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

I. DETECTION OF TESTOSTERONE PHENYLPROPIONATE ADMINISTRATIONS TO EQUINE MALE CASTRATES

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SUMMARY

A gas chromatographic—mass spectrometric (GC—MS) method using three analytes to detect and confirm the administration to equine male castrates of veterinary pro-drugs based upon esters of testosterone is described. The method involves extraction of steroid conjugates from horse urine by C_{18}-bonded cartridges and fractionation into glucuronic acid and sulpho-conjugates by Sephadex LH-20 column chromatography. After deconjugation, the free neutral steroids were partially purified by thin-layer chromatography and following derivatization (methyloxime-trimethylsilyl ether) were analysed by capillary GC—MS in the selected-ion or full-scan mode. Of the three analytes, 5α-androstane-3β,17α-diol could be detected in the glucuronic acid fraction for about ten days and 5α-androstane-3β,17β-diol and testosterone could be detected in the sulpho-conjugate fraction for up to nineteen days after administration of a single therapeutic dose (50 mg) of testosterone phenylpropionate to cross-bred and thoroughbred castrated male horses. The reasons for development of such a method, its validation and its potential for the detection of neutral metabolites of other veterinary anabolic steroids in horse urine are discussed.

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INTRODUCTION

A variety of synthetic anabolic steroid preparations are available for veterinary use in the U.K. [1] and their pharmacological properties have been reviewed recently [2, 3]. The therapeutic value of these steroids in the treatment of diseased and debilitated horses has been demonstrated, but with respect to improvements in the racing performance, reports are contradictory [4—6]. The analytical methods used and the problems encountered in the control of anabolic steroid abuse in sporting events has been reviewed recently [7]. In order to implement effectively the rules governing the use of these drugs in horse racing in the U.K., it is essential to detect and confirm the presence of the drug and/or its metabolites in horse body fluids at the time of racing. In a positive case unequivocal identification of the drug using gas chromatography—mass spectrometry (GC—MS) is mandatory. The majority of GC—MS confirmatory analysis methods are based upon the use of a single analyte, the unchanged drug. In the case of anabolic steroids where extensive metabolism occurs in the horse and the pro-drugs are either esters of endogenous hormones or of structurally related steroids, the use of multiple analytes would provide more scientifically sound evidence of their administration. GC—MS has been used extensively to detect and identify a number of anabolic steroid residues in urine samples from athletes [8, 9] and in post-race horse urine [10—12]. In our laboratory initial evidence for the presence of anabolic steroid metabolites in post-race horse urine samples is provided by a radioimmunoassay screening procedure [1, 13]; GC—MS is then used to identify and confirm the individual steroids and/or their metabolites.

Studies on the metabolism of the orally active anabolic steroids containing the 17α-alkyl group have shown that in man the unchanged drugs and their metabolites are mainly excreted in the unconjugated form in urine [14, 15]. The other $C_{18/19}$ anabolic steroids and their metabolites are excreted mainly in the form of glucuronic acid and sulphate conjugates [8, 16, 17]. Previous studies on the metabolism of $C_{18/19}$ anabolic steroids (19-nortestosterone, testosterone, 1-dehydrotestosterone) in the equine male castrate have emphasised the importance of phase II biotransformation and the inability of the aryl sulphatases of Helix Pomatia digestive juice to hydrolyse certain sulphoconjugates [18—21]. Based upon studies related to testosterone metabolism in the horse [18, 21] a general method for the isolation and confirmatory analysis of anabolic steroids in horse urine using multiple analytes is presented.

Testosterone in the form of the long-acting phenylpropionate ester (Androject®) is used to provide androgenic activity in the horse over a period of up to fourteen days following a single injection. After parenteral administration of an oily preparation of [4-^{14}C]testosterone to equine castrates, it has been shown that 5α-androstane-3β,17α-diol is excreted in the urine as a major metabolite in the glucuronic acid conjugate fraction and 5α-androstane-3β,17β-diol and testosterone are major metabolites in the sulpho-conjugate fraction. These three steroids were not detected as endogenous compounds in normal pre-administration urine samples from the castrated male horses [18, 21]. The present paper describes the synthesis of [4-^{14}C]testosterone phenyl-

propionate and its administration, in the form of an oily preparation to two cross-bred castrated male ponies. The duration of urinary excretion of the drug was monitored by liquid scintillation counting and based upon metabolic studies, a procedure was developed to confirm the presence of the three metabolites by GC—MS throughout the excretory period. This procedure was evaluated further by the analysis of urine samples obtained following the administration of therapeutic doses of Androject to two thoroughbred castrated male horses.

