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Review

Separation of finasteride and analogues

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Abstract

This review is focused on the different chromatographic strategies for determination of finasteride and its analogues in biological fluids. These compounds are used for the treatment of benign prostatic hyperplasia. Particular attention is paid to high-performance liquid chromatography with spectrophotometric and mass spectrometric detection, the clean-up procedures are also included. The relationships between pharmacokinetics of finasteride, dose administered and required limit of quantitation of the chromatographic assays are discussed. Tandem mass spectrometry is recommended as the detection method for measuring concentrations <1 ng/ml, while cheaper spectrophotometric detection may be selected for determination of higher concentrations.

Keywords: Reviews; Finasteride; Epristeride; Turosteride; 4-Azasteroids

Contents

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1. Introduction

1.1. Therapeutic efficacy

Benign prostatic hyperplasia (BPH), the progressive enlargement of the prostate gland, is the most common cause of voiding dysfunction in men. Development of BIPH is an almost universal phenomenon in aging men, by the eighth decade more than 90% of men have prostatic hyperplasia at autopsy. Because the majority of men above age 60 have some degree of prostatic hyperplasia, the presence of the disorder by itself is not an indication for treatment. Many patients who receive no therapy experience no progression in symptoms over many years. Several forms of medical or surgical treatment exist for men with more advanced symptoms. Since the introduction of effective pharmacological alternatives to transurethral resection of the prostate, the surgery rate has been reduced, but surgical treatment of BPH is still the predominant therapy in terms of healthcare costs [1,2].

The development of new drugs with few adverse effects which modulate androgen activity has expanded the spectrum of medication in BPH treatment. Finasteride (**I**), a synthetic 4-azasteroid, is a potent and specific inhibitor of steroid 5α-reductase (5αR), an intracellular enzyme that converts testosterone to dihydrotestosterone (DHT). The use of finasteride for the treatment of symptomatic BPH is directed at the role of DHT in the etiology of the disease. The goal of therapy with finasteride is to reduce prostate volume, increase urinary flow-rate, improve symptoms and halt the progression of the disease [3].

Two isozymes of 5αR are known to exist, type 1 (5αR1) and type 2 (5αR2). Finasteride specifically inhibits the 5αR2 isozyme, its predominant intraprostatic form [4]. Inhibition of 5αR2 activity by finasteride results in significant reductions in prostatic (by up to 90%) and circulating (by up to 60–80%) DHT levels compared with baseline [3]. Much of the residual DHT could be due to production by the 5αR1 which is not effectively inhibited by finasteride. Inhibitors of 5αR1 in combination with inhibitors of 5αR2 could lower residual DHT further and thus might afford a more effective clinical treatment of BPH. Moreover, the localization of the 5αR1 in the sebaceous gland of skin [5] and the implication that DHT is one of the prerequisites for the onset of acne suggests that inhibition of this isoenzyme may be useful also in the management of this disorder. The 5αR1 inhibitors under development include (5α,7β,16β)-16[(4-chlorophenyl)-oxy]-4,7-dimethyl-4-aza-androstan-3-one (**II**, L-751 788) and (4,7β-dimethyl-4-azacholestan-3-one) (**III**, MK-0386).

The other finasteride analogues tested for the treatment of BPH include 17β-benzoyl-4-aza-5α-androst-1-ene-3-one (**IV**, MK-434), 5α-23-methyl-4-aza-21-nor-chol-1-ene-3,20-dione (**V**, L-654,066), epristeride (**VI**) and turosteride (**VII**). All these compounds are 4-azasteroids with the exception of epristeride.

Finasteride and its analogues are also used for the treatment of androgenetic alopecia (male pattern hair loss), because DHT is the androgen responsible for male pattern hair loss in genetically predisposed men [6].

