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Identification of anabolic steroids and derivatives using bioassay-guided fractionation, UHPLC/TOFMS analysis and accurate mass database searching

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ABSTRACT

Biological tests can be used to screen samples for large groups of compounds having a particular effect, but it is often difficult to identify a specific compound when a positive effect is observed. The identification of an unknown compound is a challenge for analytical chemistry in environmental analysis, food analysis, as well as in clinical and forensic toxicology. In this study bioassay-guided fractionation, ultra high performance liquid chromatography combined with time-of-flight mass spectrometry (UHPLC/TOFMS) and accurate mass database searching was tested to detect and identify unknown androgens. Herbal mixtures and sport supplements were tested using an androgen bioassay and modifications in sample preparations were carried out in order to activate inactive pro-androgens, androgen esters and conjugated androgens to enable their detection in the bioassay. Two of the four herbal mixtures tested positive and bioassay-guided fractionation followed by UHPLC/TOFMS of positive fractions resulted in the identification of nortestosterone phenylpropionate, testosterone cyclohexanecarboxylate and methyltestosterone. Three of the four sport supplements reacted toxic in the bioassay or gave inconclusive results and were further investigated using UHPLC/TOFMS in combination with data processing software and an accurate mass database having approximately 40,000 entries. This accurate mass database was derived from the PubChem database on the internet and coupled to the TOFMS software. This resulted in the tentative identification of several androgens, including methylboldenone, testosterone and the androgen esters methyltestosterone propionate or testosterone isobutyrate, testosterone buciclate and methylenetestosterone acetate. The study showed that bioassay-guided fractionation in combination with UHPLC/TOFMS analysis is a useful procedure to detect, isolate and identify unknown androgens in suspected samples. As an alternative, the use of data processing software in combination with an accurate mass database and coupled on-line with the TOFMS instrument software enabled the identification of androgens and androgen esters in the chromatogram even without bioassay-guided fractionation.

1. Introduction

The identification of an unknown compound is a challenge for analytical chemistry in environmental analysis, food analysis, as well as in clinical and forensic toxicology. Following the usual course of general unknown screening, samples will initially be analyzed by immuno- or bioassays [1–4] followed by a confirmation analysis in case of positive findings. Sports doping is an area where the identification of unknown androgens is of special interest, especially in the analysis of sport supplements. New drugs are designed by the addition or change of position of some functional groups in an active "parent" compound, e.g. by structural modification. These modifications are meant to improve the characteristics of the drug or to circumvent control analysis. The recently discovered "designer" modifications [5–9] are representatives of this trend and have led to the development of alternative methods capable of detecting the presence of drugs belonging to the same class in human urine [5–7,10–12]. Such detection methods are often effect-based bioassays in combination with instrumental techniques that focus on specific mass spectral features common to a specific class of compounds. An example of this approach is the bioassay-guided fractionation for the detection of anabolic steroids in dietary supplements [13]. However, a screening method using liquid chromatography in combination with tandem mass spectrometry (LC-MSMS) for the detection of synthetic glucocorticosteroids in human urine based on the recognition of one or more recurrent fragments of the molecular structure of synthetic glucocorticosteroids [14], and a screening method based on UHPLC/TOFMS and MetaboLynx software for the determination of in silico predicted designer modifications of anabolic steroids [15] have been proven successful too. In a recent review showing cross-contamination of nutritional supplements with doping substances,

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mass spectrometric strategies for the detection of new "designer" steroids are presented [16].

While many methods have been used for analyte separation in extracts, it is safe to state that liquid chromatography (LC) is the most generic of these, and since the introduction of ultra high performance LC (UHPLC) also one of the most selective. Coupling of mass spectrometry (MS) to LC is the best possibility to increase the range of compounds amenable to MS [17]. Especially full mass scan MS techniques like time-of-flight MS (TOFMS) or OrbitrapMS provide high specificity due to both, high mass accuracy and high mass resolution and theoretically allow the analysis for an unlimited number of compounds [18]. Furthermore, data can be acquired and reprocessed without any a priori knowledge about the presence of certain compounds; that is, no analyte-specific information is required before injecting a sample and the presence of newly identified compounds can be confirmed in previously analyzed samples simply by reprocessing the data. The use of UHPLC/TOFMS generates an enormous amount of data, potentially allowing the identification of unknown compounds, but also causing problems for identification. The large number of peaks in a chromatogram may easily result in a "forest of peaks", making it difficult to distinguish between true compounds of interest and matrix compounds or endogenous compounds in biological matrices. One way to pinpoint a bioactive but unknown compound is mentioned above and generally described as bioassay-guided fractionation. The sample extract is then fractionated with the help of LC and the eluent is split in a dual collection system with 96-well micro-titre plates. Collection is usually carried out as a function of time and a fraction volume of up to 300 μL is used depending on the type of bioassay [19–22]. The LC/MS data of the duplicate plate are then matched and correlated with the bioactivity profile from the separate fractions in the first plate in order to identify peaks of bioactive compounds in the bioactivity chromatogram. Finally, this information can be used for a search of compound databases and a tentative identification of the bioactive unknown compounds. While this procedure is primarily used in the identification of natural products [23–25], it has also been used in the analysis of steroids in surface water [26], urine [3,7], dietary supplements [13] and veterinary drug residues in food [27]. A disadvantage of LC–MS is that searchable databases for compound identification, as the well known National Institute for Standards and Testing (NIST) mass spectral library for GC/MS, are virtually absent for LC/MS [28]. However, since accurate mass measurements as performed by high resolution TOFMS are specific and universal for every compound regardless of the instrumentation used, TOFMS potentially enables the use of accurate mass databases for compound identification. Thurman used this approach, a combination of TOF data and the Merck Index to identify pesticides in food, including degradation products, without the initial use of primary standards [29,30]. Another interesting approach was used by Laks who identified street drugs based on accurate mass determination and a target database of 735 exact monoisotopic masses. Theoretical monoisotopic exact masses of compounds were calculated from their molecular formula, e.g. without the use of primary reference standards, in order to prepare that database [31]. A similar procedure was used for the screening of drug residues but included isotopic pattern matching as an orthogonal criterion for compound identification [32]. Of course, without primary reference standards identifications have only a tentative character and the identity should be confirmed using the actual standard compounds in a suitable confirmation method.

