*Theses of doctoral dissertation*

# Purity test of steroid pharmaceuticals by chromatographic techniques

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## 1. OBJECTIVES

The group of the steroid compounds plays a very important role on the traditional product assortment of Gedeon Richter Plc. These compounds are utilized in contraceptive, hormone supplementing products, but they are exported in active substance forms, too.

Among steroids the norsteroids having less by one methyl group at the base skeleton are of special interest. These are manufactured by multistep syntheses from the same starting materials and at the branching of the technological procedure different end-products are formed. So in consequence of the common starting materials impurity profiles of these steroids correlate.

The object of my work was to study in detail the more important compounds (nandrolone decanoate, norethisterone, allylestrenol are the better known products) of the steroid total synthesis according to the following aspects:

- By means of following the single technological steps, elucidating the common impurities and their precursors in the different steroids, searching for analogies.
- Working out purity test methods, which make possible detecting steroid impurities not showing UV absorbance, so passing unobserved by an HPLC method using UV detection. The planar chromatographic methods (TLC, OPLC) render possible this by the application of special visualising reagents.
- Optimization and comparison of the usages of different visualising reagents.
- Elaboration of rapid checking methods, which are suitable also for testing reaction mixtures and intermediates.
- Comparison of the performance characteristics of different chromatographic test methods.
- Elaboration of a screening test system for the identification of any possible cross contaminants.

## 2. EXPERIMENTAL METHODS AND EQUIPMENT

The TLC methods were developments in saturated normal chambers in each case in a 22 x 22 x 12 cm Desaga chamber. I have used silicagel sorbent of normal particle size (Merck Art. No. 5554) applied to aluminum foil support and of fine particle size (Merck Art. No. 5548) (20 x 20 cm Kieselgel 60 $F_{254}$) for the developments in normal chambers and by OPLC.

I have performed the OPLC developments by an overpressured layer chromatograph P-OPLC BS 50. Edging of the layer necessary for the OPLC development was carried out by the OPLC-NIT Ltd. In each case I have applied 300 μl of starting rapid mobile phase volume and 50 bar external pressure.

I have applied a motor-driven vaporizer (Merck Art. No. 8540) for spraying the sorbents with visualising reagents and I have heated them on Camag hot plate (TLC Plate Heater III).

I have used a Camag VideoStore 2 equipment for the documentation of the chromatograms, and a Camag VideoScan software for the videodensitometric evaluation. I have used a UV-2A, as well as a CC-30Y colour filter in the case of the images recorded at 366 nm light. I have used the green one from the built in filters of the VideoScan software at the videodensitometric evaluation.

We have carried out TLC, OPLC, HPLC and GC measurements in the case of *nandrolone* among the examinations connected to the **norsteroid synthesis**. I have employed a spotwise application at the TLC-measurement, n-hexane – ethyl acetate 1+2 (V/V) mixture was the mobile phase and 15 cm was the development distance. I have applied the compounds to be examined in 8 mm bands onto the sorbent at OPLC measurement, with cyclohexane – ethyl acetate – chloroform 50+25+25 (V/V) mixture, and I have worked with 2000-3000-4000 μl multiple development, drying the chromatograms with air stream at room temperature for 5 minutes among the visualisations, each. We have used C18 stationary phase of 3.5 μm (Eclipse XDB, 150 x 3.0 mm) at the HPLC method and we have performed the separations with the mixtures of water – acetonitrile – methanol in different ratios, applying a gradient program. The detection has been performed at 210 and 225 nm. We have used an MDN-5S column of 30 m x 0.25 mm x 1.0 μm film, thermostated to 270°C, with helium carrier gas of 20 cm/sec flow rate and with flame ionization detection at the gaschromatographic measurement.

In the case of *dienolether* I have used TLC and OPLC methods. At the TLC examination I have made run the compounds applied in spots with cyclohexane – acetone 7+3 (V/V) mixture, the running distance was 14 cm. At OPLC examination I have applied the substances onto the sorbent in 8 mm bands, and I have carried out the separation with cyclohexane – butyl acetate – ethyl acetate 8+1+1 (V/V) mixture, as well as with 8000 μl overrun.