1.2. Chemical properties

The structures and names of finasteride and its analogues reviewed in this paper are shown in Fig. 1. In this review the compounds bearing generic names (finasteride, epristeride and turosteride) are designated by these names while for the other compounds Roman numerals listed in Fig. 1 are used. Formerly known as MK-906, finasteride (Chemical Abstract registry number 098319-26-7) belongs to the 4-azasteroid structural class of compounds. Its synthesis was published by Rasmusson et al. in 1986 [7]. The molecular formula of finasteride is $C_{23}H_{36}N_2O_2$, relative molecular mass 372.56. Finasteride is freely soluble in chloroform, dimethylsulfoxide, ethanol, methanol, *n*-propanol and very slightly soluble in HCl (0.1 mol/l), NaOH (0.1 mol/l) or water (solubility 11.68 mg/l at 25°C). The hydrophobicity of finasteride is further documented by its log *P* (octanol–water) value 3.03 [8,9]. The p*K* value of the carboxy group in epristeride is 4.8 [8].

2. Pharmacokinetics and metabolism

Finasteride is administered orally in the treatment of either BPH or male pattern baldness. In man, a single 5-mg oral dose produces a rapid reduction in

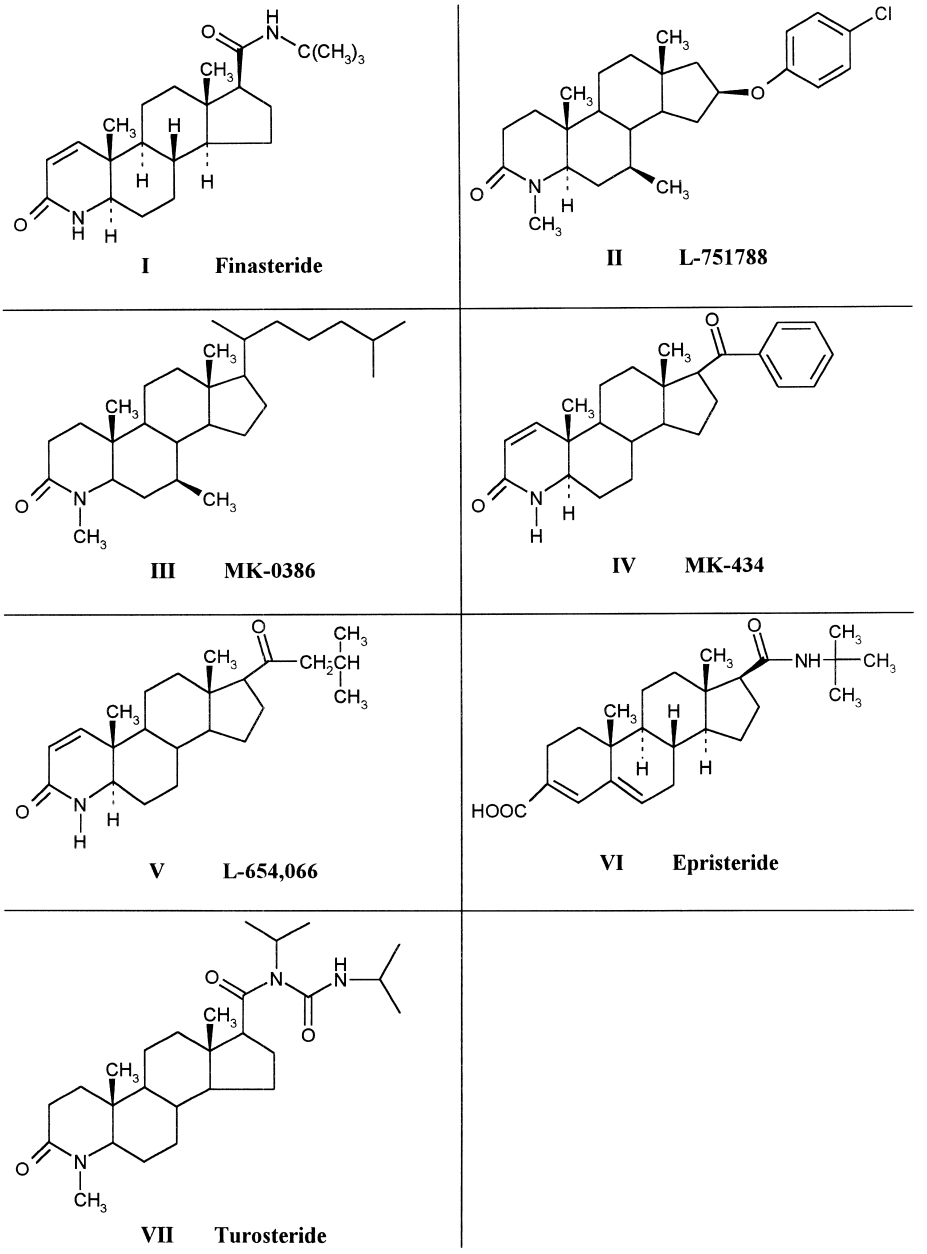

Fig. 1. Chemical structures of finasteride and its analogues.

serum DHT concentrations by as much as 70%, with the maximum effect observed 8 h after the first dose [10]. The effect lasts for at least 24 h, so once daily dosing is appropriate. The mean bioavailability following a single dose of finasteride is about 63–80% [3]. Approximately 90% is bound to plasma proteins; yet the drug has been found to cross the blood–brain barrier. Following an oral dose of finasteride in man, a mean of 39% of the dose was excreted in the urine in the form of metabolites, 57% was excreted in the feces. The major compound isolated from urine was the monocarboxylic acid metabolite. The *tert*.-butyl

side chain monohydroxylated metabolite has been isolated from plasma. The mean plasma elimination half-life of finasteride is 4.7–7.1 h [3], the elimination rate is decreased in the elderly: the terminal half-life was approximately 8 h in subjects >70 years [10].

