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Development of the fast, simple and fully validated high performance liquid chromatographic method with diode array detector for quantification of testosterone esters in an oil-based injectable dosage form

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ABSTRACT

Counterfeit steroids are available on the black market, ultimately to consumers who believe they are buying a legitimate pharmaceutical item from the labeled company. In many cases, counterfeit steroids can contain lower doses or some products can be overdosed. This can unwittingly expose users to a significant health risks. The mixture of testosterone propionate, phenylpropionate, isocaproate and decanoate in an oil-based injectable dosage form belongs to the one of the most misused illicit drugs by a variety of athletes.

This study developed a new, fast, simple and reliable HPLC method combined with a simple sample preparation step to determine testosterone propionate, phenylpropionate, isocaproate and decanoate in an oil-based injectable dosage form without the use of sophisticated and expensive instrumentation. The developed analytical procedure provides high throughput of samples where LC analysis takes only 6 min and sample preparation of oil matrix in one step takes approximately 10 min with precision ranging from 1.03 to 3.38% (RSD), and accuracy (relative error %) within ±2.01%. This method was found to be precise, linear, accurate, sensitive, selective and robust for routine application in screening of commercial pharmaceutical products based on content of mentioned testosterone esters in their oil-based injectable dosage form for counterfeit drugs.

This method was successfully applied to the analysis of nine samples of commercial testosterone mixtures purchased from various sources and will be further used as an effective screening method for determination of previously mentioned testosterone esters in samples confiscated by Institute of Forensic Science (Slovakia) during the illegal trade.

1. Introduction

The use of anabolic androgenic steroids (AAS) has changed over time. The phenomenon of abusing the AAS is no more restricted to athletes in sport competitions, but the misuse of AASs is still increasing among gym customers for whom body appearance is a priority [1]. There are numerous websites which sell AAS supplements, regardless of the legal ban on use of AAS except for medical purposes because of various health risks. The products sold on such websites are often counterfeited and adulterated. In many cases, the counterfeiters never even use any active steroids at all. In other cases, they substitute them with lower doses or cheaper steroids. However, some products are actually significantly overdosed, which can put consumers' health in danger [2]. The largest amount of scientific papers is focused on the development and application of analytical techniques for the detection of AAS and their metabolites in biological matrices [3–8]. In recent years, various analytical techniques include mainly liquid chromatography (LC) and gas chromatography (GC) usually coupled with mass spectrometry (MS) for the analysis of AAS in counterfeit products have been previously reported [2,9–11]. The basic anabolic steroid is testosterone, which is usually administered intramuscularly under its ester forms in lipophilic excipient (different oils) providing longer biological availability and lasting effects [12]. A number of different synthetic esters of testosterone exist on the market today.

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The mixtures of testosterone propionate, phenylpropionate, isocaproate and decanoate in an oil-based injectable dosage form at a total concentration of 250 mg/mL are currently one of the world's most misused illicit drugs by a variety of athletes. They have become so popular among anabolic steroid cycles that they are now sold by a large number of manufacturers with the same formula under different brand names like Sustamed, Sustanon, Induject, etc. Despite the fact that some reports can be found in the literature dedicated to the analysis of such esters in biological samples [13–15], there exists only a few papers focused on analysis of testosterone esters in an oil-based injectable dosage form. Determination of the mentioned mixture of testosterone esters was reported by Thevis et al. using LC–MS/MS [2], by Doue et al. using atmospheric solids analysis probe mass spectrometry [16] and by Musharraf and Gulzar using thin-layer chromatography [17]. Testosterone esters (specific mixture not mentioned) were analysed mainly by LC-UV [18], LC-UV-particle beam MS [19] or LC–MS [9]. This work aims at the development of a new effective LC method applicable to quantitation of testosterone propionate, phenylpropionate, isocaproate and decanoate in an oil-based injectable dosage form which would be able to provide accurate and precise results. This method was successfully applied to the analysis of nine samples of commercial testosterone mixtures purchased from various sources and will be further used as an effective screening method for determination of previously mentioned testosterone esters in samples confiscated by Institute of Forensic Science (Slovak Republic) during the illegal trade.

