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Article

Quantitative Assay Validation for Oxandrolone in Human Plasma Using LC–MS-MS

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A high-performance liquid chromatography–tandem mass spectrometry (LC–MS-MS) method for the determination of oxandrolone concentration in human plasma (0.5 mL) was developed and validated according to the 2001 FDA Bioanalytical Guidelines. Oxandrolone is an anabolic steroid used to promote weight gain for cachectic patients with severe burn injuries, HIV/AIDS, hepatitis C and other wasting syndromes. The assay procedure involved a liquid–liquid extraction of oxandrolone and methyltestosterone (the internal standard, IS) from plasma with *n*-butyl chloride. The organic layer was clarified by centrifugation and evaporated to dryness under a stream of air. The residue was reconstituted in a solution containing 25% methanol and 75% Milli-Q water, and injected onto a Luna C18 reversed-phase HPLC column (30 mm × 2.0 mm, 2 μm). Separation of oxandrolone and methyltestosterone was achieved with a mobile phase starting composition of 55% methanol and 45% ammonium formate buffer at a flow rate of 0.1 mL/min. The total run time was 21 min per sample. Selected reaction monitoring mode was used for quantifying oxandrolone (*m/z* 307 → 271) and the IS, methyltestosterone (*m/z* 301 → 149). To the authors' knowledge, this is the first LC–MS-MS method validated for oxandrolone quantification in human plasma. This method can be used in future pharmacokinetic studies involving oxandrolone.

Introduction

Oxandrolone (5α-androstan-2-oxa-17α-methyl-17β-ol-one) is a synthetic testosterone analogue (Figure 1A). It is an anabolic androgenic steroid that has been used in the treatment of multiple wasting syndromes for over 40 years (1). A major advantage of oxandrolone over other anabolic steroids is the addition of an alkyl group to the C17-α position of the steroid nucleus, which improves its oral bioavailability profile and allows it to be orally administered (2).

Oxandrolone has proven effective in reversing muscle catabolism in cachectic hepatitis (3), HIV/AIDS (4) and burn patients (5). Additionally, it has been used off-label to improve quality of life in cases of Duchenne dystrophy (6), cystic fibrosis (7) and childhood hereditary angioedema (8). It is the only oral anabolic steroid approved by the US Food and Drug Administration (FDA) for use in severe weight loss associated with injury or disease (1, 9). Oxandrolone can be used in both adults and children because of its favorable safety profile and efficacy in promoting weight gain in a wide variety of conditions (1).

Several methods have been developed for the detection of oxandrolone and its metabolites in human urine (10, 11) in response to oxandrolone's use as a performance-enhancing drug (12). In addition, Karim *et al.* (13) developed a thin-layer chromatography/scintillation spectrometry method for quantification of oxandrolone in plasma in 1973, but no validated quantitative liquid chromatography/mass spectrometry-based methods have been reported until now. Relatively little is known about the pharmacokinetics of oxandrolone in humans, and quantification in plasma has not been well described using modern analytical techniques. To that end, an LC–MS-MS method was developed and validated according to the 2001 FDA Bioanalytical Guidelines for the detection and quantification of oxandrolone in human plasma using methyltestosterone (Figure 1B), another 17α alkyl testosterone derivative, as an internal standard (IS) (14). This method, based off of an assay for structurally similar steroids and steroid-like molecules reported by Reilly and Crouch (15), and the results of the validation are presented in the following sections.

Experimental

Human plasma for method development and validation

The human plasma used for method development and validation (with the exception of selectivity and matrix effects) was a pool of plasma collected from >20 males and females by the University of Utah Hospital Blood Bank. This study was reviewed and approved by the Institutional Review Boards at the University of Utah.

Chemicals and reagents

Oxandrolone and methyltestosterone were purchased from Steraloids, Inc. (Newport, RI, USA). These materials exhibited one major chromatographic peak, as established by high-performance liquid chromatography (HPLC) analysis with detection at 230 nm, and exhibited the appropriate mass-to-charge ratio predicted for the respective compounds.

