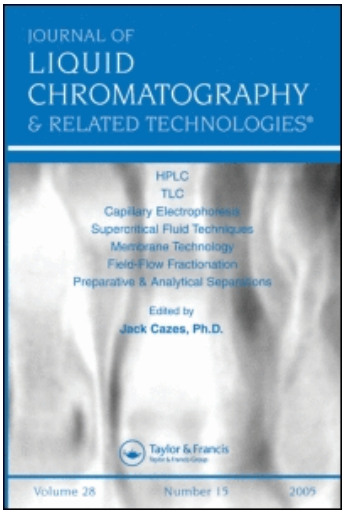

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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I. Cendrowska[a]; B. Buszewski[b]
[a] Pharmaceutical Research Institute, Warsaw, Poland [b] Department of Environmental Chemistry and Ecoanalytics, Faculty of Chemistry, Nicholas Copernicus University, Toruń, Poland

Online publication date: 13 January 2005

To cite this Article Cendrowska, I. and Buszewski, B.(1999) 'DETERMINATION OF FINASTERIDE AND RELATED COMPOUNDS BY REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY. I. CHOOSING THE MOBILE PHASE COMPOSITION', Journal of Liquid Chromatography & Related Technologies, 22: 15, 2259 — 2270
To link to this Article: DOI: 10.1081/JLC-100101799
URL: http://dx.doi.org/10.1081/JLC-100101799

J. LIQ. CHROM. & REL. TECHNOL., 22(15), 2259–2270 (1999)

DETERMINATION OF FINASTERIDE AND RELATED COMPOUNDS BY REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY. I. CHOOSING THE MOBILE PHASE COMPOSITION

I. Cendrowska,[1] B. Buszewski[2,]*

[1] Pharmaceutical Research Institute
8 Rydygiera Str.
PL-01 793 Warsaw, Poland

[2] Department of Environmental Chemistry and Ecoanalytics
Faculty of Chemistry
Nicholas Copernicus University
7 Gagarin Str.
PL-87 100 Toruń, Poland

ABSTRACT

High performance liquid chromatography in reversed-phase system was used to control the synthesis of *Finasteride* {17β (N-tert-carbamoyl)-4-aza-5α-androst-1-ene-3-one} - the drug used in the therapy of prostatic carcinoma. The objective of these investigations was the finding of such composition of mobile phase which would separate the semi-products obtained in individual stages of synthesis from final products and UV detection at 210 nm. For this purpose we adopted the method of triangle optimisation.

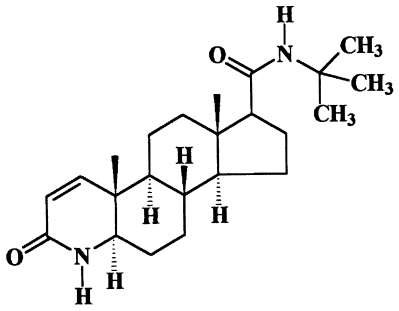

Figure 1. Chemical structure of Finasteride.

INTRODUCTION

Finasteride i.*e*., 17β- (N-tert-butyl-carbamoyl)-4-aza-5α-androst-1-ene-3-one is the drug known under the following names: Proscar (Merck Sharp and Dohme), Andozac, Chibro-Proscar, Finaspros, Procure, Prostide, YM-152.[1] (Figure 1). This compound deactivates selectively 5α-reductase - the enzyme taking part in reduction of testosterone to less hormonally active dihydrotestosterone.

It reduces the content of dihydrotestosterone in blood and prostate. It is intended for therapy of mild prostate overgrowth. It reduces the prostate overgrowth and alleviates the disease symptoms.[2]

In the literature numerous methods are described for the synthesis of *Finasteride*.[1,3-8] In Pharmaceutical Research Institute (Warsaw, Poland) efforts were made for laboratory method of preparation of this drug.

Choosing the synthesis pathway, we have taken into account the economy and degree of difficulty of the process for the environment. Moreover, we have taken into account such factors as: limitation of number of synthesis steps, commercial availability of reagents, costs of starting materials, properties of used reagents and possibilities of waste utilisation.