Maximum finasteride plasma concentration after 5-mg oral dose averaged 37–41 ng/ml. Daily dosing causes accumulation to occur, with maximum plasma concentrations increasing by 50% over those observed from a single dose. Mean trough concentrations after 17 days of dosing were 6–8 ng/ml [3,10]. Finasteride 5 mg/day is used in the treatment of BPH while lower doses 1 mg/day have been shown to be effective in men with male pattern hair loss.

The pharmacokinetic properties dictate the requirements for analytical methods. For therapeutic drug monitoring or for assessment of pharmacokinetics in the steady-state after oral doses ≥5 mg the limit of quantitation (LOQ) >5 ng/ml should be achieved. The samples from pharmacokinetic studies after single oral dose of ≥5 mg finasteride should be analysed with a method having LOQ in the range 1–5 ng/ml. More sensitive assays (LOQ 0.2–1 ng/ml) are required to completely map the plasma concentration time courses following oral administration of finasteride at 0.2- and 1-mg doses.

3. Extraction from biological matrices

It is not possible in the techniques described here to use direct injection of the plasma or serum sample, mainly because this diminishes the lifetime of chromatographic columns and for the analysis in the ng/ml range the samples must be preconcentrated. Consequently some form of extraction process is necessary. Finasteride and its analogues are lipophilic compounds and can be extracted into an organic solvent from aqueous solutions. This approach has been chosen in several methods [11–15]. The extraction efficiency for 4-azasteroids is not pH-dependent: compound **III** was extracted from plasma both at pH 2.8 and at physiological pH [14]. However, the low pH was needed to efficiently extract the carboxylic metabolite of **III**. In other procedures finasteride was extracted at physiological pH [11,13], and finasteride, **II** and turosteride at alkaline pH [12,13,15]. The sample was basified in the latter assays to reduce the amount of compounds extracted from the matrix, not to increase the recovery of the drugs. The recovery of turosteride was almost quantitative under acidic, neutral or basic conditions [15]. Systematic extraction studies were not performed, among solvents employed for extraction methyl *tert.*-butyl ether [12–14], diethyl ether [15], hexane after protein precipitation with acetonitrile [14] and hexane–isoamylalcohol (98:2, v/v) [11] were used.