EXPERIMENTAL

Solvents and chemicals

Analar-grade hexane, ethyl acetate, chloroform, toluene and methanol were obtained from BDH (Poole, U.K.) and were redistilled before use. Pyridine was dried over anhydrous calcium chloride, redistilled and stored over molecular sieve (type 5A, BDH). Thionyl chloride was obtained from Hopkin and Williams (Chadwell Heath, U.K.) and was freshly redistilled before use. [4-^{14}C]Testosterone (specific activity 51 mCi/mmol) was obtained from Amersham International (U.K.) and 2-phenylpropionic acid was purchased from Koch-Light Labs. (Slough, U.K.). 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), testosterone, 5α-androstane-3α,17β-diol, 5α-androstane-3β,17β-diol and methoxylamine hydrochloride were purchased from Sigma (Poole, U.K.). 5α-Androstane-3β,17α-diol was a gift from Professor D.N. Kirk (M.R.C. Steroid Reference Collection, London, U.K.). Silica gel S13F for thin-layer chromatography (TLC) was purchased from Whatman (Maidstone, U.K.), Kieselgel H (type 60) from Anderman & Co. (East Molesey, U.K.) and Helix pomatia digestive juice (Industrie Biologique Française) was obtained through Uniscience (London, U.K.). Androject (testosterone phenylpropionate, 10 mg/ml in arachis oil) was obtained from Intervet Labs. (Science Park, Cambridge, U.K.).

Liquid scintillation counting

A Packard Tricarb liquid scintillation spectrometer (Model 3320) was used. Determinations were carried out in polythene vials using a triton—toluene scintillant (10 ml) as described previously [22].

Synthesis of [4-^{14}C] testosterone phenylpropionate ester

A solution of 2-phenylpropionic acid (150 mg; 1.0 mmol) in freshly redistilled thionyl chloride (1.5 ml) was refluxed for 0.5 h. After cooling, the excess thionyl chloride was removed under reduced pressure. A solution of a mixture of testosterone (100 mg; 0.35 mmol) and [4-^{14}C] testosterone (50 μCi) in dry redistilled pyridine (1.2 ml) was then added to the acid chloride and the mixture was left overnight at room temperature with constant stirring. Excess pyridine was removed under reduced pressure, ice-cold deionised water (50 ml) was added and the product extracted with ethyl acetate (2 × 50 ml). TLC, using hexane—ethyl acetate (7:3, v/v), of the extract indicated that 80% of the testosterone had been converted to the phenylpropionate ester. The bulk of the

ethyl acetate was then removed under reduced pressure and the crude residue chromatographed on a Kieselgel H column (10.0 g; column diameter 2.0 cm) using toluene—chloroform (3:1, v/v) as eluent; the radioactivity was recovered between 150 and 200 ml of eluate. The total yield of [4-^{14}C]testosterone phenylpropionate was 35.6 μCi (71.2%). The purity of the product was checked by TLC (absorbance at 254 nm; radioactivity localisation using the Beta-Graph (Panax Equipment, Mitcham, U.K.). The electron-impact mass spectrum of the compound showed a molecular ion *m/z* 420 (relative intensity, 15%) with fragment ions *m/z* 378 (loss of ketene, 5%), *m/z* 271 (loss of $C_6H_5CH_2CH_2COO^{\bullet}$, 65%) and *m/z* 105 (corresponding to $[C_6H_5CH_2CH_2]^+$, 85%).

