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# CAPILLARY GAS CHROMATOGRAPHIC–MASS SPECTROMETRIC DETECTION OF ANABOLIC STEROIDS

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## SUMMARY

A procedure is described for the detection of anabolic steroids in urine by using capillary gas chromatography–mass spectrometry. The method is suitable for doping control and has a sensitivity as low as 1 ppb*. The excretion mode and the metabolism of ten commercial anabolic steroids is reported.

## INTRODUCTION

Anabolic steroids can be used not only for therapeutic purposes, but also as doping agents in sports. Athletes take these steroids, even in large doses, over long periods of training, with the aim of improving their performance. To prevent this abuse and the consequent dangerous effects of these drugs, in Italy, as well as in other countries, many sports events are monitored by specialized antidoping laboratories, where the athletes' urine samples are analyzed to detect the presence of traces of doping compounds. Many methods have been described for the analysis of anabolic steroids by gas chromatography (GC) for doping control[1–4] or for the analysis of residues in meat[5,6].

In recent years, great improvements have been made in steroid analysis by high-efficiency capillary columns[7], and with fused-silica columns connections to modern mass spectrometers are facilitated. The high degree of deactivation and thermal stability of these columns also permits the analysis of some steroids without derivatization[8].

## EXPERIMENTAL

### *Materials*

The anabolic steroids were mainly extracted from pharmaceutical products, and for excretion studies the same products were given orally to volunteers, except for the first two products of Table I, which were injected.

* Throughout this article, the American billion ($10^9$) is meant.

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The silylating reagents were purchased from Pierce, β-glucoronidase aryl sulphate from Merck and Sep-Pak-$C_{18}$ cartridges from Waters.

*Apparatus*

All gas chromatograms were carried out with a Hewlett-Packard (HP) gas chromatograph (Model 5840 A), connected to a HP mass spectrometer (Model 5985), and equipped with a HP data system. Fused-silica HP capillary columns were used (25 m × 0.31 mm I.D.), coated with SE-54 (film thickness = 0.52 μm) and siloxane-deactivated. The flexible columns were connected to a split/splitless injection system and interfaced to the mass spectrometer by direct insertion of the capillary in the ion source. Helium was used as a carrier gas at a flow-rate of 3 ml/min and a splitting ratio of 5:1; column, injector and transfer line temperatures, 260°C; ion source temperature, 200°C.

Spectra were obtained by electron impact (70 eV) and in some cases by chemical ionization with methane, for a better identification of metabolites. One μl of the purified sample solution was injected in the split/splitless system or 3–4 μl with the splitter opened. Where possible, full spectra were recorded, and for samples where small amounts of drugs or metabolites were present, multiple ion detection was used with at least three or four selected masses.

*Procedure*

All urine samples were analyzed before and after enzymatic hydrolysis. For the detection of the free steroids, 5 ml of urine were directly extracted with diethyl ether (2 × 5 ml). The solvent was dried over sodium sulphate, evaporated at room