In the case of *estradiol methylether acetate* we have carried out OPLC and GC measurements. At the OPLC examination I have worked with toluene – chloroform – cyclohexane – butyl acetate 55+3+40+2 (V/V) mixture and 4000 – 8000 µl twofold development. We have used fused silica stationary phase of 30 m x 0.25 mm of 0.5 µm particle size, thermostated to 240°C, helium carrier gas and flame ionization detection at 280°C at the gaschromatographic examination.

In the case of *molone* we have carried out OPLC and HPLC measurements. At the OPLC measurement I have performed the development with toluene – ethyl acetate – butyl acetate 8+1+1 (V/V) mixture and 8000 µl overrun. At the HPLC method we have used 5 µm C18 stationary phase and at first we have used methanol – water – acetic acid 70+30+0.3 (V/V) mixture as mobile phase, then we have modified the ratio to 40+60+0.3 (V/V) for the sake of the separation of an impurity unknown up to then. We have carried out the detection at 265 nm in both cases.

I have used the solution of the tested steroids of 0.02% concentration in chloroform as a model mixture for the **visualisation experiments** performed with the steroid mixture, and I have applied 5 µl from that solution onto silica gel sorbent of fine particle size in 5 mm bands. I have used the mixture of cyclohexane – ethyl acetate – chloroform in 3+1+1 (V/V) ratio as the mobile phase, which gave a suitable separation for each component of the model mixture. I was working with 400 µl/min flow rate of the mobile phase and with 4200 µl developing mobile phase volume. I have applied 2-5 µl volumes from the solutions of different concentrations, which related to substances of different amounts in the range of 0.1 µg and 0.0075 µg onto the sorbent for the detection limit tests performed with various visualising reagents.

At the determination of the **performance characteristics** I have carried out the development with toluene – ethanol 9+1 (V/V) mixture, using 15 cm of running distance in the case of the TLC measurements of *ethynil estradiol*. At OPLC determination I have separated the components from each other with cyclohexane – ethyl acetate – chloroform 3+1+1 (V/V) mixture, with 7000 µl overrun. At HPLC measurement we have used a 5 µm column of C18 stationary phase and water – acetonitrile – metanol 50+30+20 (V/V) mixture, with 280 nm detection.

When *norethisterone* has been examined the mobile phase of the TLC method was chloroform – acetone 9+1 (V/V). However at the OPLC measurement I have used a two-step, isocratic method: at first n-hexane, then -without interposed drying– I have continued the development with butyl acetate – chloroform 85+15 (V/V) mixture. At HPLC method we have used a 3.0 μm stationary phase with C18 filling and a six step gradient program with acetonitrile – water mixtures in different ratios. The detection was at 242 nm.

At the OPLC examination of *gestodene* cyclohexane – ethyl acetate – chloroform 3+1+1 (V/V) was the mobile phase, the development was carried out with 6500 μl overrun. When applying HPLC we have used a column filled with 5 μm particle size of C18 stationary phase and as mobile phases acetonitrile – water mixtures. The detection wavelength was 210 nm.

At **screening tests** I have performed OPLC measurements. I have applied 5-5 μg onto the sorbent using spotwise applications from the twenty examined steroids, each. I have used four mobile phase mixtures with different polarities, which were the following in order of increasing polarity: cyclohexane – butyl acetate 9+1 (V/V), toluene – ethyl acetate – chloroform 5+1+4 (V/V), cyclohexane – ethyl acetate – chloroform 3+1+1 (V/V) and cyclohexane – butyl acetate 1+1 (V/V).

## 3. NEW RESULTS

The steroids are manufactured by semi synthesis or total synthesis in the Gedeon Richter Plc. The norsteroid final products, manufactured by total synthesis, examined in this work are mainly active substances of the contraceptive, hormone supplementing, and anabolic preparations. Elucidation of impurity profiles of intermediates originated in the „methyl line" of the norsteroid synthesis was the object of my study.

