.

2.2.9 | LC-HRMS

As mobile phases, 0.1% formic acid (A) and ACN/0.1% formic acid (B) were used. Peptides were first trapped on an M/Z Symmetry C18 trap column (180 µm × 20 mm, 5 µm, 100 Å) from Waters (8 µL/min, 1% B, 3 min) and then separated on an analytical column from Agilent (ZORBAX 300SB-C18, 3.5 µm,

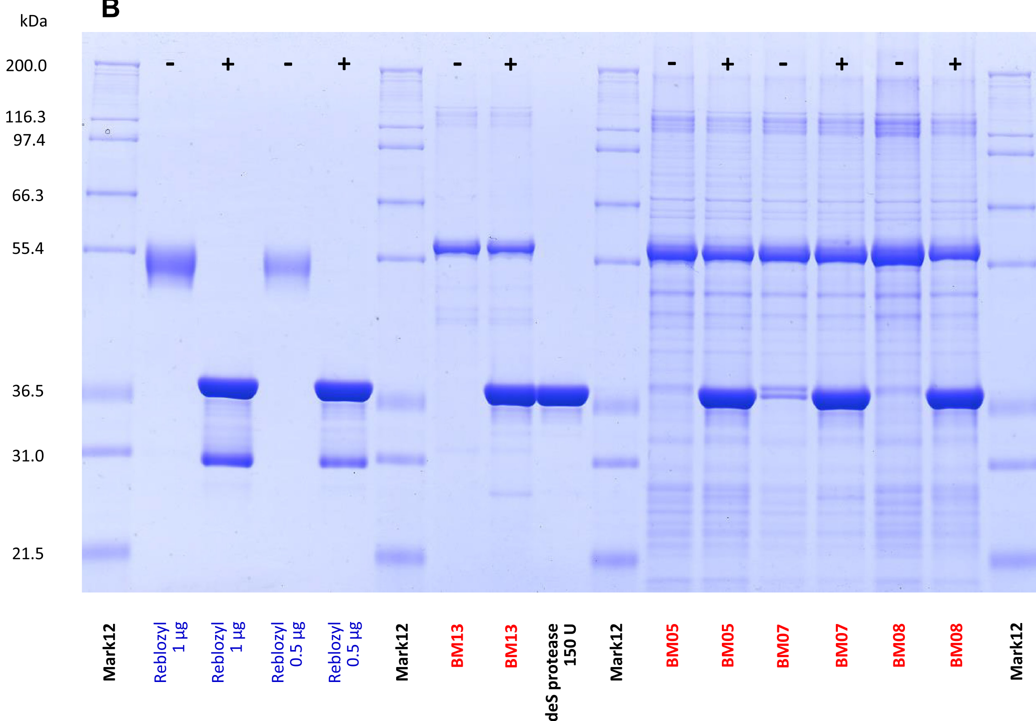

FIGURE 7 | (A) Amino acid sequence of human activin receptor type-2B (1–18: signal peptide (green), 19–137/138–158/159–512: extracellular/transmembrane/cytoplasmatic domains; see numbered amino acids in the sequence). [23] Sequences identified by mass spectrometry are marked in red (underlined: spectra shown in Figure 8). (B) IdeS protease digest of several BM ACE-031 products on SDS-PAGE with Coomassie stain. Compared with the ACVR2B-Fc fusion protein luspatercept (Reblozyl), no cleavage was observed for the BM products. ACE-031 (red) was adjusted to match the amount of Reblozyl (0.5–1.0 µg; blue). Without/with IdeS protease: −/+.

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0.10 × 150 mm; 700 nL/min). The applied gradient was (1) 1% B to 60% B in 120 min and (2) 60% B to 90% B in 10 min. Then, the column was washed for 5 min at 90% B followed by re-equilibration to 1% B. Injection volumes were 1 μL.