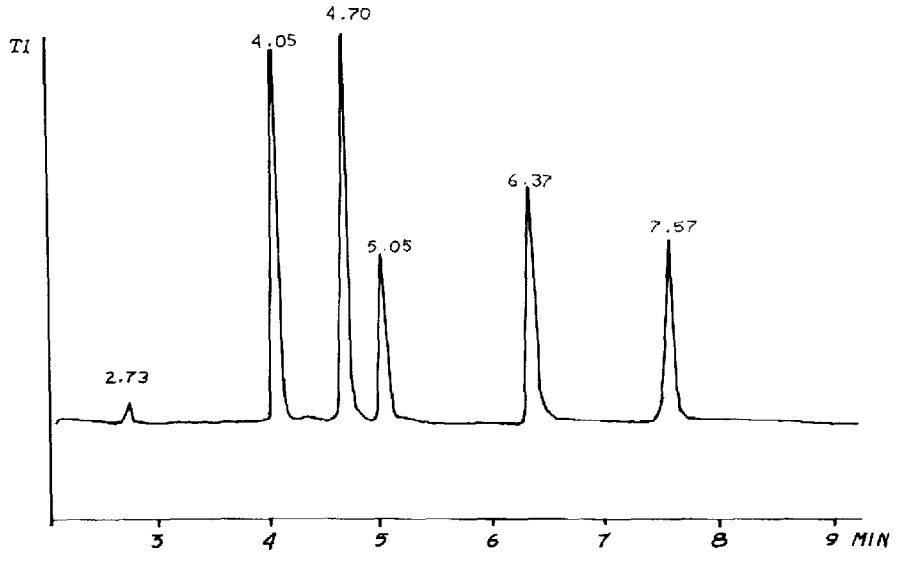


Fig. 1. Gas chromatogram, from total ion monitor of mass spectrometer. Conditions: fused-silica capillary column (25 m × 0.31 mm I.D.), SE-54, temperature = 260°C; carrier gas (helium) flow-rate, 3 ml/min; splitting ratio, 5:1. Standard mixture: 2.73, noretiocholanone; 4.05, mesterolone; 4.70, methandienone; 5.05, norethandrolone; 6.37, hydroxymethandienone; 7.57, chloromethandienone.

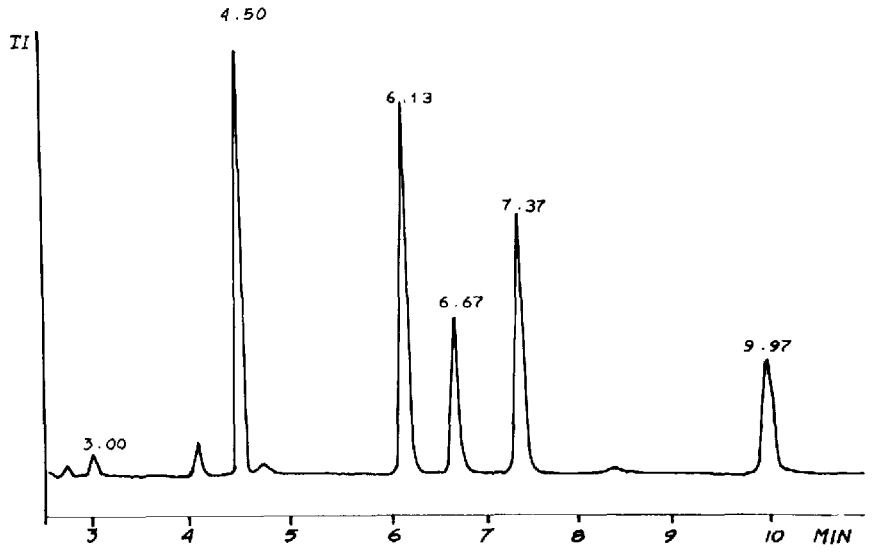


Fig. 2. Gas chromatogram of the same standard mixture after derivatization.

temperature with nitrogen and the residue was dissolved in 30 50 $\mu$l of methanol and injected.

For the analysis of conjugated steroids, 5 ml of urine were passed through a Sep-Pak-$C_{18}$, washed with 10 ml of water and eluted with 2 ml of methanol. The methanol was evaporated, the residue was dissolved in 2 ml of 1 *M* acetate buffer at pH 5.5 and 0.1 ml of enzyme was added. The conjugated steroids were hydrolyzed for 12–24 h at 40°C. This solution was brought to pH = 8.5 with sodium bicarbonate and extracted with diethyl ether, as above. The residue, after evaporation of the ether, was derivatized with 0.1 ml of a solution of N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA), N-trimethylsilylimidazole (TSIM) and trimethylchlorosilane (TMCS), in the ratio 100:2:5, at 70°C for 30 min. The silylating reagent was evaporated, and the residue was dissolved in cyclohexane and injected into the gas chromatograph.

## RESULTS AND DISCUSSION

Fig. 1 shows a gas chromatogram of a standard mixture of anabolic steroids, obtained from the total ion monitor of the mass spectrometer, with the fused-silica capillary column. The underivatized steroids separated under isothermal conditions at 260°C in about 12 min. Fig. 2 shows the chromatogram of the same mixture after derivatization. Comparing the two chromatograms, it appears that the compounds are always eluted with symmetrical peaks, and the changes in the retention times between the free and the silylated steroids are very useful for the identification of the compounds in the urine samples. The use of capillary columns facilitates the analysis of the anabolic steroids, as it permits the elution at lower temperatures in a short time and with a high resolving power.