This study reports on the development of a procedure for the identification of unknown androgens and androgen derivatives in samples suspected of containing illegal substances. For testing purposes a number of herbal preparations and sport supplements, also called ergogenic aids, were tested. The identification procedure contains the application of an existing bioassay-guided fractionation procedure in combination with advanced UHPLC/TOFMS techniques and the development and use of an accurate mass database for compound identification.

2. Materials and methods

2.1. Chemicals, reagents and samples

All solvents were of HPLC-grade or higher. Acetonitrile, methanol acetone, isooctane and water used for LC/MS measurements were purchased from Biosolve (Valkenswaard, The Netherlands). Water used for sample preparations was purified using a Millipore Milli-Q system (Bedford, MA, USA). Sodium acetate, sodium (bi)carbonate and leucine-encephalin were purchased from Sigma (Zwijndrecht, The Netherlands). Ethyl acetate, formic acid, acetic acid, sodium acetate, sodium carbonate, sodium chloride, disodium hydrogen phosphate dihydrate, sodium dihydrogen phosphate monohydrate, ammonium sulphate, magnesium chloride, potassium chloride, tris(hydroxymethyl)aminomethane (Tris), dimethylsulphoxide (DMSO), hydrochloric acid and acetic acid were purchased from Merck (Darmstadt, Germany). Glucose-6-phosphate, NADH disodium salt, NADP disodium salt and NADPH tetrasodium salt were from Roche Diagnostics (Almere, the Netherlands). Dextrose and yeast nitrogen base without amino acids and without ammonium sulphate were from Difco (Detroit, MI, USA). L-Leucine, bovine serum albumin (BSA), hydroxysteroid dehydrogenase originating from Pseudomonas, testosterone and NAD sodium salt were purchased from Sigma (St. Louis, MO, USA). The samples used in this study were four herbal mixtures, H1 to H4, and four sport supplements, S1 to S4, and were received from sport schools and intermediates.

2.2. Sample preparation for yeast androgen bioassay

The sample preparation method is comparable to that used in some recent publications [33–36]. A sample of 3 g is mixed with 6 mL methanol and 6 mL sodium acetate buffer (0.25 M; pH 4.8) and shaken by hand. Next, it is placed 10 min in an ultrasonic bath followed by 15 min in a head-over-head apparatus. Finally, the mixture is centrifuged for 15 min at 3500 × g and 6 mL of the supernatant is collected in a glass tube. To this 150 μL acetic acid (4.0 M) is added and the pH is checked to be about 4.8. For the first SPE purification a Varian C_{18} cartridge (1000 mg, 6 mL) is conditioned with 4 mL methanol followed by 3 mL of a 1:1 mixture of methanol and sodium acetate buffer (0.25 M; pH 4.8) before applying the 6 mL extract to the cartridge. The cartridge is washed in succession with 3 mL of a 1:1 mixture of methanol and sodium acetate buffer (0.25 M; pH 4.8), 6 mL milli-Q water, 3 mL of a sodium carbonate solution (0.25 M), 3 times 6 mL milli-Q water and 2 times 4 mL of a 1:1 mixture of methanol and milli-Q water. The cartridge is dried for 10 min by applying vacuum and eluted with 2 times 4 mL acetonitrile. Again, the eluates are collected in a glass tube to avoid contamination from plastic materials. For the second SPE purification an Isolute NH_2 cartridge is activated with 4 mL of acetonitrile. The 8 mL eluate of the first SPE is brought on top of the cartridge and the eluate is collected in a glass tube. Finally, the eluate is evaporated to dryness under a stream of nitrogen gas in a TurboVap apparatus at 40 °C and the residue reconstituted in 3 mL of acetonitrile.

2.3. Sample pre-treatment for hormone glycosides

Hormone glycosides like genistin are almost inactive. Their free aglycons are the active form, genistein in case of genistin. An enzymatic digestion of the sample, adapted from enzymatic digestions of urine [15], is included as a pre-treatment in order to remove