HPLC-grade (>99% purity) methanol and *n*-butyl chloride were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Ammonium formate (≥99% for mass spectrometry) and formic acid (≥98% purity) were purchased from Sigma-Aldrich Co., LLC (St Louis, MO, USA). Milli-Q water (specific resistance of 18.2 MΩ cm) was prepared using a Millipore Milli-Q Plus water purification system (Millipore Corp., Burlington, MA, USA).

Sample preparation

Stock solution preparation

Stock solutions of oxandrolone were prepared in methanol at a concentration of 1,000 ng/μL by dissolving 5 mg oxandrolone

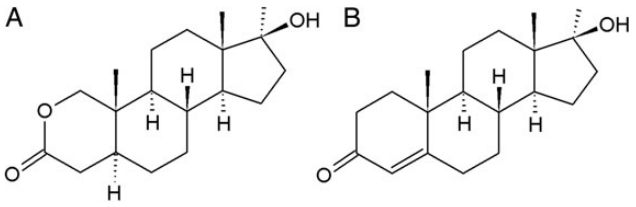

Figure 1. Chemical structure of oxandrolone (A) and the IS, methyltestosterone (B).

in 5 mL of methanol. Stock solutions of IS were prepared in the same manner at a concentration of 1,000 ng/μL.

Intermediate solution preparation

Intermediate solutions of oxandrolone were prepared at concentrations of 10, 1 and 0.1 ng/μL by performing serial dilution on the 1,000 ng/μL stock solution. Intermediate solutions of IS were also prepared by serial dilution to concentrations of 100 and 10 ng/μL. All stock and intermediate solutions were stored in unsilanized glass tubes at −20°C for the duration of the study.

Calibrator and quality control solution preparation

Calibrator and quality control (QC) solutions were prepared from intermediate solutions at the time of sample preparation and analysis. Calibration standards were prepared by aliquoting 0.5 mL of human plasma into 13 × 100 mm glass screw cap tubes and fortifying with IS to a concentration of 100 ng/mL (i.e., 5 μL of 10 ng/μL IS). A total of 10 human plasma samples were used for the calibration curve and blanks, all of which were prepared with each batch of samples. As controls, one sample was left completely blank, and one was fortified with only IS. The remaining samples were fortified with IS and oxandrolone at final concentrations of 2, 5, 10, 25, 50, 75, 100 and 200 ng/mL. Liquid–liquid extraction was performed by adding 5 mL of *n*-butyl chloride to each sample and vortexing for ∼10 s. Samples were then centrifuged in a Beckman GPR centrifuge with a 20.4 cm rotor (GH-3.7) at 3,000 rpm (∼2,050 × *g*) for 10 min. The top organic supernatant was transferred into a new set of glass tubes and dried under a constant flow of air. The dried residues were reconstituted in 150 μL of 25% methanol/75% Milli-Q water. After briefly vortexing, reconstituted samples were then transferred to an autosampler vial for analysis.

QC samples were prepared in the same manner as the calibration standards at analyte concentrations of 6, 90 and 180 ng/mL, corresponding to low, medium, and high QCs.

Apparatus

LC–MS-MS analysis was performed using an Agilent 1100 series HPLC (Agilent Technologies, Wilmington, DE, USA) with a PerkinElmer Series 200 injector and an autosampler, coupled with a Thermo-Finnigan TSQ AM tandem MS (ThermoQuest Instruments, San Jose, CA, USA). Separation of oxandrolone and IS was achieved chromatographically using a Luna C18 (30 mm × 2.0 mm, 3 μm) reversed-phase HPLC column (Phenomenex, Torrance, CA, USA). The column was equilibrated at a flow rate of 0.1 mL/min with a mobile phase consisting of 55% methanol and 45% 1 mM ammonium formate buffer (pH 3.5) at room temperature (∼23°C). Upon sample injection, the concentration of methanol was increased at a constant rate to 63% over 14.5 min. At 14.6 min, methanol was increased to 95% and the flow rate was increased to 0.2 mL/min for 2.5 min. After this time, the methanol gradient was reduced to 55% and after an additional 2.4 min, the flow rate was reduced to 0.1 mL/min. A 1-min re-equilibration preceded injection of each successive sample. The autosampler was not temperature-controlled; meaning samples were at room temperature for the duration of the run. The injection volume for each sample was 15 μL.