Fig. 3 shows the gas chromatogram of a urine blank and of a hydrolyzed urine

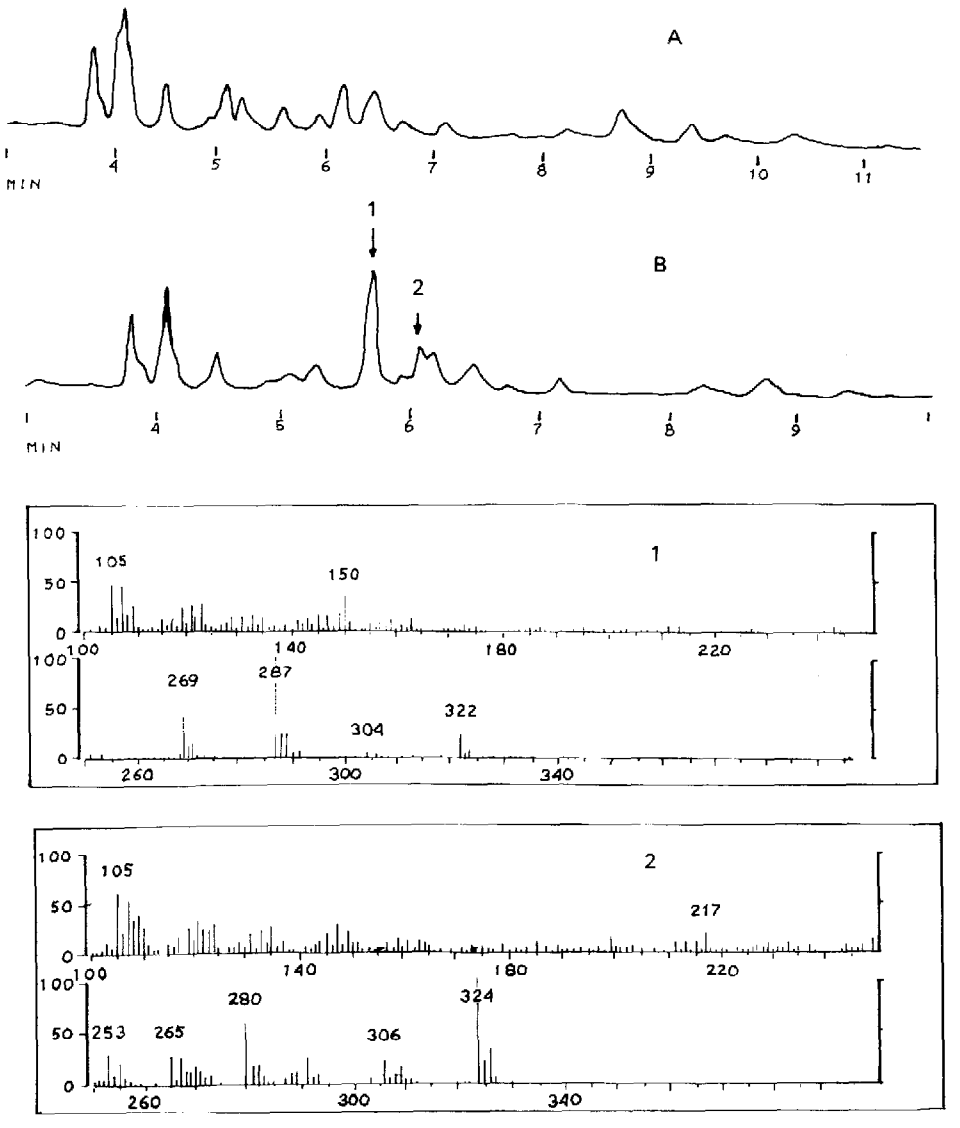


Fig. 3. Gas chromatograms of urine extracts, blank (A) and sample (B), 8 h after ingestion of 4-chlorotestosterone (20 mg). Mass spectra of the steroid as such (1) ($M^+$ = 322) and of its metabolite (2) ($M^+$ = 324) are also shown.