The mass spectrometer was operated in data-dependent acquisition (DDA) mode; that is, one full scan (*m/z* 300–2000) at a resolving power of $R = 120{,}000$ (at *m/z* 200) was followed by MS/MS scans of the 15 most intense ions at $R = 15{,}000$. Dynamic exclusion was set to 15 s and at a mass tolerance of ±10 ppm. A Nanospray Flex Ion Source (Thermo Fisher Scientific) was used to hyphenate mass spectrometer and HPLC. Positive ion mode and a spray voltage of 2.5 kV were used. The capillary temperature was 325°C. Polydimethylcyclosiloxane background ion (*m/z* 445.120025) was used as lock-mass [17].

Search parameters in Proteome Discoverer were defined as follows: trypsin (fully enzymatic); maximum number of missed cleavage sites 2; 10 ppm and 0.01 Da precursor and fragment mass tolerances. Dynamic and fixed modifications were oxidized methionine and carbaminomethylcysteine, respectively. Spectra were searched against UniProt protein database (UP000005640_9606) and a decoy database for false discovery rate estimation.

3 | Results and Discussion

3.1 | Electrophoretic Properties of BM ACE-031 on SDS-PAGE

Of the 14 tested ACE-031 products, 12 contained proteins with similar mass distribution (most intense band ca. 58.4 kDa; Figure 3). This apparent molecular mass corresponded to the mass observed for similar ACVR2B-Fc fusion proteins like luspatercept [18], but the products were very impure. One product (BM04) also contained proteins but with different mass profile (ca. 40.8 kDa). In another product (BM14), no proteins were found (Figure 3, Lane 15). Instead, a growth hormone secretagogue (Ipamorelin) was identified by mass spectrometry (data not shown).

After immunoblotting with an antibody specific for the N-terminal region, the presence of ACVR2B was confirmed for the 12 products (Figure 4A). BM04 was identified as BM follistatin 344 (Figure 4B) by incubating the blot with an anti-follistatin antibody. Incubation with an anti-His antibody further revealed that of the 12 ACVR2B-positive ACE-031 products, all except BM03 contained His-tags as already observed for BM follistatins (Figure 4C) [19].