We have chosen the adaptation of six-steps synthesis proposed earlier by Prous.[1] The scheme of this synthesis is presented in Figure 2.

During the synthesis, performed according to the scheme presented in Figure 2, there are formed (in fifth and sixth steps) two additional and undesirable compounds (i. e., β - stereoisomer of the compound #5 and β - stereoisomer of the compound #6).

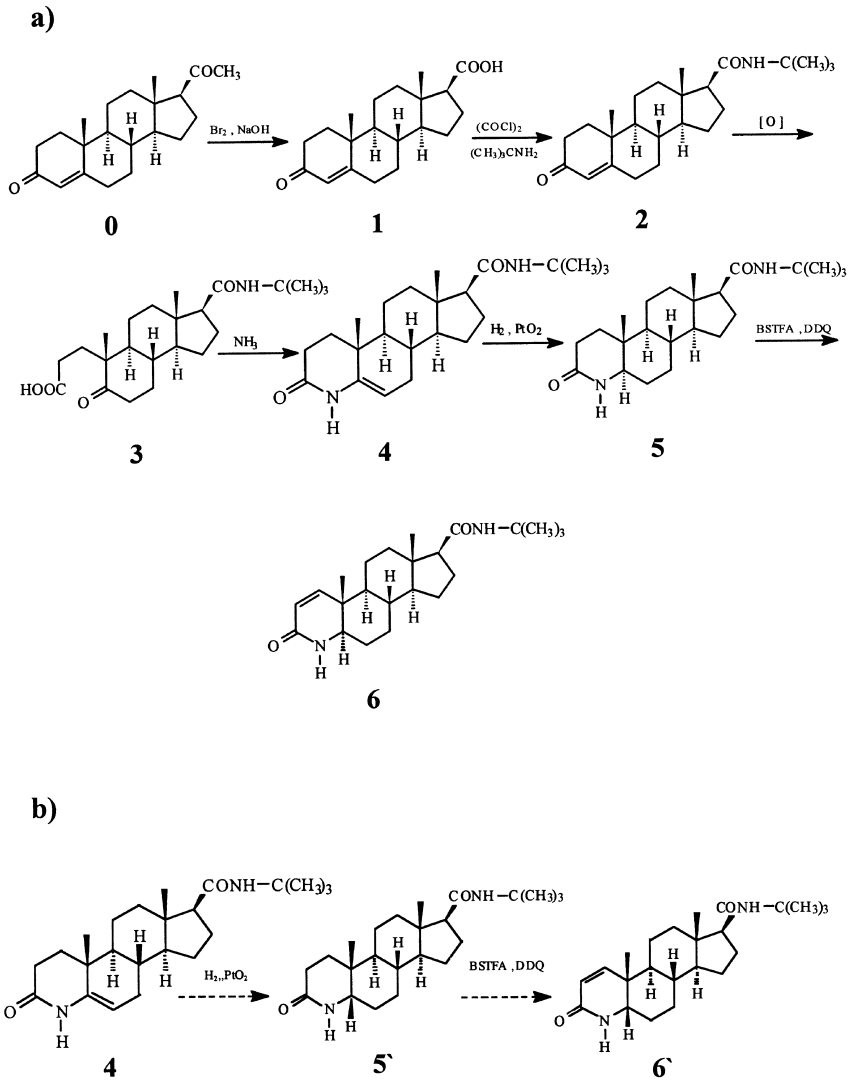

Figure 2. Scheme of Finasteride synthesis, where: a) 0 = progesterone; 1 = 3-oxoandrost-4-ene-17β-carboxylic acid; 2 = N-t-butyl-3-oxoandrost-4-ene-17β carboxylic acid amide; 3 = 17β (t-butylcarbamoyl)-5-oxo-4-nor, 3, 5-seco-androstano-2-carboxylic acid; 4 = N-t-butyl-oxo-4-aza-androst-5-ene-17β carboxylic acid amide; 5 = N-t-butyl-3-oxo-4-aza-5α-androst-17β carboxylic acid amide; 6 = 17β-(N-t-butylcarbamoyl)-4-aza-5α-androst-1-ene-3-one; b) 4 = N-t-butyl-oxo-4-aza-androst-5-ene-17β carboxylic acid amide; 5' = N-t-butyl-3-oxo-4-aza-5β-androstan-17β carboxylic acid amide; 6' = 17β-(N-t-butylcarbamoyl)-4-aza-5β-androst-1-ene-3-one.