collected 8 h after the ingestion of a single therapeutic dose of 20 mg of 4-chlorotestosterone. Comparing the two chromatograms, it appears that in the urine there are many interfering compounds, but from the mass spectra, recorded on the corresponding peaks, the presence of the 4-chlorotestosterone and of its metabolite, 4-chloronorandrosterone, is clearly shown. Fig. 4 shows the gas chromatograms of the same urine extracts after drug ingestion, derivatized as described. The silylated 4-chloro-

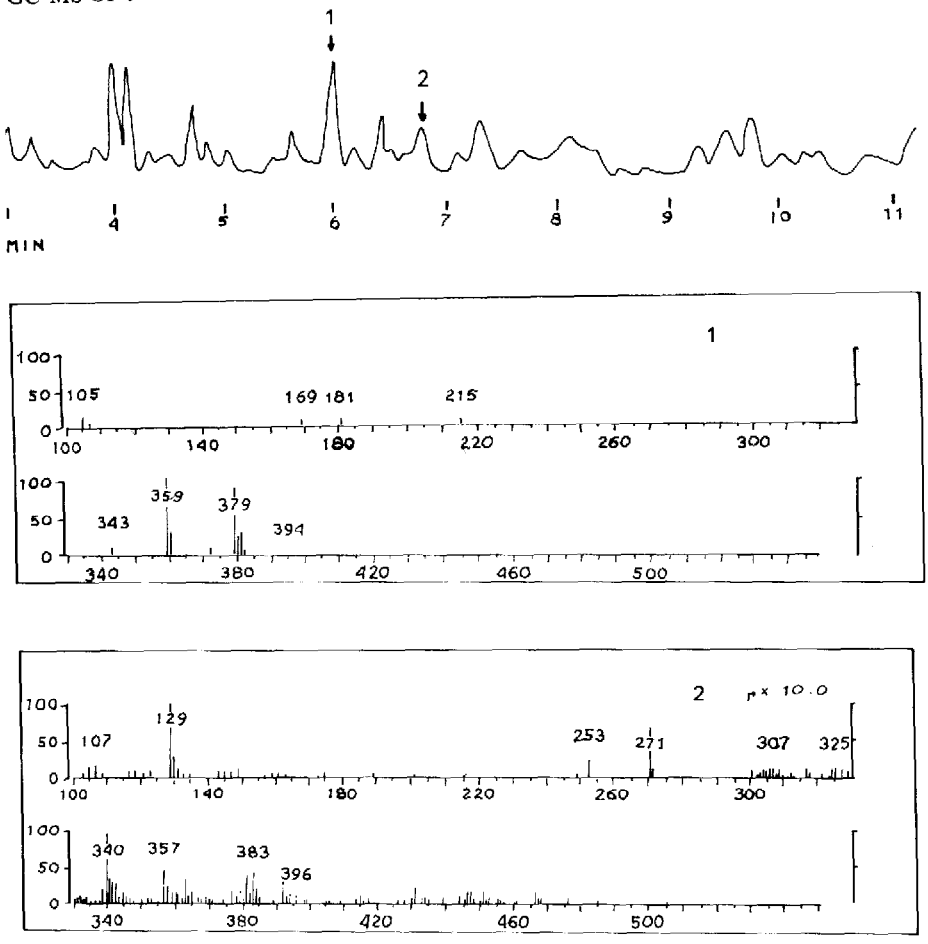


Fig. 4. Gas chromatogram of the same urine extract as in Fig. 3 after derivatization. Mass spectra o silylated 4-chlorotestosterone (1) ($M^+$ = 394) and of its metabolite (2) ($M^+$ = 396).