Administration to animals and collection of urine

Before administration of the drug, five isolated urine samples were collected from two cross-bred castrated male horses. Intramuscular administrations of [4-^{14}C]testosterone phenylpropionate were then made to each of the two horses (horse A: 265 μg/kg, 29.4 μCi; horse B: 286 μg/kg, 21.7 μCi) at a single site in the neck. Each dose was dissolved in ethanol (0.3 ml) to which was added arachis oil (7 ml) containing benzyl alcohol (10%, v/v) and cresol (0.3%, v/v). Following administration, all urine samples from each animal were collected up to 240 h. Subsequently all voided urine was collected from 8 a.m. to 8 p.m. for a further ten days. The individual samples for each 12-h period were combined and labelled as days 11 to 21 for the purpose of analysis. Of each specimen two 0.1-ml aliquots were taken to determine the total radioactivity excreted. For days 11 to 21, when urine was collected only for a 12-h period, the total radioactivity excreted per day was calculated on the basis of the average daily urine volume excreted over the first ten-day period. Before administration of the drug, ten isolated urine samples were obtained from two thoroughbred castrated male horses. Intramuscular administrations of Androject (50 mg) were then made to the two animals (body weights 440 and 470 kg) at a single site in the neck. Following administration, two individually voided urine samples from each animal were collected daily over a period of three weeks.

Extraction and analysis of urine samples

Selected urine samples from day 1 to day 21 for each administration were analysed using a modification of the method described previously [19]. Solid-phase extraction of steroid conjugates from urine (10 ml) was carried out using a primed reversed phase (C_{18}-bonded cartridge) as described by Shackleton and Whitney [23]. The conjugates were then separated into glucuronic acid and sulpho-conjugate fractions using a modification of the Sephadex LH-20 column chromatographic method described previously [19]. The Sephadex LH-20 column (2.0 g; column diameter 1.0 cm) was slurry-packed in a solvent mixture of chloroform—methanol (17:3, v/v) made 0.01 *M* with respect to sodium chloride. After collection of the glucuronides in the first 30 ml of eluate, the solvent was changed to chloroform—methanol (1:1, v/v) and the sulpho-conjugates were collected in the next 50 ml of eluate.

Enzymic hydrolysis of the glucuronides, solvolysis of the sulphates and the

isolation of the neutral factions were carried out as described previously [18]. The neutral extracts were purified by TLC using chloroform–ethyl acetate (1:1, v/v) and the areas corresponding to 5α-androstane-3α,17β-diol as a marker [11] were isolated and eluted. The residues eluted from TLC were treated with methoxylamine hydrochloride in pyridine (2.5%, w/v; 50 μl) and the mixture was heated at 70°C for 30 min. After removal of the solvent at 40°C under a stream of nitrogen, the residues were dissolved in BSA (50 μl) and TMCS (25