The MS was equipped with an electrospray ionization source and operated in selected reaction monitoring (SRM), positive ion mode for the detection of precursor-to-product ion transitions representative of oxandrolone (m/z 307 → 271) and methyltestosterone (m/z 301 → 149). Previous methods of oxandrolone detection in urine have used a product m/z of 289 (10, 11), but the fragment with an m/z of 271 was determined to have a greater signal-to-noise ratio in human plasma, potentially due to the presence of other endogenous steroids with similar transitions at 289, variation in the efficiency of water loss or simply the fact that different mass spectrometers can have different fragmentation patterns. An ionization energy of 15 V was used for the oxandrolone transition while an ionization energy of 20 V was used for the IS transition. Precursor and product ion resolutions were set such that 50% of the peak height was observed within ± 1.5 a.m.u. Additional instrument settings were as follows: capillary sheath temperature of 325°C, sheath gas and auxiliary gas (nitrogen) pressures of 10 psi and spray voltage of 9 eV.

Integration and quantitation of the LC–MS-MS data were performed using Xcalibur LCQuan® v. 2.5 SP1 (ThermoQuest). Peak areas for oxandrolone and IS were automatically integrated using a Genesis model, with a minimum peak height of 2.0% and a tailing factor of 2.0. The real-time peak detection was set at 9.5 min for oxandrolone (271.0 m/z) and at 10.5 min for methyltestosterone (149.0 m/z), with a detection window of 90 s for both. A calibration curve comparing the peak area ratio with the concentration of the analyte was generated using a quadratic log-log regression fit with equal weighting. Only calibration curves with an R^2 of ≥0.995 were used for quantitation.

Validation Methods

This assay was developed to quantitate oxandrolone concentrations in human plasma. The analytical concentration range extended from a lower (LLOQ) to an upper limit of quantitation of 2 to 200 ng/mL, respectively. Sample concentrations were determined from analyte to IS peak area ratios and calibration curves.

Selectivity

The selectivity of this assay for oxandrolone in human plasma was tested by evaluating blank samples from plasma of six unique individuals. Specifically, six blank samples were tested for interference and peak area responses, which were compared with six samples fortified at 2 ng/mL to ensure selectivity at the LLOQ. To accept selectivity at the LLOQ, the analyte response at this level was required to be at least five times the response of the blank samples.

Intra- and inter-assay accuracy and precision

Accuracy and precision were determined on both an intra- and inter-assay basis. The mean percent difference between the

calculated and expected analyte concentrations ($n = 5$) was used as a measure of intra-assay accuracy for QC samples fortified at 6, 90 and 180 ng/mL. Intra-assay precision was expressed as the percent coefficient of variation (%CV) and calculated for each batch using the standard deviation of the QC concentrations for each analyte divided by the mean concentration ($n = 5$). Inter-assay accuracy and precision were determined in a similar manner from three sets of each of the low (6 ng/mL), medium (90 ng/mL) and high (180 ng/mL) QCs ($n = 15$ at each QC level) run on 3 non-consecutive days. To accept the accuracy of the assay, each mean QC concentration was required to be within 15% of the target concentration, while assay precision necessitated a %CV of $<15\%$.

Recovery and matrix effects

Recovery of the analytes from human plasma was determined using oxandrolone and IS fortified standards ($n = 5$) at 6, 90 and 180 ng/mL. Recovery was expressed as the ratio of the concentrations obtained from extracted samples to the corresponding concentrations for unextracted samples containing 6, 90 and 180 ng/mL, which were prepared by spiking 5 mL of *n*-butyl chloride directly from the intermediate solutions. Therefore, the ratio of the two concentrations (extracted/unextracted) represented the proportion of the analyte recovered. Similarly, recovery for the IS was calculated by comparing the methyltestosterone raw responses (peak areas) of extracted and unextracted samples. All oxandrolone concentrations were calculated from a calibration curve generated with unextracted samples (i.e., *n*-butyl chloride directly fortified with oxandrolone and IS). Matrix effects were additionally assessed at low and high QC levels ($n = 6$) for oxandrolone and at 100 ng/mL for the IS ($n = 6$) using the post-extraction addition method.