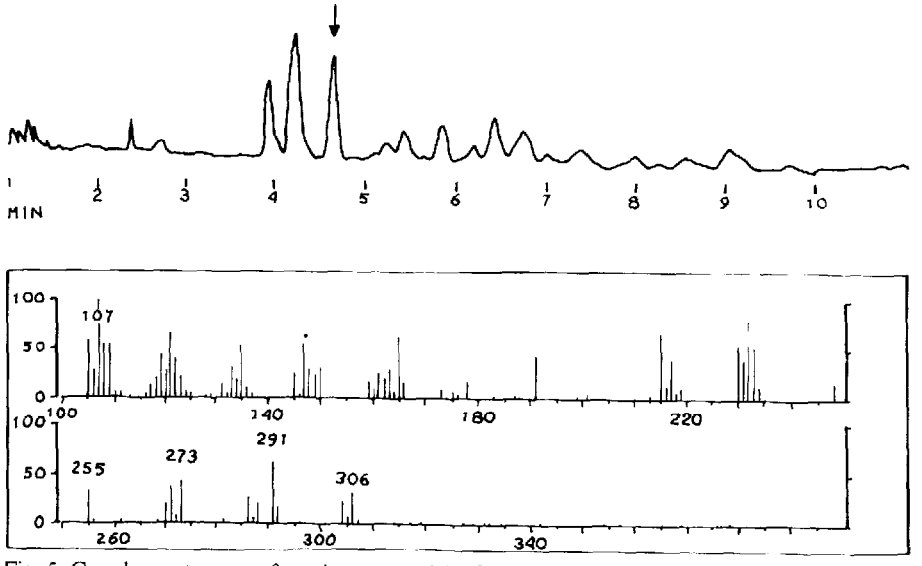


Fig. 5. Gas chromatogram of a urine extract 8 h after ingestion of methyltestosterone (20 mg). Only the metabolite ($M^+$ = 306) is detectable.

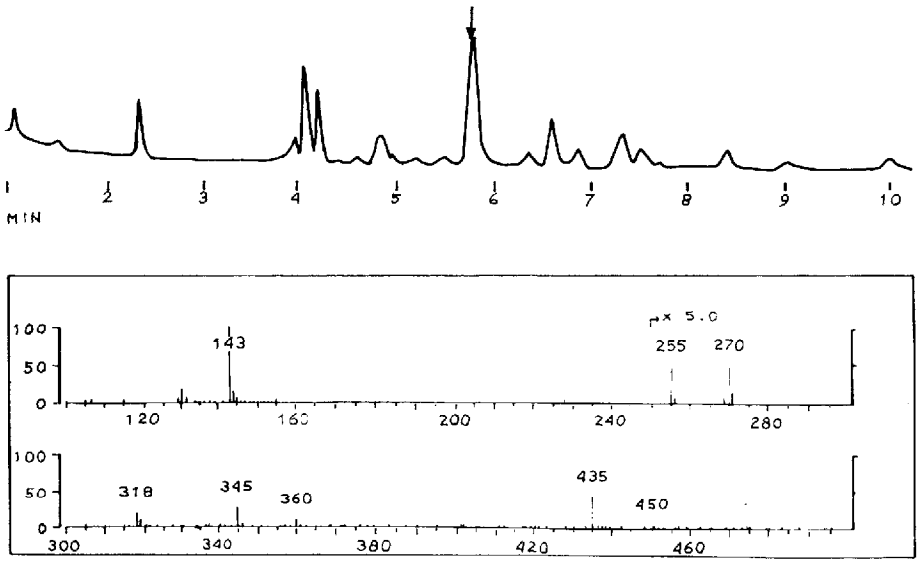


Fig. 6. Gas chromatogram of the same urine extract as in Fig. 5 after derivatization. Mass spectra of silylated metabolite ($M^+$ = 450).

testosterone and its metabolite have retention times different from the underivatized steroid, but in both cases they are easily detectable. The presence of the metabolite confirms the assumption of the anabolic steroid.

Figs. 5 and 6 show the chromatograms and corresponding mass spectra of underivatized and derivatized urine samples. In this case, after ingestion of methyltestosterone only a metabolite is detectable in the hydrolyzed urine ($M^+$ = 306) with