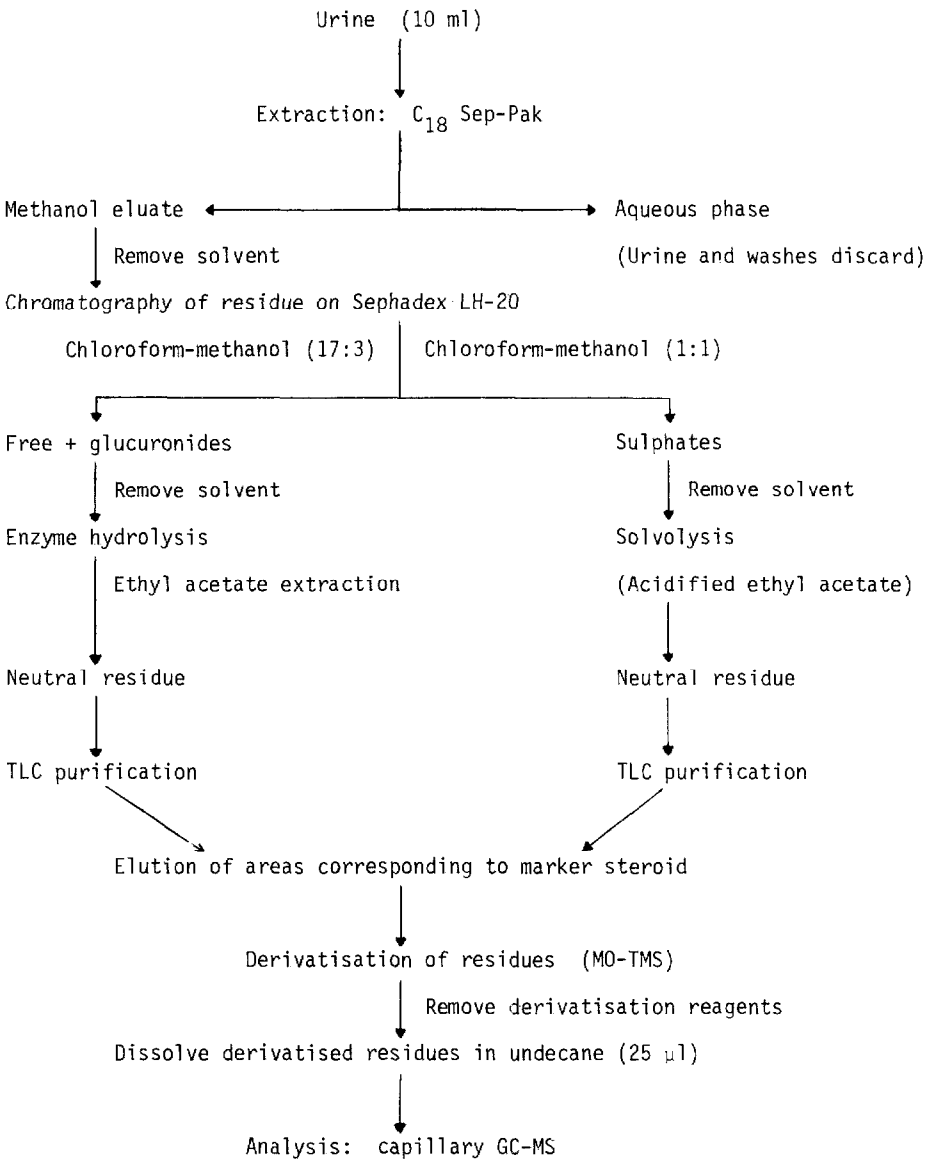

Fig. 1. Flow chart of the analytical procedures for the extraction, purification and identification of neutral analytes in horse urine after intramuscular administration of anabolic steroids.

μl) and heated at 60°C for 2 h. The bulk of the silylation reagents were removed in a stream of nitrogen at 40°C. The residual material was eluted through a short column of Sephadex LH-20 (2.0 cm × 0.5 cm) slurry-packed in a pasteur pipette using chloroform—hexane (1:1, v/v) as eluent. The steroid derivatives (methyloxime-trimethylsilyl, MO-TMS) were eluted in the first 2.5 ml of the solvent. The solvent was removed under nitrogen at 40°C and the derivatised residue dissolved in undecane (25 μl) for analysis by capillary column GC—MS. Pre-administration urine samples were similarly processed for GC—MS analysis. A flow diagram of the entire procedure is shown in Fig. 1.

Capillary column gas chromatography—mass spectrometry and selected-ion monitoring (GC—MS—SIM)

GC—MS was carried out using a Finnigan Series 4000 instrument interfaced to a Finnigan 6110 data system. A bonded-phase fused-silica capillary column (OV-1, 30 m × 0.32 mm I.D., 0.3 μm film thickness; Alltech Assoc., Carnforth, U.K.) was used. The column was passed through the GC- MS interface oven and terminated approx. 5 mm from the ion source. The transfer line temperature was 280°C and the source temperature was 220°C. Helium was used as a carrier gas (linear gas velocity 40 cm/s). On-column injections (sample volume 1—2 μl) were made at a column temperature of 205°C. After injection, the column oven was programmed up to 280°C at 5°C/min; the final temperature of 280°C 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. In addition to full-scan data, urine samples from the Androject administrations were also analysed by GC—MS—SIM using ions *m/z* 241, 256, 331 and 346 for the bis-TMS derivative of isomers of androstane-3,17-diol and ions *m/z* 153, 268, 358 and 389 for the MO-TMS derivative of testosterone.

