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N-Isobutyloxycarbonylation for improved detection of 3′-hydroxystanozolol and its 17-epimer in doping control

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An improved screening method was developed for 3′-hydroxystanozolol and its 17-epimer in human urine involving gas chromatography–mass spectrometry (GC-MS) with *N*-isobutyloxycarbonyl (isoBOC) and *O*-trimethylsilyl (TMS) derivatization. A procedure was reported previously for the pentane extraction of many steroids from urine in doping control, but it was not suitable for the detection of stanozolol metabolites. Compared with the n-pentane extraction method, which gave a poor recovery (< 10%), isoBOC extraction resulted in a good recovery (> 80%). The sensitivity and specificity of mixed *N*-isoBOC–*O*–TMS derivatization were adequate for the detection of 3′-hydroxystanozolol and its 17-epimer when 3 ml of urine was used with spiking at a level of 2 ng ml^{-1}. When applied to a stanozolol-positive urine sample, the proposed method allowed rapid and sensitive screening for the detection of 3′-hydroxystanozolol and its 17-epimer.

1. Introduction

Screening methods for steroid-doping control are usually performed on urine extracts, where anabolic steroids, or their main metabolites, have to be distinguished from numerous endogenous steroids and other polar substances. To achieve exact identification with low detection limits in complex matrices, the extraction technique must provide a good yield and selectivity. Two effective methods, additional amino-column purification[1] and extraction with n-pentane,[2] have been proposed with general extraction procedures as described elsewhere[3–9] to remove interfering backgrounds. However, these clean-up steps are not suitable for the detection of metabolites of stanozolol (17α-methyl-17β-hydroxy-5α-androstano[3,2-*c*]pyrazole), which has been one of the most abused anabolic steroids in sports for the purpose of enhancing performance. Therefore, other clean-up techniques, along with the good recoveries for metabolites of stanzolol, and other urinary steroids, were required.

Sometimes direct derivatization is transferred to the protection of amino or aromatic hydroxyl groups in aqueous solutions to make them extractable by various organic solvents. Mainly alkyloxycarbonylation (AOC) in an alkaline aqueous solution with alkyl chloroformates has been used for derivatization.[10–13] In extractive AOC procedures, derivatization and extraction of the target compounds, which contained active hydrogen atoms on amino and phenolic hydroxyl groups, are achieved in nearly the same time as in aqueous solution.

In our studies of efficient screening methods in steroid-doping control, this work has been focused on the improved recovery of 3′-hydroxystanozolol and its 17-epimer as the main metabolites of stanozolol in human urine.[4–6,8] The method is based on the extractive isobutyloxycarbonylation (isoBOC reaction), combined with subsequent pentane extraction instead of diethyl ether extraction, which is followed in the general extraction procedure. Structures of the derivatives that are newly reported were confirmed by their mass spectral patterns. The application of the method to stanozolol-positive samples was studied using urine.

2. Experimental

2.1. Chemicals

3′-Hydroxystanozolol (17α-methyl-17β-hydroxy-5α-androstano[3,2-*c*]pyrazole) and 3′-hydroxy-17-epistanozolol (17β-methyl-17α-hydroxy-5α-androstano[3,2-*c*]pyrazole) were obtained from Cologne Laboratory (Institute of Biochemistry, German Sports University, Germany). 17α-Methyltestosterone (17α-methyl-17β-hydroxy-4-androsten-3-one) as an internal standard (IS), *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA), ammonium iodide (NH_4I), dithioerythritol (DTE) and isobutyl chloroformate (isoBCF) were purchased from Sigma (St. Louis, MO, USA). Serdolit Pad-1 resin (particle size 0.1–0.2 mm) was supplied by Serva (Heidelberg, Germany) and washed with acetone, methanol and distilled water before use. β-Glucuronidase from *E. coli* was purchased from Boehringer Mannheim (Mannheim, Germany).

2.2. Instrumental conditions

The GC-MS system (Model 5973MSD combined with a Model 6890 Plus gas chromatograph; Hewlett-Packard, Avondale, PA, USA) was used in both scan and selected-ion monitoring (SIM) modes. The electron energy was 70 eV and the ion source temperature was 230 °C. Samples were injected into an Ultra-2 (SE-54 bonded phase) fused-silica capillary column (Hewlett-Packard) (17 m × 0.2 mm id, 0.33 μm film thickness) in the split-injection mode (5 : 1) at 230 °C. The oven temperature was initially 230 °C, then raised at 5 °C min^{-1} to 290 °C (held for 3 min) and finally at 3 °C min^{-1} to 320 °C. Helium, as the carrier gas, was set to a column head pressure of 103 kPa (column flow rate 0.6 ml min^{-1} at 230 °C).