Control of the individual synthesis steps was determined initially by thin layer chromatography (TLC) using E. Merck TLC plates, GF 254, 0.25 mm.[9] Ternary benzene-methanol-acetic acid mixture (90:5:5, v/v) was used as the mobile phase. However, using this technique did not give good results, because it has failed to separate those compounds, which form additionally during fifth and sixth steps. In further investigations, we have concentrated our attention on application of high performance liquid chromatography (HPLC), which is characterised by higher selectivity and performance than TLC. Moreover, HPLC technique in reversed-phase mode (RP) appears to fulfil all the requirements necessary for the separation of the compounds characterised by specific and complex structures containing different functional groups.[10]

Our investigations described in this paper were concentrated mainly on the selection of optimal composition of hydro-organic mobile phase. For this purpose we used the solvophobic model, in which the interactions between individual solutes and stationary phase are neglected. In consequence we have assumed, that the main factor determining the retention as well as the selectivity of separation process is the type and composition of mobile phase. Thus the exchange of the organic solvent causes the change in retention and resolution of the substances of differentiated structure and chemical nature. Because the use of binary hydroorganic systems, even with modification of pH values has not given satisfactory results, it was necessary to use a multicomponent system for separation of the individual compounds formed during synthesis of *Finasteride*. For this reason the use of commercial computer programs (DryLab® and ChemLab®)[11,12] has appeared less effective and less useful. In this connection the optimisation of mobile phase composition was made on the basis of optimisation triangle mode.[13,14] This paper is dedicated just to such optimisation for the utilisation in routine laboratory practice.

EXPERIMENTAL

Chemicals and Reagents

The following solvents (all HPLC grade) were selected as components of the mobile phase: acetonitrile, tetrahydrofuran (S. Witko-J. T. Baker, Łódz, Poland). Water was purified by means of Milli Q-RO apparatus (Milipore, El Passo, TX, USA) and trifluoroacetic acid (Fluka, Buchs, Switzerland). The following semi-products and final product of Finasteride synthesis were used as test compounds: progesterone (compound #0), 3-oxo-androst-4-ene-17β-carboxylic acid (compound #1), N-t-butyl-3-oxoandrost-4-ene-17β carboxylic acid amide (compound #2), 17β-(t-butylcarbamoyl)-5-oxo-4-nor 3,5-seco-androstano-2-carboxylic acid (compound #3), N-t-butyl-3-oxo-4-aza-androst-5-ene-17β carboxylic acid amide (compound #4), N-t-butyl-3-oxo-4-aza-5α-androst-17β carboxylic acid amide (compound #5), N-t-butyl-3-oxo-4-aza-5β-

androstan-17β carboxylic acid amide (compound #5'), 17β-(N-t-butylcarbamoyl)-4-aza-5α-androst-1-ene-3-one (compound #6), 17β-(N-t-butylcarbamoyl)-4-aza-5β-androst-1-ene-3-one (compound #6').

These compounds were synthesised in the Pharmacy Institute (Warsaw, Poland). Identity of individual compounds was confirmed by IR, ^{1}H NMR and UV-Vis spectroscopy.[15]

Chromatographic Conditions

The HPLC system consisted of model LC- 6 A pump, model SPD-6 AV UV-Vis detector operating at 210 nm, model CR-6A integrator (Shimadzu Europa, Duisburg, Germany) and a Rheodyne Model 7125 injection valve (Berkeley, CA, USA) equipped with 20 μL loop. A Supelcosil LC-18 DB column (250 x 4.6 mm ID) (Supelco Inc., Bellofonte, PA, USA) was used in all chromatographic investigations. Flow rate of the mobile phase was 1 mL/min., at room temperature.

