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DEVELOPMENT OF A GAS CHROMATOGRAPHIC—MASS SPECTROMETRIC METHOD USING MULTIPLE ANALYTES FOR THE CONFIRMATORY ANALYSIS OF ANABOLIC STEROID RESIDUES IN HORSE URINE

II. DETECTION OF ADMINISTRATION OF 19-NORTESTOSTERONE PHENYLPROPIONATE TO EQUINE MALE CASTRATES AND FILLIES

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SUMMARY

Esters of 19-nortestosterone form an important group of anabolic preparations used in veterinary practice. Based upon results from detailed metabolic studies for 19-nortestosterone in the horse, a method to confirm the administration of anabolic preparations of this steroid to castrated male horses and fillies is described; the method is based upon the use of multiple analytes. Following administration of the anabolic preparations, solid-phase extraction of urinary conjugates and the separation of the conjugate groups prior to hydrolysis allow for the determination of specific metabolites conjugated with either glucuronic acid or sulphate. Following hydrolysis of the conjugates, purification of the free neutral steroids on thin-layer chromatography, derivatisation and gas chromatographic—mass spectrometric analysis, the presence of the major metabolites, estrane-3,17α-diol in the glucuronic acid fraction and 19-nortestosterone and two isomers of estrane-3,17-diol in the sulphate fraction, could be confirmed for 17—18 days after administration of Nandrolin (19-nortestosterone phenylpropionate).

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INTRODUCTION

In a previous publication [1] a method, based upon the use of multiple analytes, was described to confirm the administration to horses of proprietary anabolic preparations of testosterone. Based upon evidence derived from detailed metabolic studies this method has now been applied to confirm the administration of proprietary preparations of 19-nortestosterone.

19-Nortestosterone is the parent steroid of a number of proprietary anabolic preparations used in veterinary practice, its metabolism has been studied in man [2–6], the rabbit [7] and the horse [8—10]. Detailed metabolic studies in the horse have shown that the major urinary metabolites are estrane-3,17α-diol and estran-3-ol-17-one excreted as glucuronic acid conjugates and estr-4-en-17β-ol-3-one (19-nortestosterone) and an isomer of estrane-3,17α-diol and estrane-3,17β-diol excreted as sulphate conjugates. By comparison of retention time data and co-injection with authentic standard steroid derivatives under combined gas chromatographic—mass spectrometric (GC—MS) conditions the stereochemistry of the estranediol isomers in the glucuronic acid and sulphate fractions has been established [8, 10]. The analysis method described is based upon confirming, by GC—MS, the presence of estrane-3,17α-diol in the glucuronic acid fraction and 19-nortestosterone and the isomers of estrane-3,17-diol in the sulphate fraction.

EXPERIMENTAL

Solvents and chemicals

Analar-grade hexane, ethyl acetate, chloroform and methanol were obtained from BDH (Poole, U.K.) and were redistilled before use. Sep-Pak C_{18} cartridges were obtained from Waters Assoc. (Milford, MA, U.S.A.). Sephadex LH-20, N,O-bis(trimethylsilyl)acetamide (BSA), trimethylchlorosilane (TMCS), 19-nortestosterone, 5α-androstane-3α,17β-diol and methoxylamine hydrochloride were purchased from Sigma London (Poole, U.K.). Precoated silica gel plates (Kieselgel $60F_{254}$, 0.2 mm thickness) for thin-layer chromatography (TLC) were purchased from BDH and *Helix pomatia* digestive juice (Industrie Biologique Français) was obtained through Uniscience (London, U.K.). 5α-Estrane-3β,17α-diol was obtained from Organon (Oss, The Netherlands) and Nandrolin (19-nortestosterone phenylpropionate; 50 mg/ml in arachis oil) was obtained from Intervet Labs. (Cambridge, U.K.).

Administration to animals and collection of urine

Before administration of the proprietary preparation, ten isolated urine samples were collected from each animal. Intramuscular administrations of Nandrolin (400 mg) were made to three castrated thoroughbred male horses (body weight 554, 436 and 460 kg). Following administration all urine samples were collected for 48 h. Subsequent samples were collected between 8.00 a.m. and 8.00 p.m. for a further 19 days. The individual urine samples for each 12-h period of collection were combined and an aliquot was taken for analysis.