An alternative to liquid–liquid extraction of 4-azasteroids is solid-phase extraction. A variety of extraction columns packed with C_{18} [16–20] and CN [15,19,21,22] bonded silica and with graphitized carbon black [23] were used.

Guarna et al. [16] washed the C_{18} SPE columns after application of plasma with finasteride with hexane and hexane–ethyl ether to selectively remove testosterone and DHT from the sample. Finasteride was then eluted with ethyl acetate, mean recovery was 90%. The method could be applied to the simultaneous determination of the drug and the two hormones by GC–MS [16]. Washing the C_{18} columns with hexane was employed also for clean-up of plasma containing epristeride; this steroid analogue was then eluted with a mixture of hexane–dichloromethane-isopropanol–acetic acid, mean recovery was also 90% [20]. Similarly, in the analytical procedure for determination of turosteride a CN cartridge was washed with hexane–cyclohexane mixture and the drug was eluted with acetone. However, this extraction procedure was complicated, because it was preceded by liquid–liquid extraction and followed by washing the residue after SPE with hexane [15].

In other SPE procedures **IV** and **V** were eluted from the C_{18} cartridges directly with methanol after washing the columns with water [17,18], the recovery of extraction was high. In an older assay a combination of C_{18} and CN cartridges was required to achieve a limit of quantitation 10 ng/ml for finasteride using spectrophotometric detection, the drug was eluted from the CN column with dichloromethane [19]. This procedure was simplified in more recent papers [21,22]. The use of a single CN cartridge washed with a 10% acetone–water mixture

effectively removed major plasma endogenous interferences, finasteride was eluted from the CN cartridge with methanol. The limit of quantitation was reduced from 25 to 1 ng/ml [22].

Carlucci and Mazzeo claimed that graphitized carbon black cartridges provided purer extracts than those obtained with other methods [23]. In their method finasteride was eluted from the cartridges with a mixture of chloroform–methanol (20:80, v/v), recovery was nearly quantitative, but the limit of quantitation was not superior to the other methods (5 ng/ml).

An alternative to off-line solid-phase extraction is a column-switching technique described by Takano and Hata [24]. Diluted plasma samples are injected on a small CN column, finasteride is retained while proteins and polar compounds are eluted to waste. Then finasteride is back-flushed to the C_{18} analytical column and separated from remaining interferences. Only 75 μl of plasma is needed to achieve limit of quantitation 1 ng/ml. Although this system is fully automated it is very complex: four pumps and four columns are necessary, two additional columns are required to eliminate impurities in the mobile phases.

4. Separation methods

In the literature an approximately equal number of papers is devoted to liquid chromatography (LC) with spectrophotometric detection [11,15,19,20,22–24] and to LC with tandem mass spectrometry [12–14,17,18,21]. The former methods are cheaper, the detector response is stable and little maintenance of the detector is required. However, more time should be devoted to method development, especially extraction of azasteroids from biological fluids and separation from interfering compounds. Owing to spectral properties of finasteride and its analogues, LC methods with spectrophotometric detection cannot measure concentrations <1 ng/ml in plasma. The equipment for LC–MS–MS methods is expensive, but the methods are fast, specific and no alternative is available for determination of subnanogram concentrations in biological fluids. A paper describing a GC–MS method was also published [16]. The individual methods are discussed in more details below and are summarized in Table 1.

4.1. Liquid chromatography

4.1.1. Separation conditions

The majority of separations are carried out with bonded-phase columns in the reversed-phase mode. The stationary phases include C_{18} [11–13,15,17,18,21], C_8 [14,19,22] and C_4 [24] bonded silica.