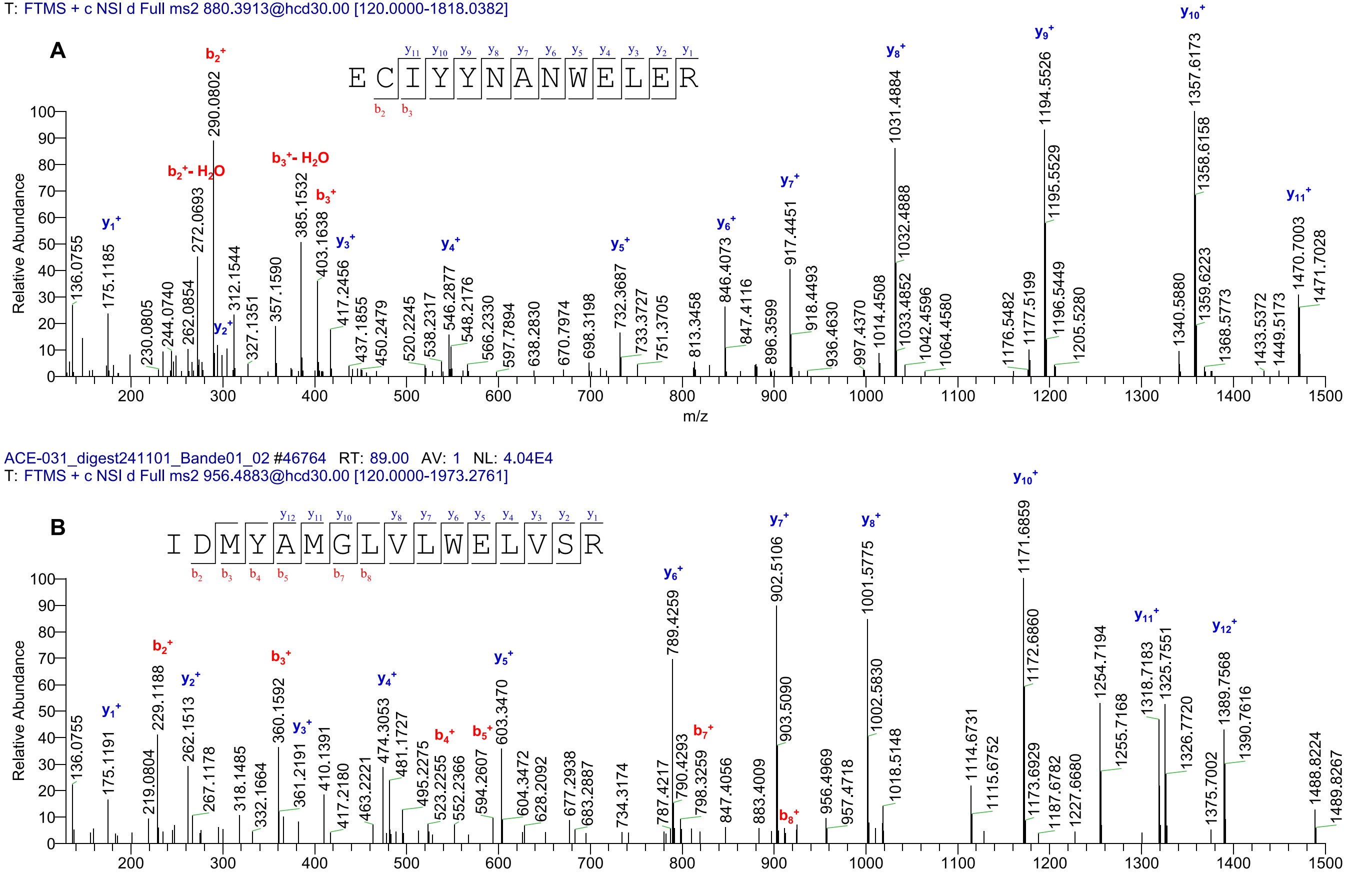

FIGURE 8 | Examples of two MSMS spectra obtained by DDA of the tryptic in-gel digest of BM ACE-031. (A) Precursor ion *m/z* 880.39136 Da ($z = 2$) was identified as carbamidomethylated peptide EC$_{cam}$IYYNANWELER, and (B) *m/z* 956.48828 Da ($z = 2$) as IDMYAM$_{ox}$GLVLWELVSR. Product ions *y* and *b* are annotated in blue and red.