The same preparation (400 mg) was also administered to a thoroughbred

filly (body weight 440 kg). Following administration two naturally voided urine samples were collected daily for 28 days. The two samples for each day were combined and an aliquot was taken for analysis. The animals were fed and exercised in the normal manner.

Extraction and derivatisation procedures

The detailed extraction and derivatisation procedures were described in a previous publication [1] and are summarised in the flow diagram shown in Fig. 1. Five pre-administration urine samples, five individual samples obtained during the first 48 h following administration and each of the combined daily urine samples collected between days 3 and 21 post-administration for each of the three castrated male horses were analysed. Similarly for the filly ten pre-administration urine samples and each of the combined daily urine samples were analysed.

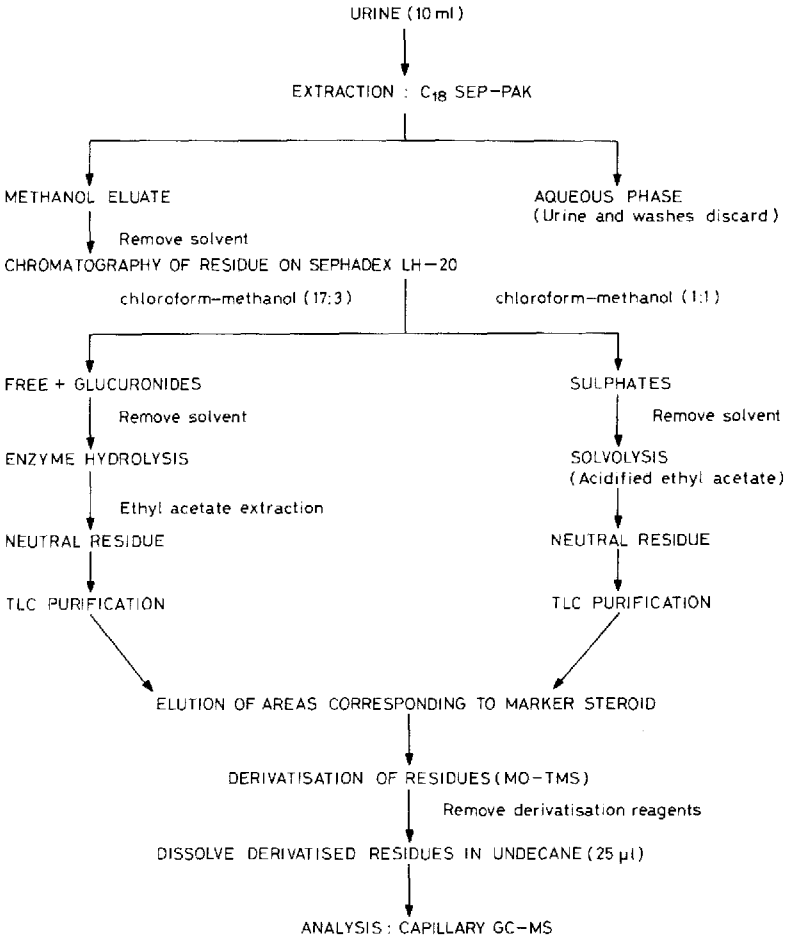

Fig. 1. Flow chart of the analytical procedure.

Capillary column gas chromatography—mass spectrometry

Combined GC—MS was carried out using a Finnigan Series 4000 instrument interfaced to a Finnigan 6110 data system. A Hewlett-Packard methyl silicone

fused-silica column (25 m × 0.2 mm I.D.) was used. The column passed through the gas chromatographic–mass spectrometric interface oven and terminated approximately 5 mm from the ion source. Helium was used as a carrier gas (linear gas velocity 40 cm s^{-1}) and the injector was maintained at 250°C. Splitless injections were made at a column temperature of 150°C which was held for 1 min, programmed up to 220°C at 20°C min^{-1}, and then at 5°C min^{-1} to a final temperature of 270°C which was held for 10 min. The mass spectrometer was operated in the repetitive scan mode with a scan time of 1 s for the mass range between 100 and 450 a.m.u. Data were also obtained in the selected-ion monitoring (SIM) mode.