2.3. Sample preparation

2.3.1. General extraction procedure. An aliquot of urine (3 ml) was taken and 17α-methyltestosterone solution (1

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μg ml^{-1} × 50 μl) as an IS was added. The aqueous Serdolit Pad-1 slurry was filled into a Pasteur pipette to a bed height of 1.5 cm. The column was washed with 3 ml of distilled water and the mixture was loaded on to a Serdolit Pad-1 resin cartridge. The column was washed with water (5 ml) and eluted with methanol (1.5 ml × 2) into a test-tube; the eluate was evaporated to dryness (40 °C under nitrogen). The residues were dissolved in 1 ml of sodium phosphate buffer (pH 7.0, 0.2 mol l^{-1}). In order to hydrolyze the glucuro-conjugated form, 25 μl of enzyme solution were added to the buffer and the solution was heated at 55 °C for 1 h. The solution was cooled to room temperature and the pH was adjusted to 10–11 by adding 200 mg of K_2CO_3. The urinary steroids were extracted with 5 ml of diethyl ether on a mechanical shaken for 5 min. After centrifugation (5 min at 2500*g*), the organic layer was separated by placing it in a dry-ice–acetone bath and then evaporated. The residue was dried in a vacuum desiccator over P_2O_5–KOH for at least 30 min prior to derivatization.

2.3.2. *N*-Isobutyloxycarbonylation. The urine samples were prepared as described above, but the extraction step involved an extractive isoBOC reaction instead of diethyl ether extraction. A 50 μl volume of isoBCF and 1 ml of n-pentane were added, then the mixture was vortex mixed for 10 min. In addition, 2 ml of pentane were added. The mixture was shaken for 5 min with subsequent centrifugation. The organic layer was separated and evaporated.

2.3.3. Derivatization. The dry residue is derivatized with MSTFA–NH_4I–DTE (50 μl, 1000 : 2 : 5 v/w/w). The mixture was placed in a heating block (60 °C) for 15 min. An aliquot of the TMS-derivatized sample solution was injected into the GC-MS system.

2.4. Method validation

The extraction recovery was calculated by the analysis of urine samples spiked with 10 and 50 ng ml^{-1} of 3′-hydroxystanozolol and its 17-epimer, and also other target compounds for steroid-doping control. 17α-Methyltestosterone was used as an IS (50 μl of a methanolic solution of 1 μg ml^{-1}). Samples for intra- (n = 3) and inter-day (n = 3) assays and for recovery tests were prepared at two different concentrations, *e.g.*, 5 and 15 ng g^{-1}, using urine samples in which 3′-hydroxystanozolol and its 17-epimer had been spiked. They were analyzed on the same day for intra-day assays and every other day for inter-day assays. Spiked urine samples containing 3′-hydroxystanozolol and its 17-epimer at various concentrations (0.5–20.0 ng ml^{-1}) were subjected to isoBOC reaction with a subsequent TMS derivatization, as described above, to determine the linearity and the limit of quantification (LOQ). The LOQ was estimated by establishing the minimum concentration at which the analytes could be detected with a signal-to-noise (S/N) ratio of the diagnostic ion for each compound greater than 3 : 1.

2.5. Data acquisition

The peak identification of compounds was performed based on the relative retention time (RRT) and comparison with diagnostic ions. The first condition is that the difference in RRT of the analyte should not be higher than 1% compared with the same steroid present in spiked urine. For MS analysis, the evaluation must include three diagnostic ions. Each peak in the urine samples was identified by matching the peak-area ratios of three ions with those of the steroid in spiked urine.

The start time for SIM was programmed from 7.0 to 23.0 min to set up *m/z* 588, 573 and 282 (for 3′-hydroxystanozolol and its 17-epimer) and *m/z* 446 (for methyltestosterone) to be monitored. A dwell time of 120 ms was chosen in the SIM mode. The relative voltage of the electron multiplier (EM) was set to 400 eV higher than that in the scanning mode for each ion monitored.

2.6. Urine samples

Blank urine samples were collected as first-morning, drug-free urine (from a male (aged 33) and a stanozolol-positive urine (No. 98-8-6) was provided by the International Olympic Committee (IOC) for reaccreditation for the antidoping analysis test.