2. Materials and methods

2.1. Chemicals

Reference standards of testosterone propionate, phenylpropionate, isocaproate, decanoate, acetate, benzoate, cypionate, enanthate, and undecanoate were supplied by Sigma – Aldrich (St. Louis, USA). Acetonitrile (gradient grade), methanol (gradient grade), and isopropanol (gradient grade) were purchased from Merck (Darmstadt, Germany). Acetic acid (purity >99.8%) and ammonium acetate (purity >98%) were supplied by Lachner (Neratovice, Czech Republic). Benzyl benzoate (purity >99%), benzoic acid (purity >99%) were supplied by Sigma – Aldrich (St. Louis, USA). Peach oil (pharmaceutical grade) was purchased from Natures Natural (New Delhi, India). Sustamed (Balkan Pharmaceuticals, Republic of Moldova), Sustanon (Organon International, USA) and Omnadren (Jelfa, Poland) were purchased from two different sources. One package was purchased from a dealer in Slovakia. The second one was purchased from an online store. Testosterone compound injection (Genesis, Singapore), Pharmasust (Pharmacom, underground laboratory) and Sustex (DMX, underground laboratory) were purchased from an online store. The declared concentrations of testosterone esters were the same for each product as follows: testosterone propionate 30 mg/mL, testosterone phenylpropionat 60 mg/mL, testosterone isocaproate 60 mg/mL and testosterone decanoate 100 mg/mL. The deionized water used in this work was purified using Milli-Q water purification system (Millipore, Bedford, USA). Millipore 0.45 mm Nylon filters mobile phases were also used.

2.2. Preparation of solutions

Working standard solutions of testosterone esters at concentrations of 20, 40, 60, 80, 100, 120 and 140% of testosterone esters levels in commercial testosterone mix were prepared. Stock solution of analytes was prepared by dissolving accurate amount of reference standards materials into methanol in a 50 mL volumetric flask and completing the volume properly.

The optimized sample solution preparation procedure was as follows:

950 μL of isopropanol was added to 50 μL of commercial testosterone mix and diluted by 3 ml of methanol in a 5 mL volumetric flask. The sample was put in an ultrasonic bath and sonicated for 5 min with occasional shaking. Volumetric flask was filled up to the mark with methanol after sonication and cooling down at the room temperature.

Placebo solution consisted of benzoic acid, benzyl benzoate and peach oil. The amount of 0.1 mL of benzoic acid and 0.5 mL of benzyl benzoate were put into 5 mL volumetric flask, mixed properly and filled up to the mark with peach oil. Then, the placebo solution for HPLC analysis was prepared in a same way as the sample solution.

Quality control samples preparation composition corresponds to the composition in Sustamed solution. It was prepared by mixing appropriate amount of each of testosterone esters at 20, 80, 100 and 140% of the concentration levels of testosterone esters in Sustamed, 0.1 mL of benzoic acid, 0.5 mL of benzyl benzoate and 2 mL of peach oil. After the sample was kept at 50 °C for 5 min in a water bath, it was cooled down by moving to a room temperature environment (25 ± 5 °C). Finally, the 5 mL volumetric flask was filled up to the mark with peach oil and mixed well.

2.3. Instrumentation and the experimental conditions

All chromatographic measurements were carried out on HPLC system Agilent series 1260 (Agilent Technologies, Waldbronn, Germany). For selectivity verification Triple Quad 6460 tandem mass spectrometer (Agilent Technologies, Waldbronn, Germany) was used. For data acquisition and analysis, the OpenLab software was used. Two columns were tested: Poroshell HPH C18 (3.00 mm i.d. × 100 mm, 2.7 μm) from Agilent Technologies (Waldbronn, Germany) and Zorbax Eclipse Plus C18 (3.00 mm i.d. × 100 mm, 3.5 μm) from Agilent Technologies (Waldbronn, Germany). The effect of the ratio of organic solvent to water content in the mobile phase and the influence of additive type (acetic acid, formic acid, ammonium acetate and ammonium formate), its pH and concentration on separation and peak shape of testosterone esters were investigated. Final mobile phases consisted of acetonitrile and 10 mM ammonium acetate buffer. Ammonium acetate buffer was prepared by dissolving appropriate amount of ammonium acetate in deionized water and has been titrated with acetic acid required to reach pH 4.5.

2.4. Method validation

Developed and optimized method was validated according to the ICH Q2 guideline [20] in terms of linearity, limits of detection (LOD) and quantitation (LOQ), accuracy, precision, selectivity, robustness and stability of the sample and standard solution. The selectivity of the method was confirmed by analysis of the sample solvent, placebo sample and other different synthetic esters of testosterone exist on the market today, including acetate, benzoate, cypionate, enanthate, and undecanoate [14]. The peak purity test was also evaluated to confirm that no unknown impurity coelutes with the principal peaks of the testosterone esters. Selectivity was also tested by the analyzing of sample of Sustamed by the newly developed HPLC method combined with mass spectrometry detection (TripleQuad).

Linearity was established using standard solutions of testosterone esters at the levels of 20, 40, 60, 80, 100, 120 and 140% of testosterone esters concentrations in commercial testosterone mix. Linearity was evaluated statistically by linear regression anal-