Stability

Various storage and handling conditions were evaluated to determine their effect on analyte stability. Fortified QC standards ($n = 3$) prepared in 10 mL BD Vacutainer® glass sodium heparin plasma tubes (BD Diagnostics, Franklin Lakes, New Jersey, USA) at 6 and 180 ng/mL were subjected to three freeze–thaw cycles, stored on the bench top at $\sim 23^{\circ}$C for 7 h following one freeze–thaw cycle, or stored at -20°C for 3 weeks. IS was added to the samples following storage treatment, immediately prior to extraction. After extraction, the samples were prepared and analyzed as described in previously. Stability was assessed by determining the mean assayed concentration for the stored samples relative to a standard curve generated from freshly prepared standards. Intermediate solution stability and post-preparative stability were also tested. For intermediate solution stability, intermediate solutions were used to generate a calibration curve and baseline QC samples on the day they were prepared. Additional QC samples were also generated and used from the same intermediate solutions stored at -20°C for 1, 2, 3, 5, 7 and 14 days. The $\pm 15\%$ accuracy cutoff was used in some tests (e.g., freeze–thaw, short-term temperature and long-term) as a measure of stability, but was not required in others (e.g., intermediate solution stability and post-preparative stability), which used statistical tests (linear regression and Student's *t*-test respectively) to determine stability.

Application

Blood samples were obtained from five neonatal patients 30 min after treatment with oxandrolone. These samples, which had been stored at -20°C prior to analysis, were prepared as described previously.

Results and discussion

Liquid chromatography–tandem mass spectrometry

Optimization of sample preparation and analytical parameters provided adequate separation, resolution and signal-to-noise ratio for both the analyte and IS. Each molecule exhibited a single chromatographic peak that was easily discernible from the baseline noise between 2 and 200 ng/mL. Retention times were reproducible at ~9.5 min for oxandrolone and 10.5 min for methyltestosterone. An additional 10 min for each run was necessary to prevent carry-over and re-equilibrate the column between sample injections. The assay was approximately linear across the range from 2 to 100 ng/mL, but the data were best fit using a quadratic log-log regression. Calculated concentrations for all standards were within $\pm 15\%$ of their expected values.

Selectivity

Selectivity of the assay for oxandrolone was determined by comparing peak area responses of blank plasma samples and samples fortified at the LLOQ (2 ng/mL). Blank samples showed only background noise for IS and oxandrolone at the expected elution times, while samples prepared with IS and oxandrolone at the LLOQ had well-defined peaks that were easily distinguishable from the background noise (Figure 2). Comparison of the peak area responses for the LLOQ samples ($n = 6$) versus the blank samples ($n = 6$) demonstrated that the oxandrolone response at the LLOQ was at least 5-fold greater than the blank response in every sample, indicating that the assay was suitable for the detection and quantification of oxandrolone in plasma.

Accuracy and precision

Intra-assay accuracy and precision were confirmed with QC accuracy between 93–105% and %CV $<11\%$ (Table I). These samples were additionally used to determine the inter-assay accuracy and precision, in conjunction with samples from analytical runs 2 and 4 days later. Inter-assay accuracy and precision were demonstrated with the percentage of target concentrations between 100–109% and %CVs $<10\%$ (Table I).

Recovery and matrix effects

Recovery of oxandrolone from the liquid–liquid extraction was determined at low, medium and high QC concentrations (Table II). Percent recovery for oxandrolone was calculated between 80 and 90%. Recovery for the IS was calculated at 81%. The %CV for each set of extracted and unextracted QCs was $<9\%$. No significant ion suppression was noted for the analyte (low or high QCs) or IS (data not shown).