3. Results and discussion

3.1. Extractive *N*-isobutyloxycarbonylation

3′-Hydroxystanozolol and its 17-epimer have three ionizable hydrogen atoms. In order to extract the compounds and improve their GC properties, an initial effort was made to examine the AOC method using isoBCF. The injection of the *N*-isoBOC–*O*-TMS derivatives of both 3′-hydroxystanozolol and its 17-epimer, by GC-MS, showed the presence of two peaks with identical mass spectra in the electron ionization (EI) mode. The chromatograms did not show any peaks corresponding to partially derivatized or derivatized steroids with unexpected features, indicating that the derivatization reaction was complete. The difference between the total ion chromatograms of a blank urine extracted using general extraction procedure and extractive *N*-alkyloxycarbonylation is demonstrated in Fig. 1. Using extractive *N*-alkyloxycarbonylation, many interfering background peaks are reduced or eliminated.

3′-Hydroxystanozolol and its 17-epimer were extracted from urine with a lower recovery than for other urinary steroids because of their polar structure, with a pyrazole nucleus attached to the steroidal A-ring. The pentane extraction procedure[2] to removing interfering backgrounds especially offered low recovery, with about a 6% yield. Extraction with an

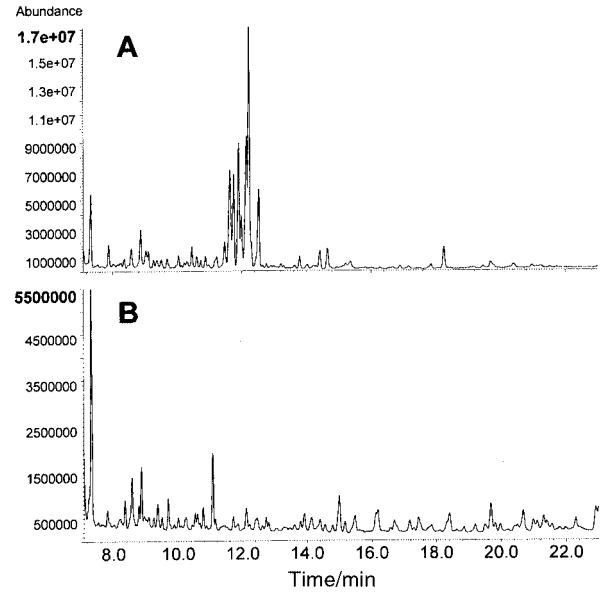

Fig. 1 Total ion chromatograms of a blank urine extracted using the general extraction procedure (A) and extractive *N*-alkyloxycarbonylation (B). Separation was performed on an Ultra-2 fused-silica capillary column (17 m × 0.2 mm id, 0.33 μm film thickness). The oven temperature was programmed at 5 °C min^{-1} from 230 to 290 °C (held for 3 min) at 3 °C min^{-1} to 320 °C. By efficient extraction of the urine sample, many polar substances are reduced or eliminated.

isoBOC reaction led to excellent recoveries of the steroids studied. The recoveries of 3′-hydroxystanozolol and its 17-epimer, and other steroid-doping substances included in the screening procedure after the extractive isoBOC reaction, ranged from 76.4 to 96.5%, except for the major metabolite of fluoxymesterone (36.4%). Using this method, the recovery of most steroids is similar to that with a pentane extraction. However, 3′-hydroxystanozolol and its 17-epimer gave recoveries of 87.6 and 81.3%, respectively. With the present method, no steroids, except for 3′-hydroxystanozolol and its 17-epimer, were converted into isoBOC derivatives.

According to the results obtained in a previous comparative study,[2] the extraction method with pentane was not suitable for stanozolol metabolites and therefore it was not recommended as a screening method in steroid-doping control. However, a significant advantage of the present method, involving pentane extraction combined with *N*-isoBOC, is that it is a very simple and useful method for the recovery of many urinary steroids, including stanozolol metabolites, and also removes interfering polar substances.

3.2. Mass spectral analysis

The EI mass spectrum of 3′-hydroxystanozolol is shown in Fig. 2. An *N*-isoBOC–*O*-TMS derivative was obtained when this pure standard was subjected to the derivatization process. Loss of a methyl group led to an abundance of M − 15 u less than that of the molecular ion. Peaks at M − 57 u, [M − $CH_2CH(CH_3)_2]^+$, and M − 100 u, [M − $CO_2CH_2CH(CH_3)_2]^+$, were observed. The isoBOC derivatives led to a tendency for expulsion of dimethylmethoxycarbonyl radicals as the most stable tertiary radicals in the EI mode.[12] Likewise, trimethylsilylation gave ions of M − 72 u, [M − $Si(CH_3)_3]^+$, M − 90 u, [M − $Si(CH_3)_3OH]^+$, and M − 105 u, [M − $Si(CH_3)_3OHCH_3]^+$, although their abundances were low. Also, other abundant ions at *m/z* 458, 282 and 182 detected in this spectrum were derived from cleavage of the steroidal D, A and C rings.