RESULTS

Using this procedure the mass spectra of the bis(trimethylsilyl) (bis-TMS) derivative of the isomer of estrane-3,17α-diol isolated from the glucuronic acid fraction and the methyloxime-trimethylsilyl (MO-TMS) derivative of 19-

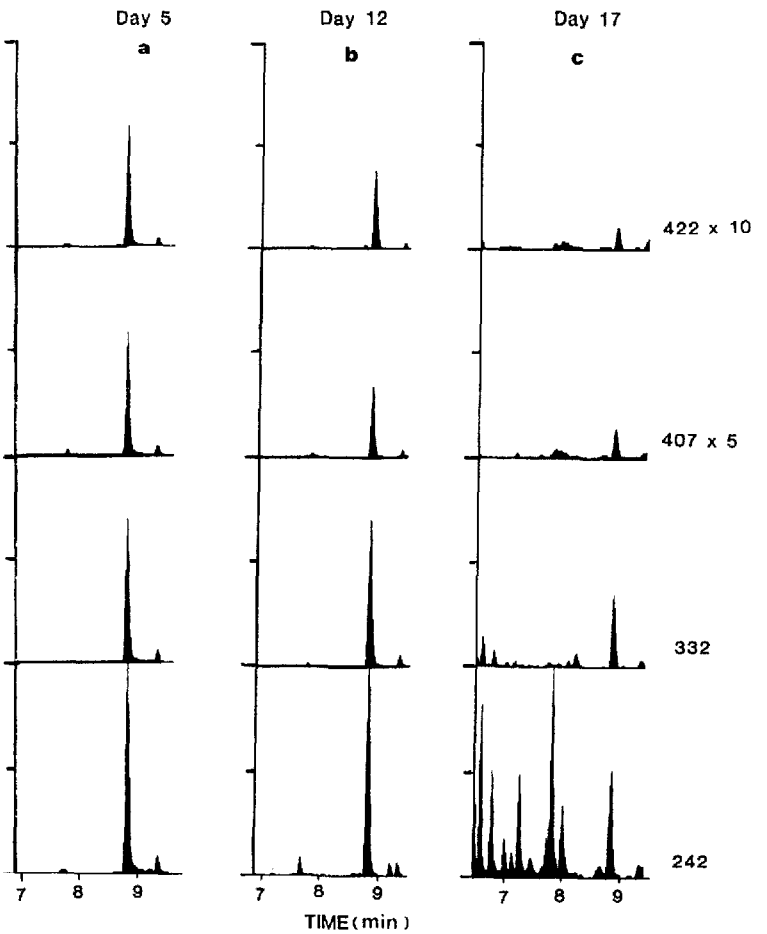

Fig. 2. SIM chromatograms to monitor estrane-3,17α-diol bis-TMS in the urinary glucuronic acid fraction following administration of Nandrolin to a thoroughbred castrated male horse.

nortestosterone isolated from the sulphate fraction for urine samples obtained 8 and 11 days after administration, respectively, corresponded to those of derivatised standard steroids.

In addition to obtaining full-scan mass spectral data on selected samples, SIM was used to monitor estrane-3,17α-diol in the glucuronic acid fraction and 19-nortestosterone and the isomers of estrane-3,17-diol in the sulphate fraction in both pre- and post-administration urine samples. The presence of isomers of the bis-TMS derivative of estranediol can be monitored using the ions *m/z* 422, 407, 332 and 242. The selected-ion chromatograms recorded for the derivatised residues isolated from the glucuronic acid fractions of post-administration urine samples obtained on days 5, 12 and 17 from one of the castrated male horses are shown in Fig. 2. The selected-ion chromatograms to monitor estranediol isomers in the residues isolated from the sulphate fractions of the same urine samples are shown in Fig. 3.