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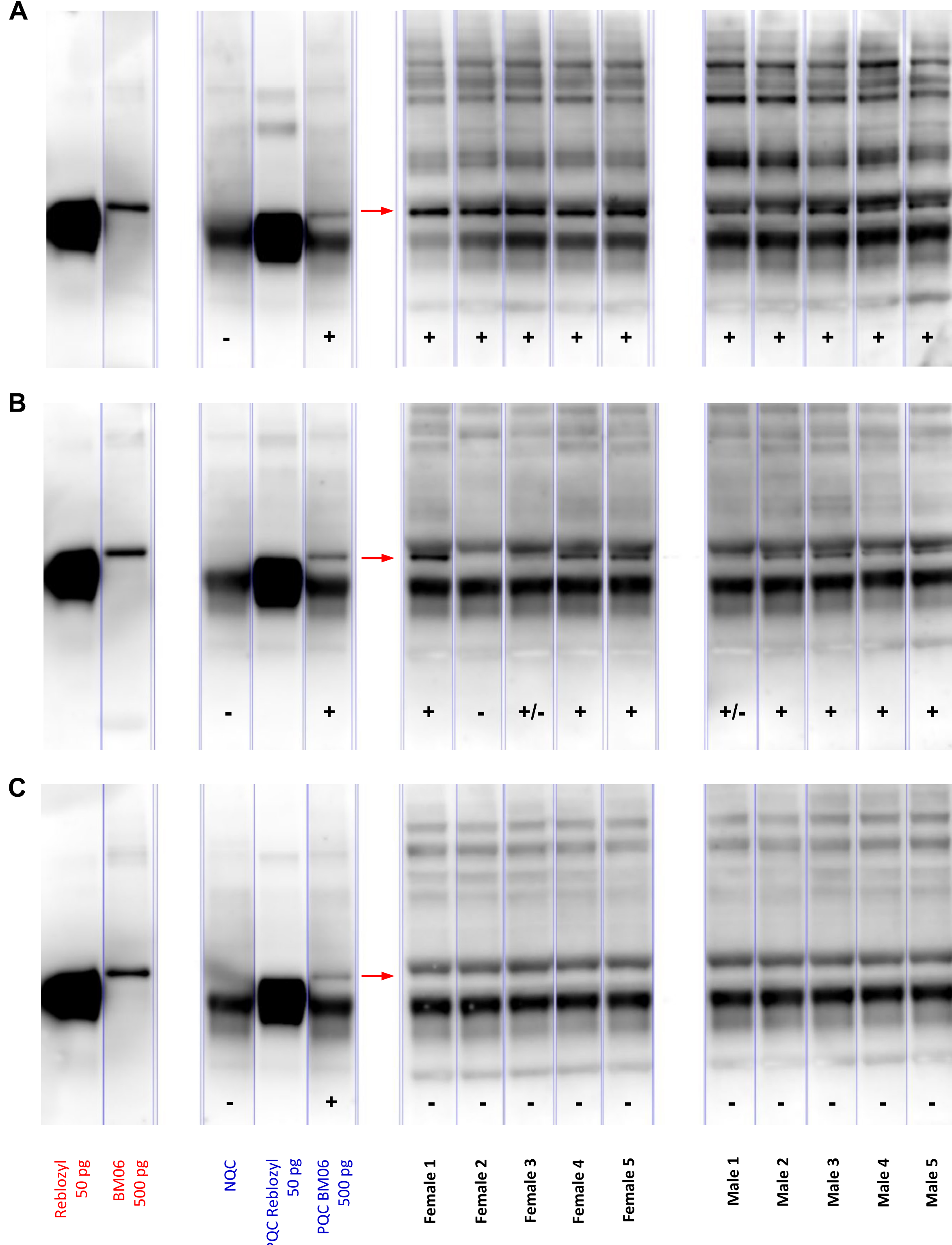

FIGURE 9 | Analysis of five female and five male rat serum samples 24/48/168 h after administration of BM ACE-031 (red arrow). IP with polyclonal anti-ACVR2B antibody followed by SDS-PAGE and immunoblot with biotinylated clone 60408 anti-ACVR2B antibody and streptavidin-POD. Note the strong difference in signal intensity between the black market (red) product and the Reblozyl (blue), which indicates that the BM product contains a much lower amount of immunoreactive ACVR2B. *NQC* (negative rat serum), *PQC* (Reblozyl/BM ACE-031 positive rat serum). Lanes marked with a "+","+/−", or "−" refer to samples in which BM ACE-031 was "clearly," "not clearly," or "not" identified.

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For the administration study, ACE-031 was bought from one and the same supplier, who was reliable enough to provide the required number of vials for the study (BM06).

3.2 | Inter-Laboratory Method Verification With Human Serum

To confirm that our previously developed detection method for luspatercept in human samples can also detect BM ACE-031 in human serum, an inter-laboratory study was performed. Eight samples containing either BM myostatin inhibitors (2×FS344, 2×GDF8, 1×ACE-031) or none (3 ×) were prepared by another WADA doping control laboratory and sent to us blind for analysis. "Sample B" was correctly identified to contain BM ACE-031 (Figure 5).

3.3 | Method Verification for Rat Specimens

As the detection method for luspatercept was originally developed for human serum and urine [15], it had to be verified whether it could also be applied to rat specimens. For that purpose, commercial rat serum (500 µL) and rat urine (5 mL) were spiked with a dilution series of BM ACE-031 and then processed as described in Figure 3.