Freeze and thaw stability

Freeze–thaw stability was tested for oxandrolone in plasma samples at low (6 ng/mL) and high (180 ng/mL) QC concentrations ($n = 3$). Samples were subjected to a 24-h freeze–thaw cycle

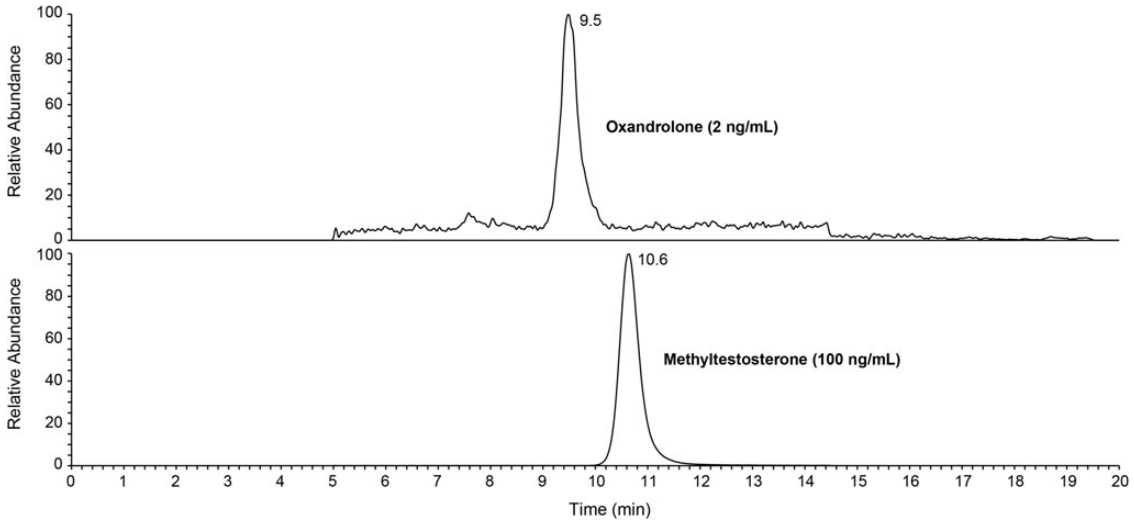

Figure 2. Chromatogram of oxandrolone (top) and methyltestosterone (bottom) peaks. The oxandrolone peak is at a concentration of 2 ng/mL (lower limit of quantitation) and methyltestosterone peak is at 100 ng/mL.

Table I
Intra- and Inter-Assay Variability for Oxandrolone in Human Plasma

Comparison	QC level	Number of samples	Accuracy ± CV (%)
Intra-assay variability	Low QC (6 ng/mL)	5	94 ± 3
	Mid QC (90 ng/mL)	5	105 ± 3
	High QC (180 ng/mL)	5	103 ± 11
Inter-assay variability	Low QC (6 ng/mL)	14[a]	100 ± 7
	Mid QC (90 ng/mL)	15	108 ± 4
	High QC (180 ng/mL)	15	102 ± 9

QC, quality control; CV, coefficient of variation.
[a]One of the low QC samples was not injected properly and could not be analyzed.

Table II
Recovery of Oxandrolone in Extracted Samples

QC level	Unextracted mean concentration (ng/mL, $n = 5$)[a]	Extracted mean concentration (ng/mL, $n = 5$)	% Recovery[b]
Low QC (6 ng/mL)	5.6	5.0	89
Mid QC (90 ng/mL)	83.8	73.6	88
High QC (180 ng/mL)	178.8	142.7	80

QC, quality control.
[a]Unextracted samples represent 100% recovery.
[b]% Recovery calculated as: extracted concentration/unextracted concentration × 100.

followed by two 12-h freeze–thaw cycles prior to analysis. Both concentrations fell within the required range for target concentration and %CV (Table III), indicating that oxandrolone was stable in plasma after three freeze–thaw cycles.

Short-term temperature stability

For short-term temperature stability, oxandrolone was fortified into plasma at low and high QC concentrations ($n = 3$). The samples were frozen for 24 h after which they were allowed to thaw at room temperature for 7 h and subsequently prepared for analysis. While the precision of the samples undergoing short-term temperature stability was adequate (%CV <7%), the concentration of oxandrolone in the samples was calculated at ~36–39% of the expected values (Table III). The lower concentration of oxandrolone in these samples suggests that oxandrolone is not stable in plasma at room temperature for this period. Therefore, in order to minimize analyte loss, samples should be stored at −20°C and only removed from the freezer to prepare them for analysis.