The MO-TMS derivative of 19-nortestosterone shows significant ions in its mass spectrum at *m/z* 375, 360, 344 and 254. The selected-ion chromatograms recorded for the presence of this steroid in residues isolated from the sulphate fraction of the same post-administration urine samples are shown in Fig. 4. By monitoring these ions it was also possible to detect the presence of estr-4-

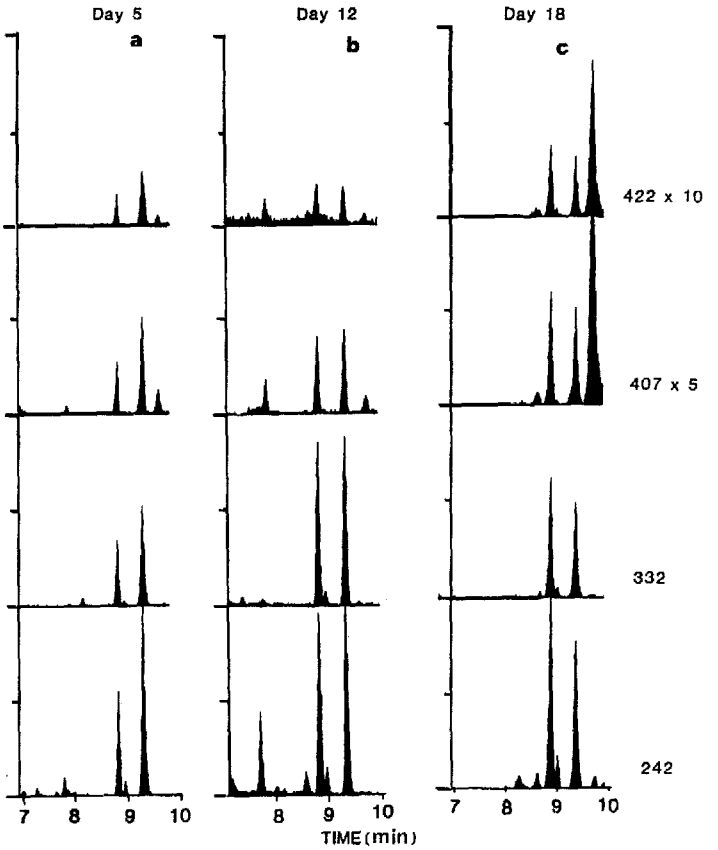

Fig. 3. SIM chromatograms to monitor isomers of estrane-3,17-diol bis-TMS in the urinary sulphate fraction following administration of Nandrolin to a thoroughbred castrated male horse.