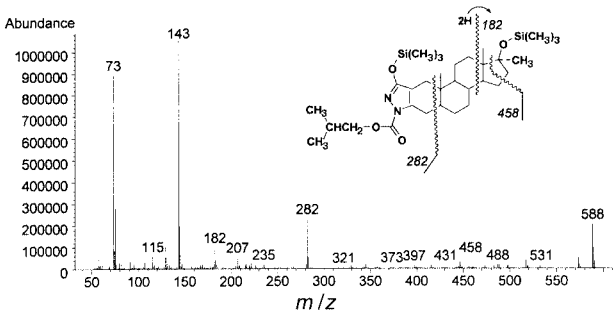

Fig. 2 EI mass spectrum of 3′-hydroxystanozolol as its *N*-isoBOC–*O*-TMS derivative obtained in the scanning mode at a rate of 0.48 scans s^{-1} with a mass range of *m/z* 50–650.

3.3. Method validation

The precision of the present method was assessed by analyses of replicate aliquots of urine specimens spiked with two different concentrations of 3′-hydroxystanozolol and its 17-epimer. The accuracy (% bias) for recovery tests of 3′-hydroxystanozolol and its 17-epimer varied from 4.6 to 10.9%, the recoveries being 87.6 and 81.3%, respectively, with precision (RSD) varying from 5.1 to 12.6% for three different runs. The LOQs of 3′-hydroxystanozolol and its 17-epimer in urine in the SIM mode were 2 and 1 ng ml^{-1}, respectively. It is noteworthy that the detection of stanozolol metabolites was improved with respect to both the diethyl ether and pentane extraction procedures. Stability was demonstrated over at least 72 h at room temperature and three freeze–thaw cycles.

3.4. Application to urine samples

When two identical urine samples, with 3′-hydroxystanozolol and its 17-epimer spiked at a level of 2 ng ml^{-1}, were analyzed using two different sample preparation techniques, both compounds were well detected without interfering peaks using the present method [Fig. 3(right)]. However, using the general extraction procedure 3′-hydroxystanozolol and its 17-epimer were not detected whereas some abundant interferences were observed [Fig. 3(left)].

When applied to a stanozolol-positive sample (No. 98-8-6), the present method provides a sensitive technique for the detection of 3′-hydroxystanozolol and its 17-epimer in urine samples (Fig. 4). This method can be employed to improve the extraction efficiency in terms of sensitivity and selectivity