Long-term stability

For long-term stability, oxandrolone-fortified plasma samples (low and high QC concentrations, $n = 3$) were stored at −20°C for 23 days, after which they were thawed at room temperature and analyzed. Both accuracy (86–103% of target) and

Table III
Oxandrolone Stability Under Various Conditions

QC level	Condition[a]	Accuracy ± CV (%)
Low QC (6 ng/mL)	Three freeze/thaw cycles	111 ± 11
	7 h room temperature	36 ± 6
	3 weeks frozen (−20°C)	104 ± 2
High QC (180 ng/mL)	Three freeze/thaw cycles	95 ± 5
	7 h room temperature	39 ± 6
	3 weeks frozen (−20°C)	86 ± 2

QC, quality control; CV, coefficient of variation.
[a]$n = 3$ for each condition evaluated.

%CV (~2%) were within the acceptable range (Table III). Therefore, it can be concluded that oxandrolone is stable in plasma for at least 3 weeks at −20°C.

Intermediate solution stability

The stability of intermediate solutions stored at −20°C was tested at 1, 2, 3, 5, 7 and 14 days after the solutions were prepared. For each analytical run, intermediate solution concentrations were averaged across four QCs with a mean %CV of 11% between the seven sample sets. Linear regression revealed no significant change in oxandrolone concentrations over a 2-week period ($F = 0.0799$, $P = 0.789$).

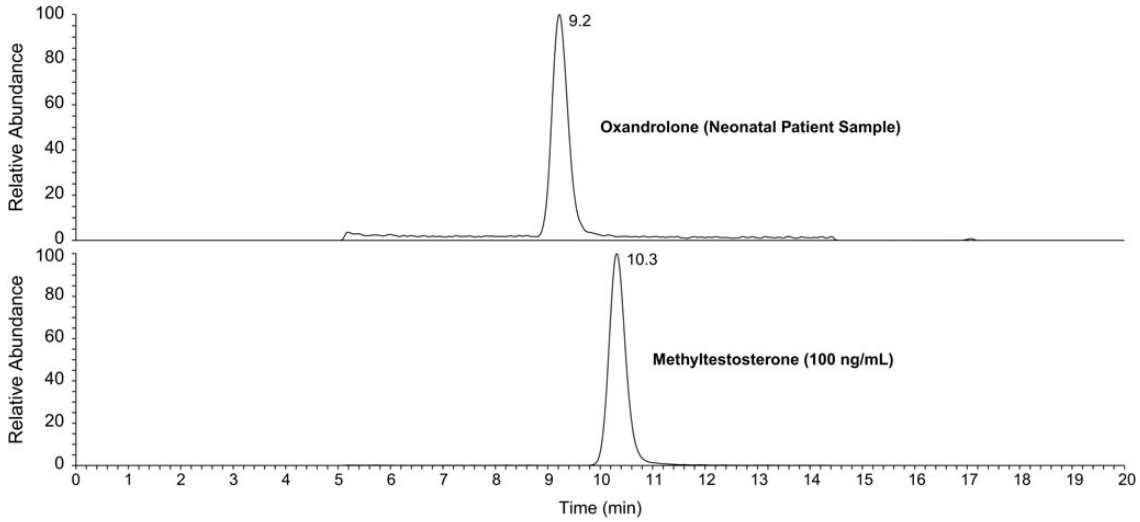

Figure 3. Sample chromatogram of a neonatal patient treated with oxandrolone.

Post-preparative stability

Post-preparative stability was determined by comparing one set of QCs assayed immediately after preparation with another set of QCs stored in the autosampler tray for ~12 h, the expected length of an analytical run. Concentrations at the low and intermediate ranges did not change significantly, while the high QC concentration increased after a 12-h period on the autosampler (Student's *t*-test, $P < 0.05$). Based on these results, there was no significant loss of analyte for prepared samples left on the autosampler for a 12-h period. There may have been a slight buildup of oxandrolone on the column or the detector over time, which may explain why the second set of high QCs had a greater average calculated concentration than the first set.

Application

Blood samples from five neonatal patients taken 30 min after treatment with oxandrolone were analyzed. An example chromatogram is shown in Figure 3. All calculated concentrations (8.9–42.0 ng/mL) fell within the calibration range.