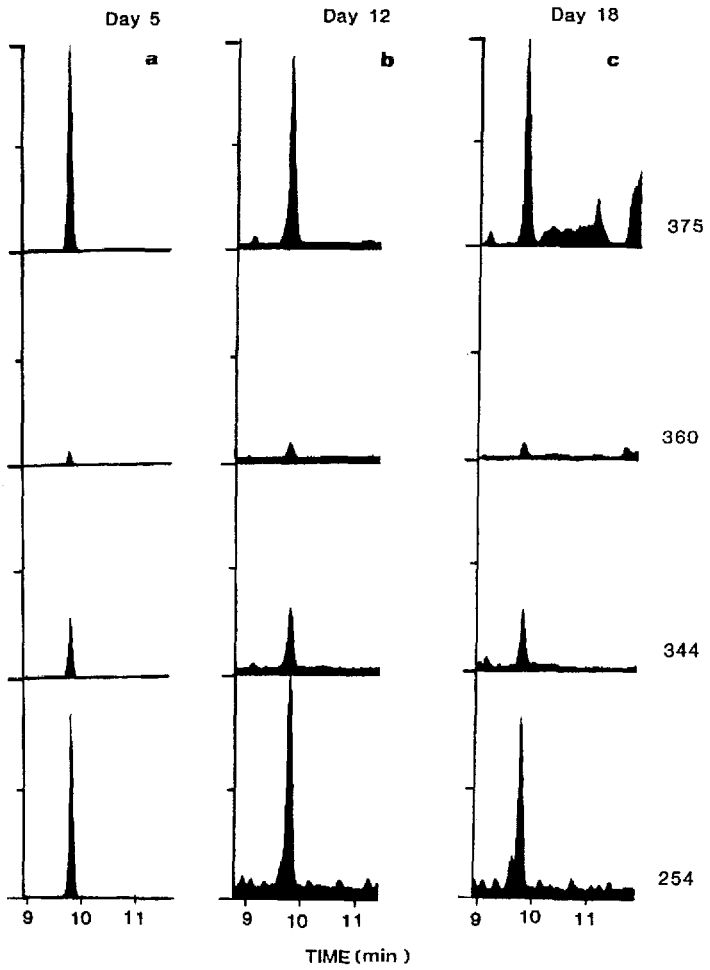

Fig. 4. SIM chromatograms to monitor 19-nortestosterone MO-TMS in the urinary sulphate fraction following administration of Nandrolin to a thoroughbred castrated male horse.

en-17α-ol-3-one (17α,19-nortestosterone) as a minor metabolite in the glucuronic acid fraction for 6—8 days after administration. Using this method an isomer of estrane-3,17α-diol was detected in the glucuronic acid fractions and 19-nortestosterone and two isomers of estrane-3,17-diol in the sulphate fractions of urine samples obtained up to 21 days after administration of 400 mg of Nandrolin to thoroughbred castrated male horses. The full-scale mass spectra of these analytes could be obtained for urine samples excreted up to 17—18 days after administration. Following the administration of Nandrolin to a filly, these analytes were detected in the urine samples for a similar period of time. None of these analytes was detected in the pre-administration urine samples from the four animals.

DISCUSSION

Based upon initial metabolic studies [8] a GC—MS method using packed columns was developed to confirm the administration of esters of

19-nortestosterone to the equine castrate [11]. Subsequently this method was modified to incorporate the use of capillary columns [12, 13]. This method was based upon the identification of a single analyte, an isomer of estrane-3,17α-diol isolated after direct enzyme hydrolysis of the urine, solvent extraction of the freed steroids and purification by TLC or column chromatography prior to derivatisation. On the basis of the results obtained from more detailed metabolic studies of 19-nortestosterone in the horse [10] a method of confirmatory analysis based upon the use of multiple analytes has now been developed. The importance of carrying out detailed metabolic studies in order to develop methods to confirm the presence of anabolic steroid residues in the equine castrate is clearly demonstrated in this study. 19-Nortestosterone is extensively metabolised in the horse with Phase II biotransformations playing an important role. 17β,19-Nortestosterone is excreted in the urine only as a sulphate conjugate and, as with the sulphate conjugates of testosterone and 1-dehydrotestosterone [14—16], this conjugate is also not hydrolysed by the aryl sulphatases of *Helix Pomatia*. Thus, to overcome this problem, the development of a method of confirmatory analysis for this steroid based upon the use of multiple analytes necessitated the separation of the glucuronic acid and sulphate conjugate groups prior to hydrolysis.

Using this procedure evidence for administration of an ester of 19-nortestosterone to the horse can thus be obtained based upon the identification of two or more analytes in the urine samples. This procedure can be applied to two categories of the racing throughbred horse, namely the castrated male horse and the filly. The analytes arising in filly and castrate urine following administration of 19-nortestosterone are known to occur endogenously in the urine of the entire sexually mature male horse [17]. These analytes are produced in the testis [18] and thus would not be expected in the urine of the normal filly and castrate. However, for forensic purposes it was necessary to demonstrate that in fillies and castrates any endogenous production of 19-nortestosterone, if it occurs at all, is at a urinary concentration that is insignificant. Such a demonstration is essential in order that the qualitative identification of these compounds in the urine of fillies and castrates by full-scan electron impact MS can constitute proof of administration of anabolic preparations of 19-nortestosterone. The failure to detect these compounds by SIM in the urine of a series of normal animals vindicates the use of this technique for the confirmation of doping. The identification of isomers of 19-nortestosterone and estrane-3,17α-diol in normal stallion urine [17] precludes their direct use as analytes to detect administrations of 19-nortestosterone to the entire male horse and an alternative procedure has now been developed for this purpose [19].

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