RESULTS

Urinary excretion of radioactivity and GC—MS analysis of individual urine samples

The urinary excretion of total ^{14}C activity from the two radioactive drug administrations is shown in Fig. 2. Between 2 and 5% of the administered dose was excreted in the urine over a period of 24 h, 48—55% being recovered over a period of 500 h (21 days). Individual urine samples were analysed by the scheme shown in Fig. 1. After initial extraction, separation and deconjugation of the metabolites, the mixture of constituents in the neutral residues was partially resolved by TLC. The areas corresponding to the marker steroid 5α-androstane-3α,17β-diol for both the aglycone and the solvolysed sulphate fractions were eluted, derivatized and analysed by GC—MS.

The presence of 5α-androstane-3β,17α-diol bis-TMS as the major metabolite in the aglycone fractions was confirmed. This analyte could be detected for up to ten days in the urine after a single administration of [4-^{14}C] testosterone phenylpropionate. The full-scan mass spectrum of the metabolite obtained from a urine sample ten days after administration corresponded to that of a published spectrum [24]. The presence of 5α-androstane-3β,17β-diol bis-TMS and the MO-TMS derivative of testosterone was confirmed in the sulphate

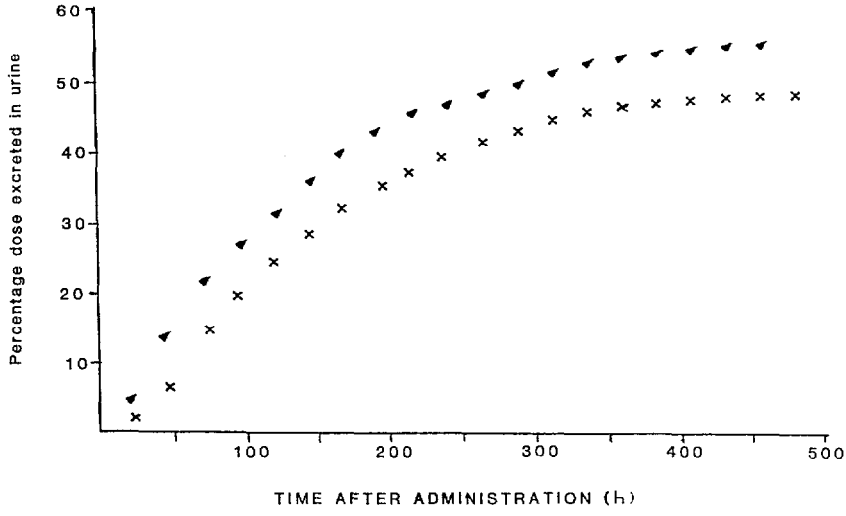

Fig. 2. Cumulative recovery of radioactivity in urine following intramuscular administrations of [4-^{14}C]testosterone phenylpropionate to two castrated cross-bred male horses.

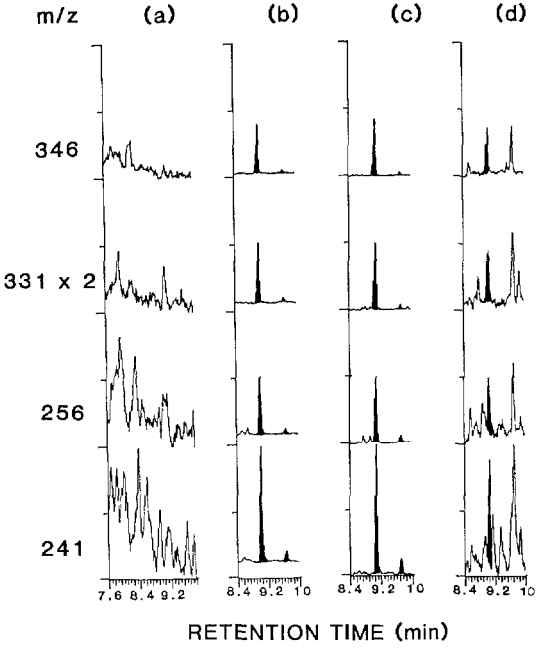

Fig. 3. Selected-ion monitoring chromatograms for the detection of 5α-androstane-3β,17α-diol bis(trimethylsilyl) ether (retention time = 9.05 min) in the aglycone fraction after administration of Androject (50 mg) to castrated thoroughbred male horses. (a) Pre-administration urine; (b—d) post-administration urine samples obtained on days 3, 7 and 10, respectively.