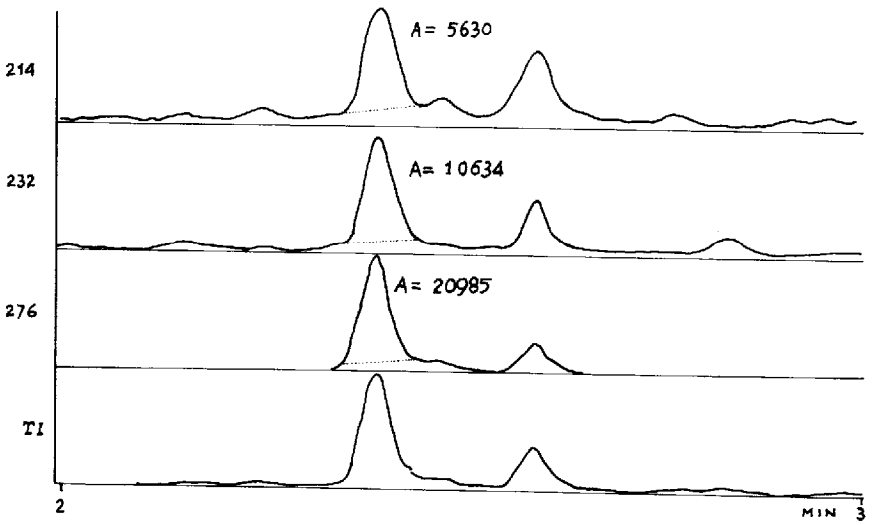


Fig. 7. Low level detection of an underivatized urine extract, 7 days after injection of a single dose (50 mg) of nortestosterone. Mass spectra recorded at three selected masses of the metabolites ($M^+$ = 276). The A values are peak areas related to ion intensities.

TABLE I

ANABOLIC STEROIDS INVESTIGATED

| *Compound* | *Dose (mg)* | *Required hydrolysis* | *Species found in the 24-h urine* | |
|---|---|---|---|---|
| | | | *Parent* | *Metabolites* |
| 1 Nortestosterone | 50 | + | – | Norandrosterone<br>Noretiocholanolone |
| 2 Methenolone | 100 | + | + | 6-Hydroxy derivative |
| 3 Mesterolone | 50 | + | + | Methylandrosterone |
| 4 Norethandrolone | 10 | + | – | Dihydro-3-hydroxy,<br>Dihydroxy-3,21-hydroxy derivatives |
| 5 Methandienone | 10 | – | + | 6β-Hydroxy derivative |
| 6 Fluoxymesterone | 10 | – | + | One non-identified,<br>6β-hydroxy derivative |
| 7 Methyltestosterone | 20 | + | – | Dihydro-3-hydroxy derivative |
| 8 Chloromethandienone | 10 | – | + | 6β-Hydroxy derivative |
| 9 4-Chlorotestosterone | 20 | + | + | Chloroandrosterone<br>Chloroetiocholanolone |
| 10 4-Chloronortestosterone | 20 | + | + | Chloronorandrosterone<br>Chloronoretiocholanolone |

four mass units more than the parent compound, and after derivatization this product contains two trimethylsilyl groups ($M^+ = 450$).

Fig. 7 shows an example of the high level of specificity and sensitivity obtained by the combination of capillary GC and mass spectrometry (MS) in doping control for anabolic steroids. A urine sample, collected 7 days after the injection of a single therapeutic dose of 50 mg of nortestosterone, after hydrolysis and without derivatization, was analyzed by SIM (multiple ion detection). The detection was carried out by selectively monitoring the same molecular ion ($M^+ = 276$) and two other characteristic fragments of two metabolites of this drug. The values of the retention times and of the ion intensities of both of these metabolites clearly verify the presence of this anabolic steroid.

Table I shows some of the more frequently used anabolic steroids with their therapeutic dosages and their main excretion forms. The last two columns indicate whether the parent compound is detectable in urine samples collected within 24 h after drug ingestion and also closely related metabolites that may be found. It is obvious that the detection limit depends on the ion intensities and on the biological background of the sample. In a normal situation about 10 ng should be injected in the gas chromatograph to obtain a full spectrum, and 50–100 pg for SIM detection. These amounts, according to the procedure described, correspond to concentrations in the urine of from 0.1 ppm to 1 ppb, respectively. The use of capillary GC–MS is of great utility in the detection of anabolic steroids and in studies of their metabolism. The possibility of analyzing derivatized and underivatized urine samples facilities the detection and identification of the compounds, because more information is obtained from the same sample.

ACKNOWLEDGEMENT

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