Short (3–5 cm) reversed-phase columns (mainly C_{18}) are commonly utilized in LC–MS–MS methods which result in chromatographic run times of 2–6 min. The eluent choice is severely restricted compared to the options available with spectrophotometric detection. The organic modifier is either methanol or acetonitrile, and volatile buffers must be used to sustain high throughput analysis without source fouling [25]. Most often the volatile buffers consist of ammonium acetate or ammonium formate and trifluoroacetic or formic acid.

The methods with spectrophotometric detection employ longer (15–25 cm) columns and consequently the run times are much longer (15–60 min). These methods were not optimized with respect to the analysis time. The typical chromatograms are clean after extraction of the drugs from biological fluids and much shorter columns could have been used to shorten the analyses.

In an LC–MS–MS method for determination of **II** the column was heated to 60°C to reduce the retention time of analytes and to improve peak shape [12]. This approach was preferred to decreasing retention time by increasing acetonitrile content in the mobile phase from 80 to 95%, because the efficiency of ionization was lower when a mobile phase with high organic content was utilized. Similar chromatographic conditions were selected for determination of finasteride by LC–MS–MS: temperature 70°C and 70% acetonitrile in the mobile phase [21] or temperature 60°C and 90% acetonitrile [13]; also the separation of **III** was conducted at higher temperature (60°C and 75–90% acetonitrile) [14]. The other groups performed separations at ambient or only slightly elevated temperatures. It follows that chromatography of azasteroids at high temperatures is not a prerequisite for obtaining good peak shape, but represents an additional mean for controlling retention and selectivity of the separation.

In addition to reversed-phase separations of 4-azasteroids, normal-phase separation of epristeride

Table 1
Chromatographic methods for determination of finasteride and its analogues in plasma

Refs.	Compound	Technique	I.S.	Sample preparation	Detection	Column	Plasma volume (ml)	LOQ (ng/ml)	Run time (min)
[11]	Finasteride	LC	Clobazam	L–L extraction with hexane–isoamylalcohol (98:2)	UV 210 nm	C_{18} (150×3.2 mm)	1	4	25
[13]	Finasteride	LC–MS–MS	Analogue	L–L extraction with methyl-*tert.*-butyl ether	MS–MS (*m*/*z* 373→317)	C_{18} (50×2 mm or 30×4.6 mm)	1	0.025	2
[16]	Finasteride	GC–MS	D_3-Finasteride	SPE on C_{18}	MS	Non-polar (12 m×0.2 mm)	1	0.25	35
[19]	Finasteride	LC	4-*N*-Methyl Analogue	SPE on C_{18} and CN	210 nm	C_8 (250×4.5 mm)	1	10	21
[21]	Finasteride	LC–MS–MS	Analogue	SPE on a CN cartridge	MS–MS (*m*/*z* 373→317)	C_{18} (33×4.6 mm)	1	0.2	3
[22]	Finasteride	LC	4-*N*-Methyl Analogue	SPE on a CN cartridge	210 nm	C_8 (150×4.6 mm) +C_8 (50×4.6 mm)	1	1	23
[23]	Finasteride	LC	4-Androstene-3,17-dione	SPE on graphitized carbon black	215 nm	Reversed-phase (250×4.6 mm)	1	10	20
[24]	Finasteride	LC	No I.S.	column switching	210 nm	C_4(250×4.6 mm)	0.075	1	38
[12]	**II**	LC–MS–MS	Finasteride	L–L extraction with methyl-*tert.*-butyl ether	MS–MS (*m*/*z* 430→114)	C_{18} (50×4.6 mm)	1	0.5	5
[14]	**III** and metabolite	LC–MS–MS	Analogue	L–L extraction with methyl-*tert.*-butyl ether or hexane	MS–MS (*m*/*z* 416→114)	C_8 (50×4.6 mm) or (150×4.6 mm)	1	0.2	6
[17]	**IV**	LC–MS–MS	Analogue	SPE on C_{18}	MS–MS (*m*/*z* 378→310)	C_{18} (50×4.6 mm)	1	0.5	5
[18]	**V**	LC–MS–MS	Analogue	SPE on C_{18}	MS–MS (*m*/*z* 358→290)	C_{18} (50×4.6 mm)	1	0.5	2
[20]	Epristeride	LC	Analogue	SPE on C_{18}	274 nm	Aminopropyl silica (220×2.1 mm)	1	2.5	16
[15]	Turosteride	LC	No I.S.	SPE on a CN cartridge	210 nm	C_{18} (250×4 mm)	1	5	60