It could be demonstrated that the method is also applicable for rat samples. The achieved detection limits were ca. 1 ng/mL and ca. 2.5 ng/mL for rat serum and urine, respectively. Figure 6A shows the results obtained for BM ACE-031 and rat serum, and Figure 6B for rat urine.

3.4 | In-Gel Digestion and Analysis by High-Resolution High-Accuracy Mass Spectrometry

The electrophoretic and immunological methods only demonstrated that the apparent molecular mass of BM ACE-031 is similar to luspatercept (Coomassie stain) and that an immunoreactive amino acid sequence from the N-terminus of human ACVR2B is present (Western blot). Because it was unclear, if BM ACE-031 is indeed an ACVR2B-Fc fusion protein, a shotgun proteomics approach was applied. It identified extracellular (i.e., N-terminal) ACVR2B tryptic peptides but also revealed the absence of the expected human IgG1 Fc-fragment. Surprisingly, many peptides from the cytoplasmatic region of human ACVR2B were identified (Figures 7A and 8). Taken together, these data demonstrate that the 12 BM products examined do not contain an ACVR2B-Fc fusion protein but instead the full length activin receptor 2B. This result was further supported by the non-cleavability of the BM products by IdeS protease, an enzyme that specifically cuts IgGs below the hinge region, releasing the Fc fragment (Figure 7B) [20–22]. Furthermore, the observed apparent molecular mass on SDS-PAGE is in good accordance with the calculated mass of (the non-glycosylated) ACVR2B protein (55.9 kDa; without signal peptide). This indicates that the BM products were produced in bacterial rather than mammalian expression systems. Otherwise, their mass would be higher.

3.5 | Analysis of Rat Serum Samples After Administration of BM ACE-031

Twenty four, 48 and 168 h after administration of BM ACE-031 to five male and five female rats, blood was collected and serum