fraction. These two analytes could be detected for up to nineteen days after administration. The full-scan mass spectrum of testosterone MO-TMS derivative obtained from a urine sample nineteen days after administration was consistent

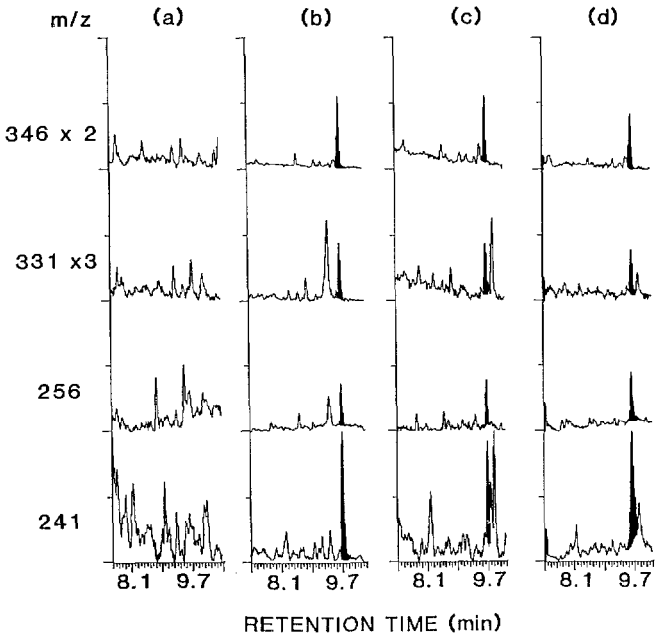

Fig. 4. Selected-ion monitoring chromatograms for the detection of 5α-androstane-3β,17β-diol bis(trimethylsilyl) ether (retention time = 9.7 min) in the sulpho-conjugate fraction after Androject administration. (a) Pre-administration urine; (b—d) post-administration urine samples obtained on days 7, 12 and 19, respectively.

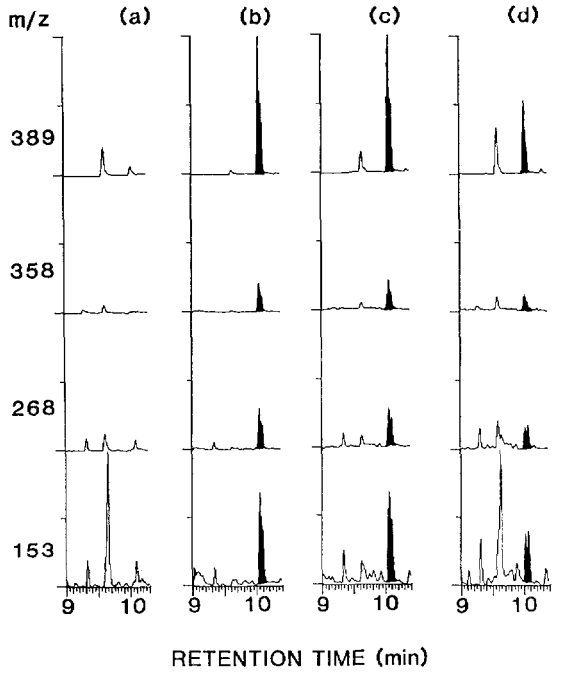

Fig. 5. Selected-ion monitoring chromatograms for the detection of testosterone methyloxime-trimethylsilyl ether (retention time = 10.1 min) in the sulpho-conjugate fraction after Androject administration. (a) Pre-administration urine; (b—d) post-administration urine samples obtained on days 7, 12 and 19, respectively.

with that of the published spectrum [24]. Absolute stereochemistry of these analytes was established by comparison of the retention times with those of authentic steroids [21].