Optimisation of Mobile Phase Composition

In order to optimise the composition of the mobile phase we have used the optimisation triangle[13,14] and selected the solvents (methanol, acetonitrile, tetrahydrofuran) localised near three triangle vertices, in order to obtain the largest differences in selectivity. The composition of mobile phase was selected in such a way to obtain, in each case, the similar range of k' coefficients for all components of the tested mixtures.

In the initial stage of the experiments, seven solvent mixtures were used, then - four successive separations with modification of mobile phase pH value. In order to establish the composition of mobile phase we have made the calculations based on the following equations:

Solution:

$$P_{mixt.} = P_1 \varphi_1 + P_2 \varphi_2 \quad (1)$$

$$P_{mixt.} = P'_{MeOH} \times \varphi_{MeOH} + (P'_{water} \times \varphi_{water}; \text{ this component is equal to zero}) \quad (2)$$

Acetonitrile (ACN) - water:

$$\varphi_{ACN} = P'_{mixt.} / P'_{ACN} \quad (3)$$

Table 1

Mobile Phases Used in the Separation of Finasteride, and Semi-Products of Its Synthesis and Potential Impurities

Mobile Phase	Preparation of Mobile Phases	Calculations	Composition of Mobile Phases
1	MeOH-H_2O (70:30, v/v)	P' =2.6x0.7+0x0.3=1.82	MeOH-H_2O (70:30, v/v)
2	ACN-H_2O	φ=1.82/3.2=0.57=57%	ACN-H_2O (57:43 v/v)
3	THF-H_2O	$\varphi_{THF} = \frac{1{,}82}{4.50} = 0.40 = 40\%$	THF=H_2O (40:60, v/v)
4	**1** + **2** (1:1, v/v)	H_2O(0.5x30)+(0.5x43)=36.5 parts MeOH: 0.5x70=35 parts ACN: 0.5x57=28.5 parts	MeOH-ACN-H_2O (35:28.5:36.5, v/v/v)
5	**2** + **3** (1:1, v/v)	H_2O: (0.5x43)+(0.5x60)=51.5 parts CAN: 0.5x57=28.5 parts THF: 0.4x40=20 parts	ACN-THF-H_2O (28.5:2:51.5, v/v/v)
6	**1** + **3** (1:1, v/v)	H_2O: (0.5x30)+(0.5x60)=45 parts MeOH: 0.5x70=35 parts THF: 0.5x40=20 parts	MeOH-THF-H_2O (35:20:45, v/v/v)
7	**1** + **2** + **3** (1:1:1, v/v/v)	H_2O: (0.33x30)+(0.33x43)+ 0.33x60)=43.9 parts MeOH: 0.33x70=23.1 parts ACN:0.33x57=18.8 parts THF:0.33x40= 13.2 parts	MeOH-ACN-THF-H_2O (23:1:18.8:13.2:43.9, v/v/v/v)
8	MeOH-TFA* H_2O		MeOH-TFA-H_2O (70:0.025:30, v/v/v); pH=3.0
9	ACN-TFA-H_2O		ACN-TFA-H_2O (57:0.025:43, v/v/v); pH=3.0
10	THF-TFA-H_2O		THF-TFA-H_2O (40:0.025:60, v/v/v); pH=3.0
11	THF-MeOH TFA-H_2O		THF-MeOH-TFA-H_2O (30:5.0.025:65, v/v/v/v); pH=3.0

* TFA = Trifluoric acetic acid.

Tetrahydrofuran (THF) - water:

$$\varphi_{THF} = P'_{mixt.} / P'_{THF} \quad (4)$$

The mode of preparation and the composition of mobile phases used for separation are listed in Table 1. We determined the capacity factor (k') and separation selectivity ($\alpha = k_2/k_1$), parameters influenced resolution (R_s) of the individual and pairs of semi-products and Finasteride utilising, optimally selected mobile phase.

RESULTS AND DISCUSSION

Selection of the Mobile Phase Composition

In order to separate Finasteride and its semi-products according to the concept of selection of solvents on the basis of optimisation triangle, the composition of mobile phase was selected in the following way, so as to obtain in each case a similar range of k' coeffcient for all compounds of the tested mixture. We selected methanol as first solvent regulating the selectivity, defining then elution strength of mobile phase of composition: 1 methanol-water (70:30, v/v) as equal to 1.82. On this basis we have established the composition of net mobile phases (Table 1).