1. I have worked out rapid purity test procedures by overpressured thin layer chromatographic method for examining *nandrolone*, *dienolether*, e*stradiol methylether acetate* and *molone (methyl-seco-olone)*. In the case of the above intermediates I have evaluated the results obtained with other chromatographic techniques, too. Simultaneous detection of *5α-4,5-dihydro-nandrolone* and *nandrolone* $\Delta^{5(10)}$*-isomer*, the two main impurities of *nandrolon*e, was successful only by OPLC. These two molecules of similar structure cannot be separated from each

other by TLC, *5α-4,5-dihydro-nandrolone* cannot be determined by HPLC with UV detection, GC can separate them adequately but the principal component decomposes to $\Delta^{5(10)}$*-isomer* during the examination, thus the result is not reliable. Concerning *dienolether* the OPLC procedure worked out by me, each known impurity can be evaluated selectively, and regarding *estradiol methylether acetate* the combination of the methods was successful. In the case of *molone* I have separated the main impurity from the principal component by OPLC, I have cleared up the arising conditions of the main impurity and promoted its identification.

2. I have optimized and compared the most often used acidic visualisation methods for steroids. I have examined the application of sulphuric acidic, phosphomolibdic acidic and phosphoric acidic reagents at the visualisation of *ethynil estradiol, dienolether, norethisterone, norethisterone acetate, norethisterone enanthate, nandrolone and nandrolone decanoate* active substances. I have established that the visualisation at higher temperatures lasting for shorter periods is generally more sensitive, however heating for longer periods at lower temperatures results in a more stable, robust visualisation comparing the different visualizing reagents.
3. I have studied and compared the efficiencies of TLC, OPLC and HPLC purity test methods developed for different steroid active substances. In the case of *ethynil estradiol* by TLC 71.5 μm, by OPLC 24.6 μm, by HPLC 19.0 μm average plate heights have been obtained; consequently the efficacies of OPLC and HPLC methods have been similar, and that of TLC method has been far inferior. According to the above the separating capabilities of the methods are similar and suitable in the case of OPLC and HPLC, while the related substances of similar structure cannot be separated by TLC from each other. I have obtained comparable experiences in the case of *norethisterone, nandrolone and gestodene*, too.
4. I have constructed a polarity series for the typical impurities of the steroids investigated and I have determined their relative retardation factors related to the main component.
5. I have worked out a screening test method for the steroids manufactured by the same plant which enables rapid examinations and identifications of the possible cross contaminants for the given compound group.

6. I have worked out a spot examination method without development which can be suitable for rapid checking the effectiveness of the washing during the cleaning of the equipment.

On the basis of my working experiences the thin layer chromatographic technique, first of all the OPLC, besides the HPLC method proposed and expected by the authorities can also be used well in the future for the purity tests of steroids in the pharmaceutical analysis and as preliminary test, detecting and proving the impurity profiles of new substances, as well as performing rapid screening tests.

## 4. PUBLICATIONS

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2. ***B. Bagócsi**, G. Rippel, M. Mezei, K. Ferenczi-Fodor*
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5. ***B. Bagócsi**, A. Laukó, S. Mahó, Z. Végh, K. Ferenczi-Fodor*
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*Sz. Nyiredy, A. Kakuk* (eds.), Proc. Int. Symp. Planar Separations – Planar Chromatography 2003, Budapest, Hungary, June 2003, Res. Inst. Medicinal Plants, Budakalász, Hungary, pp. 185-189.

6. ***B. Bagócsi**, G. Rippel, M. Mezei, K. Ferenczi-Fodor*
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7. ***B. Bagócsi**, A. Laukó, S. Mahó, S. Németh, Z. Végh, K. Ferenczi-Fodor*
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3. *L. Kocsis, J. Mink, K. Ferenczi-Fodor,* ***B. Bagócsi***

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5. *J. Kozma,* ***B. Bagócsi****, F. Kassai, K. Ferenczi-Fodor*

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