GC—MS—SIM analysis of urine samples after Androject administration

In addition to the full-scan analysis, the SIM method of detection was applied to the determination of the three analytes after Androject administration. Using this technique 5α-androstane-3β,17α-diol was detected in the aglycone fractions for a period of between seven and ten days after a single intramuscular administration of 50 mg Androject (Fig. 3). Similarly 5α-androstane-3β,17β-diol and testosterone could be detected in the urinary sulpho-conjugate fractions for a period of between fourteen and nineteen days after administration (Figs. 4 and 5). None of these three analytes was detected in the pre-administration urine samples from all four animals.

DISCUSSION

The procedures for the isolation, purification and derivatisation of urinary steroids have been developed with the aim of obtaining rapid sample throughput while maintaining acceptable efficiency and recovery. In order to isolate the neutral steroids as analytes for detection of the administrations, a technique had to be developed based upon initial solid-phase extraction of the conjugates and their fractionation into glucuronides and sulphates by Sephadex LH-20 chromatography. This was done because previous studies have shown that direct mixed enzyme hydrolysis using Helix pomatia does not hydrolyse some steroid sulphates present in horse urine. The present method permits simultaneous hydrolysis of the conjugate groups and extraction of the analytes. After purification by TLC, the residues are derivatised rapidly to form the TMS/MO-TMS derivatives.

The results presented in this paper have demonstrated that administrations of testosterone preparations to equine castrates can be confirmed using three metabolites as analytes. Analysis of post-administration urine samples have shown that 5α-androstane-3β,17α-diol could be detected in the glucuronic acid fractions for up to ten days and testosterone and 5α-androstane-3β,17β-diol for up to nineteen days in the sulphate fractions. Minor amounts of other isomers of 5-androstane-3,17-diol were also detected in the post-administration samples. Determination of the stereochemistry of these isomers is at present under investigation. The three major analytes used in this study were not detected in the pre-administration urine samples of these animals, in routine analysis of numerous post-race urine samples from thoroughbred castrates and also during our studies on the metabolism of administered 19-nortestosterone [19, 25, 26] and testosterone [18, 21]. If these three neutral steroids occur endogenously, perhaps from the adrenocortical source [27], and are excreted in the urine of the castrated male horse, the normal threshold levels are too low to enable detection by the present methods. This method can be used to confirm the administration of testosterone preparations only to the equine male castrates as the three analytes have been identified as endogenous in the normal urine of the entire male horse and the mare. The failure to detect these analytes

in the urine of the untreated castrated male horse vindicates the use of this technique to confirm administrations of testosterone esters to this category of the thoroughbred racehorse.

The effect of esterification upon the urinary excretion rate is clearly evident from a comparison of the duration of excretion of the ester to that of the parent steroid. Following the intramuscular administration of [4-^{14}C]-testosterone [28], 25% of the radioactivity was excreted in the urine in the first 24 h as opposed to only 2.5% for the phenylpropionate ester using the same drug delivery system. The period of excretion of the radioactivity in the urine was twice as long after administration of the ester at half the equivalent dose than that of the unesterified parent steroid testosterone. The duration of action of long-acting hormone preparations is dependent upon the chemical structure of the ester group and alterations in the vehicle or in the route of administration [29, 30]. It has been shown that the rate of absorption from the site of injection rather than the cleavage of the ester moiety limits the flux of testosterone into the blood stream [31]. Following absorption, the esters are hydrolysed releasing the active anabolic agent. Our unpublished studies on the metabolism of testosterone and 19-nortestosterone esters in this laboratory have shown that qualitatively esterification does not affect the overall metabolic profile. GC—MS confirmatory analysis methods for anabolic steroids using multiple analytes can therefore be developed based upon initial metabolic studies using the unesterified steroids. However, problems of detectability of these steroids are enhanced after administration of long-acting anabolic esters due to the low levels of analytes found in body fluids.

This analytical method has now been extended to confirm the administration of 19-nortestosterone preparations to horses.

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