The changes in capacity factor coefficients for individual semi-products, final products and two additional compounds depending on composition of mobile phase are presented in Figure 3.

The data presented in Fig. 3 shows that compounds #1 and #6, as well as compounds #4 and #6' were not separated in the mobile phase 1. In this case when ACN + H_2O (57 : 43, v/v) mobile phase was used, compound #1 and #5' were not resolved. When the mobile phase 3 i.e. THF + H_2O (40 : 60, v/v) was used the following compounds were not separated #5', #6 and #6'. The mixing of the phases 1 and 2 (in proportion of 1:1) did not separate compounds #3, #6, #4, #5, #1 and #5'. Also, mixing of the phases 2 and 3 in proportion of 1:1 did not give satisfactory results; compounds #4, #5, #5', #6 and #6' were not separated. So, at this stage of investigation, it was realised that acetonitrile is not a suitable solvent regulating the selectivity of tested compounds.

When the mobile phase 6 (mixture of 1 and 2 eluents in proportion of 1:1) was used, compounds #5 and #6, as well as the compounds #5' and #6', were not separated. For the mobile phase 7 (the mixture of the eluents 1, 2, and 3 in proportion of 1:1:1) compounds #4 and #6' and the compounds #5 and #6 were not separated. Moreover, we considered a factor to change selectivity of

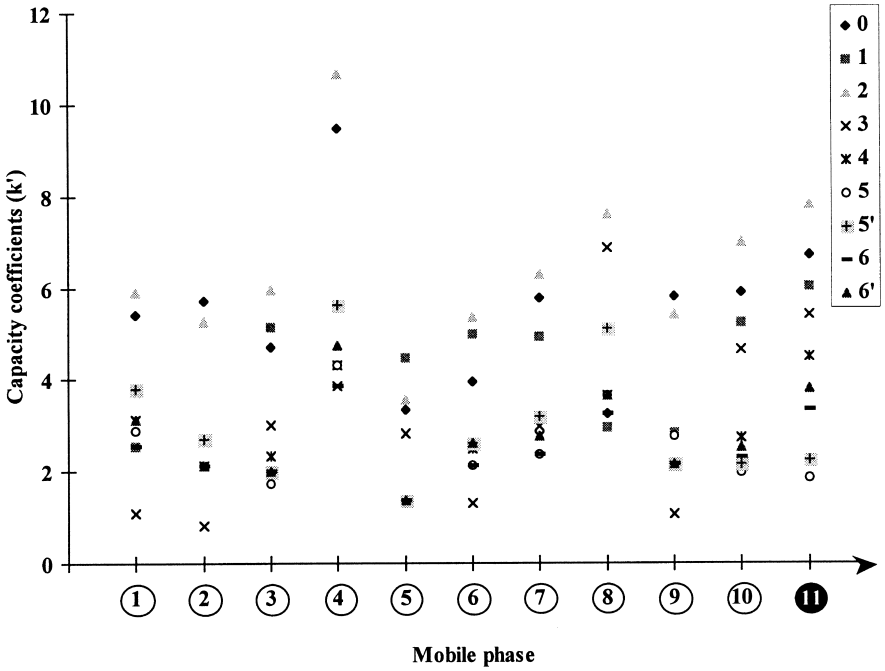

Figure 3. Comparison of capacity factor (k) determined for individual compounds formed during synthesis of Finasteride depending on the composition of mobile phase.

separation i.e. the change of mobile phase pH value from 7.0 to pH = 3.0 by addition of trifluoroacetic acid (the phases 8, 9, 10). For the mobile phase 9 containing acetonitrile, there was no improvement of separation of the tested compounds (they did not separate the compounds #4, #5, #6, #6', #5', and #1).

When the mobile phase 8 containing methanol was used, compounds #0, #6, #6', #4, and #5 were not separated.

The best separation was obtained for the mobile phase 10 which is THF-TFA-H_2O (40 : 0.025 : 60 v/v/v/v, pH = 3.0). In this case only the compounds #5' and #6 were not resolved.