on an aminopropylsilica column has also been published [20]. Use of four-component mobile phase (hexane–methylene chloride–2-propanol–acetic acid) was necessary to resolve the analyte and internal standard peaks from the endogenous plasma peaks and to obtain sharper peaks.

4.1.2. Spectrophotometric detection

The spectrophotometric detector is the only one that has been used for the detection of finasteride and its analogues. Finasteride is not easily oxidized at the low potential necessary for electrochemical detection. Also, it is practically non-fluorescent [22]. The straightforward derivatization of finasteride is not feasible, due to the lack of a functionality in its molecule which could be easily exploited for this purpose.

The lack of a useful chromophore or derivatizable functional group makes finasteride a difficult compound to assay with optimum sensitivity and specificity. The ultraviolet (UV) spectrum of finasteride in methanol indicated the presence of a strong absorption band with the maximum at 204 nm (the molar absorptivity ε=15 900 M^{-1} cm^{-1}) and a shoulder between 220 and 270 nm (ε=2400–600) [22]. Similar spectral properties were described for **III** and turosteride. The absorption of finasteride at longer wavelengths is not of sufficient intensity for sensitive detection. Therefore, the detection wavelength of 210 nm (ε=14 700 M^{-1} cm^{-1}) was chosen in the majority of published assays [11,19,22,24]. A typical separation of finasteride from internal standard and endogenous interferences monitored at 210 nm is shown in Fig. 2. In one method column eluate was monitored at 215 nm, but the LOQ of the method was high (10 ng/ml) [23].

The presence of chlorophenoxy group in **II** enhanced the absorption of **II** in comparison with other azasteroids. The UV absorbance spectra of **II** showed two absorption bands with the maxima at 204 and

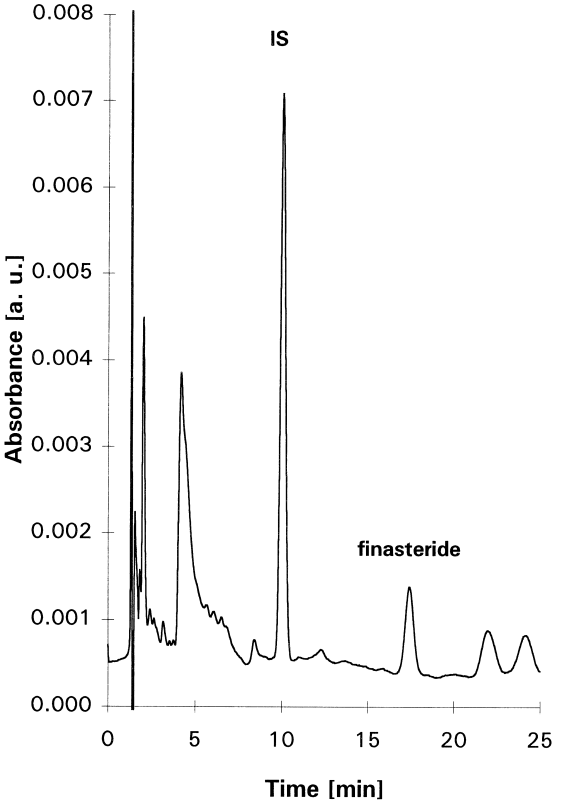

Fig. 2. Chromatogram of a plasma sample from a volunteer 11 h after administration of 10 mg of finasteride, spectrophotometric detection at 210 nm. The respective concentration was 19.7 ng/ml. Reproduced from Ref. [11].