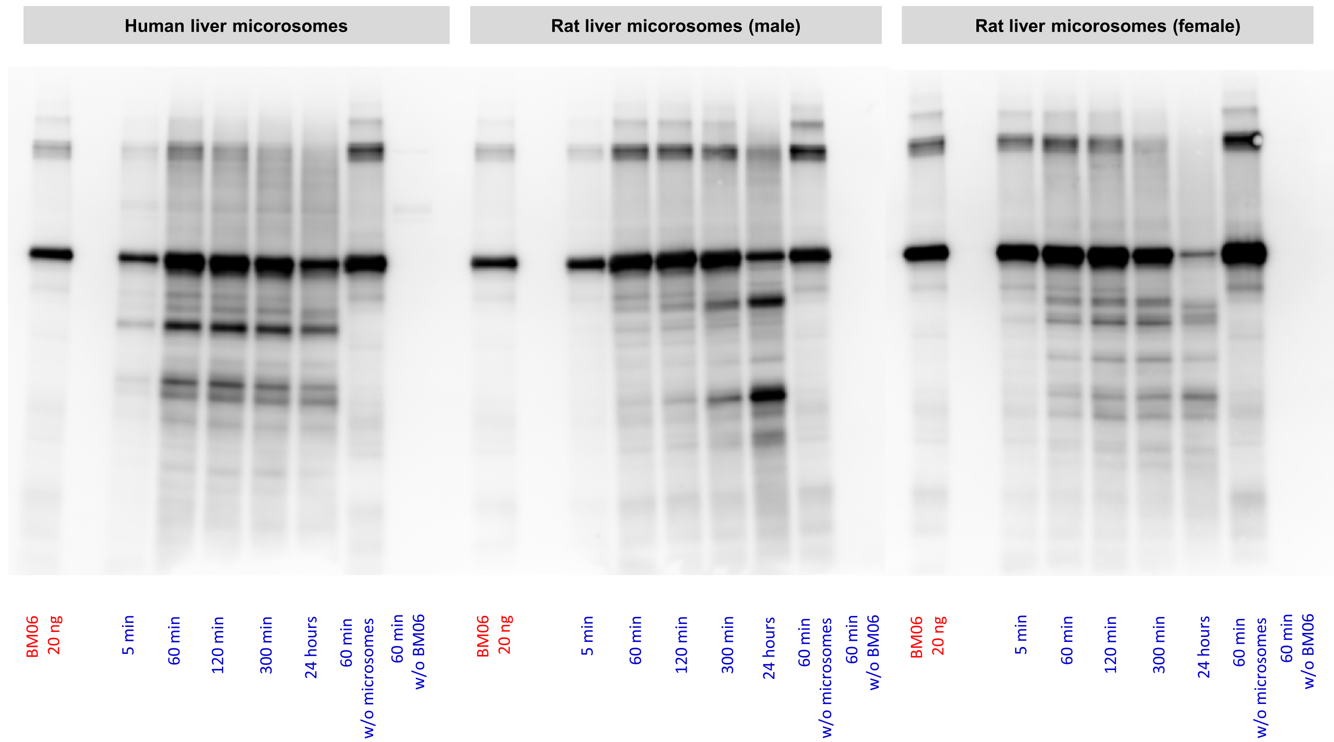

FIGURE 10 | In vitro study of BM ACE-031 using human and rat liver microsomes. SDS-PAGE and Western blot with biotinylated clone 60408 anti-ACVR2B antibody and streptavidin-POD.

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analyzed as described above. BM ACE-031 was detectable in all male and female samples 24 h post administration (Figure 9A). After 48 h, the product was still detectable in most of the male and female samples (Figure 9B) but was no longer detectable after 168 h (Figure 9C).

3.6 | Analysis of Rat Urine Samples After Administration of BM ACE-031

Because the collected urine volume of each rat was quite low, samples of each group were pooled. The lowest amount of pooled urine (5 mL) was obtained 24 h post administration. Hence, also, 5 mL of pooled urine were investigated of the samples collected after 48 and 168 h. At all three time points, ACE-031 was undetectable in the pooled urines (data not shown).

3.7 | In Vitro Metabolism Study of BM ACE-031

To investigate if metabolites in rat are different from those in humans, BM ACE-031 was incubated with human and rat liver microsomes according to a standard protocol. Samples were taken after 5/60/120/300 min and 24 h and analyzed by SDS-PAGE. After 24 h, a significant decrease in signal intensity of the main band of ACE-031 was observed both for incubations with human and rat microsomes. Before that, the intensity of the main band was hardly affected by the microsomal enzymes (Figure 10). This shows that human and rat liver microsomes are not ideal systems for representing in vivo metabolism in this case.

4 | Conclusion

Our study of 14 BM ACE-031 products revealed that none of the products contained ACE-031, a fusion protein composed of human activin receptor IIB- and IgG1 Fc- fragments (ACVR2B-Fc). Instead, the full-length receptor without the Fc-fragment was found in 12 products and BM follistatin and ipamorelin in the other two. Because our electrophoretic detection method for luspatercept (another ACVR2B-Fc fusion protein) targeted the extracellular domain of the receptor, it was also applicable for the detection of BM ACE-031. Furthermore, the observed apparent molecular mass is in line with the mass of the non-glycosylated protein. Due to this difference and because ACVR2B is a transmembrane protein, confusion with possible traces of the endogenous protein in blood can be ruled out. The presence of a His-tag may be used as additional evidence for the exogenous origin of the compound.

After a single-dose administration of BM ACE-031 to rats (10 mg/kg body weight), the protein was detectable in all serum samples after 24 h. While it remained traceable in the majority of the 48 h samples, it was undetectable after 7 days (168 h). No ACE-031 was found in the pooled urine samples at all three time-points. However, due to the relatively high dose applied and possible differences in metabolism, the detection window may be different in humans.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available upon reasonable request.

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