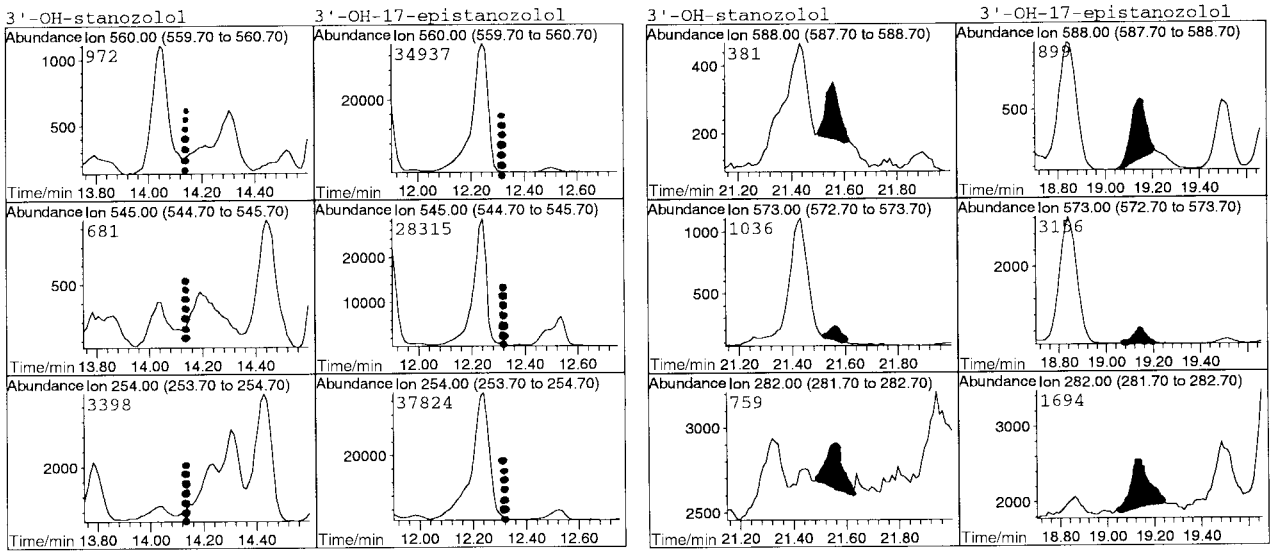

Fig. 3 SIM chromatograms for detection of 3′-hydroxystanozolol and its 17-epimer spiked into urine at a level of 2 ng ml^{-1} using the general extraction procedure (left) and extractive *N*-alkyloxycarbonylation (right). The characteristic ions (*m/z* 560, 545 and 254 for tri-TMS derivatives and 588, 573, and 282 for *N*-isoBOC–*O*-TMS derivatives) were monitored for detection of 3′-hydroxystanozolol and its 17-epimer. A dwell time of 120 ms was chosen in the SIM mode. The relative voltage of the EM was set to 400 eV higher than that in the scanning mode for each ion monitored.

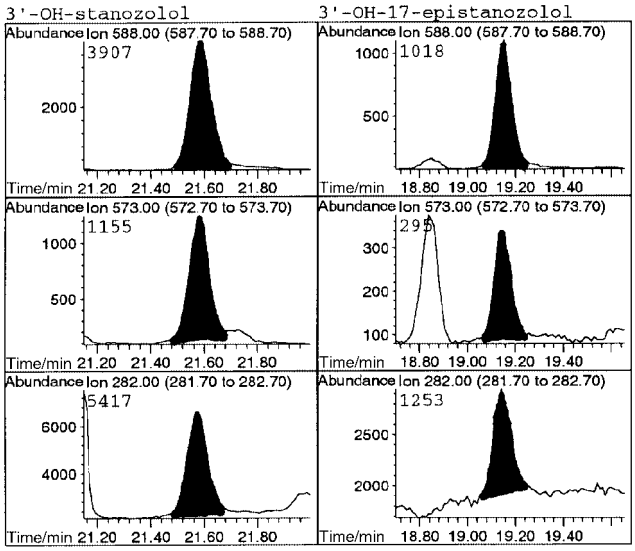

Fig. 4 SIM chromatograms of a stanozolol-positive urine extracted using the proposed method. Most of the interfering background peaks were diminished and 3′-hydroxystanozolol and its 17-epimer were clearly detected.

within a reasonable time frame for stanozlol metabolites, and also other anabolic and endogenous steroids in human urine.

References

1 A. Leinonen, L. Savonen and K. Kuoppasalmi, in *Recent Advances in Doping Analysis: Proceedings of the 11th Cologne Workshop on Dope Analysis*, ed. W. Schänzer, H. Geyer, A. Gotzman, U. Mareck-Engelke and S. Rauth, Sport und Buch Strauss, Cologne, 1994, pp. 25–31.

2 H. Geyer, U. M. Engelke, W. Schänzer and M. Donike, in *Recent Advances in Doping Analysis: Proceedings of the 11th Cologne Workshop on Dope Analysis*, ed. W. Schänzer, H. Geyer, A. Gotzman, U. Mareck-Engelke and S. Rauth, Sport und Buch Strauss, Cologne, 1994, pp. 97–103.

3 M. Donike, J. Zimmermann, K. R. Bärwald, W. Schänzer, V. Christ, K. Klostermann and G. Opfermann, *Dtsch. Z. Sportmed.*, 1984, **35**, 14.

4 R. Massé, C. Ayotte, H. Bi and R. Dugal, *J. Chromatogr.*, 1989, **497**, 17.

5 W. Schänzer, G. Opfermann and M. Donike, *J. Steroid Biochem.*, 1990, **36**, 153.

6 B. C. Chung, H. P. Choo, T. Kim, K. D. Eom, O. S. Kwon, J. Suh, J. Yang and J. Park, *J. Anal. Toxicol.*, 1990, **14**, 91.

7 W. Schänzer and M. Donike, *Anal. Chim. Acta*, 1993, **275**, 23.

8 W. Schänzer, *Clin. Chem.*, 1996, **42**, 1001.

9 M. H. Choi, B. C. Chung, M. Kim, J. Choi and Y. Kim, *Rapid Commun. Mass Spectrom.*, 1998, **12**, 1749.

10 A. P. J. M. de Jong and C. A. Cramers, *J. Chromatogr.*, 1983, **276**, 267.

11 K. R. Kim, M. J. Paik, J. H. Kim, S. W. Dong and D. H. Jeong, *J. Pharm. Biomed. Anal.*, 1997, **15**, 1309.

12 M. H. Choi, K. R. Kim and B. C. Chung, *Analyst*, 2000, **125**, 711.

13 M. H. Choi, K. R. Kim and B. C. Chung, *J. Chromatogr. A*, 2000, **897**, 295.