After the analysis of acquired results, using the optimisation triangle and pH changes we selected the mobile phase of the composition: THF + MeOH + TFA + H_2O (30:5:0.025:65, v/v/v/v) permitting the separation of all semi-

Table 2

List of Retention Data Obtained for Optimum Mobile Phase*

Compound	Retention Time (t_R)	Capacity Factor (k')	Relative Retention (α)
#5	6.33	1.84	---
#5'	7.25	2.26	1.23
#6	10.01	3.35	---
#6'	11.04	3.80	1.13
#4	12.67	4.51	---
#3	14.75	5.54	1.29
#1	16.15	6.02	---
#0	17.75	6.72	1.12
#2	20.25	7.80	---

* TFH + MeOH + TFA + H_2O (30:5:0.025:65, v/v/v/v).

products, β - stereoisomer of the compound #5, β - stereoisomer of the compound #6 and the final product i.e., Finasteride. Retention data obtained for separated compounds utilising optimum mobile phase are listed in Table 2, and the optimal chromatogram is shown in Figure 4.

Quantification of Recommended RP HPLC Method

Based on the reported results (final chromatogram from Figure 4) this method permitted the qualitative and quantitative determination of all nine compounds of Finasteride synthesis (Table 3).

The repeatability of peak area measurements was in the range 0.1-0.5% relative standard deviation (RSD) (n>5) for 5 μg Finasteride injected, 0.7-1.5% for solutes at the 25 ng level and below 4.8 % when 5 ng were applied to the column. The linearity was studied for Finasteride. Peak areas were proportional to the concentration within ± 0.7% from 0.1 to 10 μg injected amount.

Estimated detection limits, expressed as the amount of substance injected corresponding to a peak height equal to three times the noise, were in the range 0.3-1.1 ng for all solutes.

The favourable characteristics as regards selectivity and quantitative performance make the recommended method suitable for analysis of these compounds.

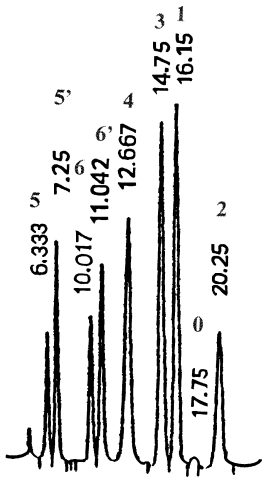

Figure 4. Typical chromatogram for a mixture of all compounds*:* #0 - progesterone; #1 - 3-oxoandrost-4-ene-17β-carboxylic acid; #2 - N-t-butyl-3-oxoandrost-4-ene-17β carboxylic acid amide; #3 - 17β (t-butylcarbamoyl)-5-oxo-4-nor, 3, 5-seco-androstano-2-carboxylic acid; #4 - N-t-butyl-oxo-4-aza-androst-5-ene-17β carboxylic acid amide; #5 - N-t-butyl-3-oxo-4-aza-5α-androst-17β carboxylic acid amide; #5' - N-t-butyl-3-oxo-4-aza-5β-androstan-17β carboxylic acid amide; #6 - 17β-(N-t-butylcarbamoyl)-4-aza-5α-androst-1-ene-3-one; #6' - 17β-(N-t-butylcarbamoyl)-4-aza-5β-androst-1-ene-3-one, obtained with utilization of the 11 mobile phase composition (for detail see in the text).

Table 3

Quantitative Estimation of Semi-Products and *Finasteride*

Compound	Purity Determined by HPLC (Normalization Method)	Content Determined by HPLC (External Std. Method)
1	89.20%	90.31%
2	80.80%	89.10%
3	94.00%	61.90%
4	96.60%	75.00%
5	96.10%	97.30%
5'(β-stereoisomer)*	---	0.99%
6	99.60%	99.80%
6' (β-stereoisomer)*	---	0.10%

* Additional compounds which may be formed during performed synthesis.

ACKNOWLEDGMENT

The authors are grateful to Dr. Haleem J. Issaq (NCI-Frederic Cancer Research & Development Center) for his helpful comments and suggestions.

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Received January 15, 1999
Accepted January 27, 1999
Manuscript 4963

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