Conclusion

An LC–MS-MS method was developed with sufficient accuracy and precision to quantify plasma concentrations of oxandrolone between 2 and 200 ng/mL. To the authors' knowledge, this is the first LC–MS-MS method for the quantification of this drug in plasma, and it has the advantage of leveraging more recent technology to provide improved accuracy, specificity and ease of preparation when compared with the previously published thin-layer chromatography/scintillation spectrometry method (13). The assay has been validated according to the 2001 FDA guidelines, and provides a means for quantification of oxandrolone in plasma that may be used in future pharmacokinetic studies involving oxandrolone.

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References

1. Akyurek, M., Dunn, R.M. (2006) Oxandrolone. *Plastic and Reconstructive Surgery*, **118**, 791–794.
2. Orr, R., Fiatarone Singh, M. (2004) The anabolic androgenic steroid oxandrolone in the treatment of wasting and catabolic disorders: review of efficacy and safety. *Drugs*, **64**, 725–750.
3. Mendenhall, C.L., Moritz, T.E., Roselle, G.A., Morgan, T.R., Nemchausky, B.A., Tamburro, C.H. *et al.* (1995) Protein energy malnutrition in severe alcoholic hepatitis: diagnosis and response to treatment. The VA Cooperative Study Group #275. *Journal of Parenteral and Enteral Nutrition*, **19**, 258–265.
4. Fox-Wheeler, S., Heller, L., Salata, C.M., Kaufman, F., Loro, M.L., Gilsanz, V. *et al.* (1999) Evaluation of the effects of oxandrolone on malnourished HIV-positive pediatric patients. *Pediatrics*, **104**, e73.
5. Miller, J.T., Btaiche, I.F. (2008) Oxandrolone in pediatric patients with severe thermal burn injury. *The Annals of Pharmacotherapy*, **42**, 1310–1315.
6. Fenichel, G.M., Griggs, R.C., Kissel, J., Kramer, T.I., Mendell, J.R., Moxley, R.T. *et al.* (2001) A randomized efficacy and safety trial of oxandrolone in the treatment of Duchenne dystrophy. *Neurology*, **56**, 1075–1079.
7. Varness, T., Seffrood, E.E., Connor, E.L., Rock, M.J., Allen, D.B. (2009) Oxandrolone improves height velocity and BMI in patients with cystic fibrosis. *International Journal of Pediatric Endocrinology*, **2009**, 826–895.
8. Church, J.A. (2004) Oxandrolone treatment of childhood hereditary angioedema. *Annals of Allergy, Asthma and Immunology: Official Publication of the American College of Allergy, Asthma, and Immunology*, **92**, 377–378.
9. Langer, C.J., Hoffman, J.P., Ottery, F.D. (2001) Clinical significance of weight loss in cancer patients: rationale for the use of anabolic agents in the treatment of cancer-related cachexia. *Nutrition*, **17**, S1–20.
10. Leinonen, A., Kuuranne, T., Kotiaho, T., Kostiainen, R. (2004) Screening of free 17-alkyl-substituted anabolic steroids in human urine by liquid chromatography-electrospray ionization tandem mass spectrometry. *Steroids*, **69**, 101–109.
11. Pozo, O.J., Deventer, K., Van Eenoo, P., Delbeke, F.T. (2007) Presence of endogenous interferences in the urinary detection of selected anabolic steroids by liquid chromatography/electrospray tandem mass

spectrometry. *Rapid Communications in Mass Spectrometry: RCM*, **21**, 2785–2796.
12. Kam, P.C., Yarrow, M. (2005) Anabolic steroid abuse: physiological and anaesthetic considerations. *Anaesthesia*, **60**, 685–692.
13. Karim, A., Ranney, R.E., Zagarella, J., Maibach, H.I. (1973) Oxandrolone disposition and metabolism in man. *Clinical Pharmacology and Therapeutics*, **14**, 862–869.
14. US Food and Drug Administration, Center for Drugs Evaluation and Research. (2001) *Guidance for Industry: Bioanalytical Method Validation*. Rockville, MD.
15. Reilly, C.A., Crouch, D.J. (2004) Analysis of the nutritional supplement 1AD, its metabolites, and related endogenous hormones in biological matrices using liquid chromatography-tandem mass spectrometry. *Journal of Analytical Toxicology*, **28**, 1–10.