230 nm and ε of 18 600 and 14 700 M^{-1} cm^{-1}, respectively [12]. Nevertheless, the authors have chosen LC with tandem mass spectrometry as the preferred technique due to simple sample preparation procedure and short analysis time.

The spectral properties of epristeride which is not an azasteroid like the other compounds are different. The high value of ε (>20 000 M^{-1} cm^{-1} at 274 nm) makes the compounds more suitable for analysis by LC with spectrophotometric detection [20].

4.1.3. Mass spectrometric detection

The combination of LC and mass spectrometry (MS) has been used for many years in bioanalytical chemistry. In recent years the number of scientific contributions devoted to LC with atmospheric pressure ionization (API) and tandem mass spectrometric (MS–MS) detection showed huge increase [13]. This technique is a method of choice for determination of finasteride and its analogues in biological fluids at subnanogram/ml concentrations [12–14,17,18,21]. A representative LC–MS–MS chromatogram of finasteride at concentration 0.05 ng/ml in plasma is shown in Fig. 3.

The precursor→product ion combinations for individual compounds are noted in Table 1. Although the blank plasma samples monitored at these ion combinations do not show any interfering peaks, the results may be adversely affected by lack of specificity and selectivity due to ion suppression caused by the sample matrix [13]. The undetected but co-

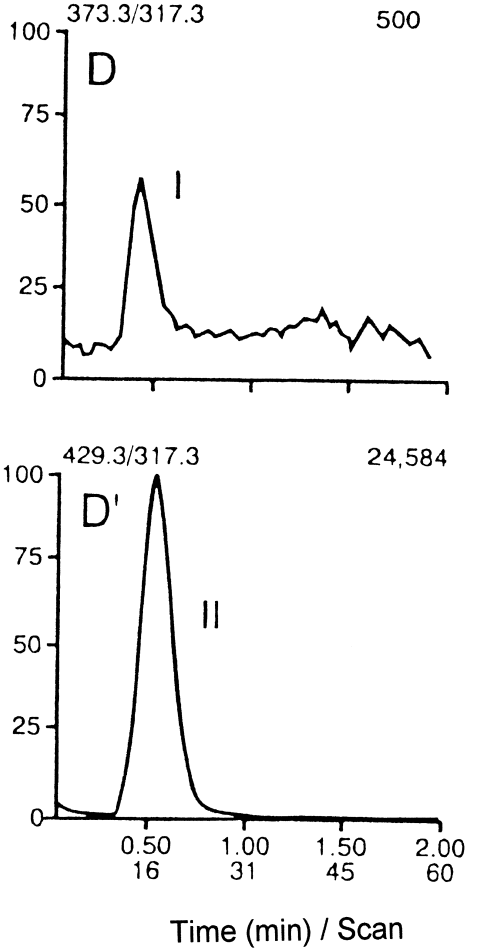

Fig. 3. Representative LC–MS–MS chromatograms of plasma extracts obtained by multiple reaction monitoring at m/z 373.3→317.3 for finasteride (upper panel, plasma spiked with 0.05 ng/ml) and m/z 429.3→317.3 for internal standard (lower panel, plasma spiked with 10 ng/ml). Reproduced from Ref. [13].

eluting endogenous impurities may affect the ionization efficiencies of the analytes. The assessment of matrix effects and assay reliability is critical when analogues rather than stable isotope-labeled parent compounds are selected for use as internal standard [12] as is the case in all methods discussed here.

The matrix effects in the method for determination of finasteride in human plasma were studied by Matuszewski et al. [13]. The assay had originally poor precision and accuracy and slope of the standard line for one set of plasma samples was substantially different from that from other plasma sources. This variability was not attributable to the differences in extraction recovery of finasteride and internal standard but it was due to significant differences in MS–MS response explained by ion suppression of the analytes in the presence of a variety of undetected matrix components coeluting with the peaks of interest. These undesired effects were eliminated by increasing capacity factor of finasteride and by using more selective extraction.

Another obstacle in the LC–MS–MS analysis of azasteroids in biological fluids may be the interference with metabolites. The chromatographic conditions (short column, fast analyses) do not necessarily lead to separation of metabolites from parent compounds. The commonly formed metabolites coeluting with parent compounds have the potential of giving fragment ions in mass spectra which may be characterized by the same m/z ratio as the protonated molecular ions of either parent compound or an internal standard. Further fragmentation of these ions originating from metabolites leads to the formation of product ions which are the same as product ions of a parent compound or an internal standard utilized for quantitation. This problem was demonstrated by Constanzer et al. [14]: the unknown metabolite interfered with the carboxylic acid metabolite of **III** and chromatographic conditions had to be modified to increase the retention and separate the interference.

4.1.4. Quantitation

The relatively complex sample preparation requires use of an internal standard method to compensate for sample losses occurring during extraction, clean-up and final chromatographic analysis and for signal changes caused by variation in experimental conditions. An ideal internal standard should resemble the analyte as closely as possible in terms of chemical and physical properties and this requirement is fulfilled when a structural analogue is selected as an internal standard [13,14,17–22]. The problem with this approach is the commercial unavailability of these analogues which precludes their use by other groups. Finasteride in determination of **II** [12], 4-androstene-3,17-dione [23] and clobazam [11] are listed among commercially available internal standards.

4.2. Gas chromatography

Several groups reported unsuccessful attempts to analyse finasteride and its analogues by gas chromatography (GC). Takano and Hata [24] observed adsorption of finasteride on fused-silica capillary columns caused by two amide groups in the molecule. The nitrogen at the 4 position could be derivatized, but no reaction with the other amide took place because of the steric hindrance caused by *tert*.-butyl group. Low volatility of **IV** and **V** precluded their analysis by GC–MS and *N*-trimethylsilyl and *N*-trifluoroacyl derivatives of these compounds were unstable [17,18]. Thermal instability of turosteride inhibited the use of GC as reported by Basileo et al. [15].

Constanzer et al. [14] mention a GC with negative ion chemical ionization mass spectrometry for determination of **III** and its metabolite but the method required double derivatization with heptafluorobutyric anhydride and diazomethane and precision and accuracy of the method was not fully assessed.

Nevertheless, a validated method for the determination of finasteride in plasma by GC–MS was presented by Guarna et al. [16]. Samples were injected into the non-polar capillary column without any derivatization, the limit of detection was 50 pg which corresponds to 250 pg/ml in plasma. The good values of accuracy and precision were enabled by using trideuterated finasteride as an internal standard. This internal standard is not commercially available; it was prepared from 3-oxo-4-aza-5-androstene-17β-carboxylic acid in a four-step procedure and this may represent a limitation in a transfer of the method to other laboratories.

5. Conclusions

Drug level monitoring of steroid 5α-reductase inhibitors is helpful to obtain quantitative information about the absorption, distribution and elimination of these drugs, and the effect of disease states on these processes, as well as information concerning the correlation of efficacy with their measured concentrations and their active metabolites in plasma and other biological material.

The purpose of this review is to present a summary of analytical techniques suitable for the determination of these drugs. With one exception all the analytical methods use liquid chromatography and again in almost all but one paper reversed-phase stationary phases are employed. The methods with spectrophotometric detection are cheaper, but the analysis times are longer and the limits of detection are higher than 1 ng/ml. The use of more selective detection (MS–MS) enabled to lower the limit of quantitation to subnanogram/ml values and to increase the speed of analyses. If the analyst if aware of possible problems caused by matrix effects and/or interferences with metabolites and the higher instrument cost can be accepted then the LC–MS–MS represents a technique of choice for the determination of finasteride and its analogues, especially at low concentrations.

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