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HPLC separation of related impurities in etiprednol dicloacetate, a novel soft corticosteroid

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Etiprednol dicloacetate (ethyl 17α-dichloroacetoxy-11β-hydroxy-androsta-1,4-diene-3-one-17β-carboxylate, code-named: BNP-166) has been prepared in a 3-step synthesis from prednisolone as starting material. The primary aim of the present work was to develop HPLC methods for the separation of all the impurities found in experimental pilot plant batches of BNP-166 at concentrations ≥0.10 area %. Besides BNP-166, a total of 19 compounds, eight of them potential impurities, were involved in the HPLC studies in which several HPLC systems were examined and tested to optimize the separation. Of the parameters influencing chromatographic behaviour column type, the nature and composition of the mobile phase and column temperature were varied, while the pH of the eluent was kept constant at 4.5, a pH value at which stability of the BNP-166 ester bonds was found to be the highest. A comparison of the RRT values obtained allowed some conclusions to be drawn concerning the physico-chemical forces governing separation. The isocratic reversed-phase HPLC system (V02) chosen to be used for various GXP studies on BNP-166 affords baseline separation of nearly all the compounds concerned, and also the quantitation of the drug candidate (BNP-166). By means of this system, it was shown that the target compound prepared by the standardized synthesis method on a pilot plant scale, never contained more than 2–3 impurities with area % values higher than 0.10.

1. Introduction

The soft corticosteroid etiprednol dicloacetate (ethyl 17α-dichloro-acetoxy-11β-hydroxy-androsta-1,4-diene-3-one-17β-carboxylate; code-named: BNP-166) was developed as a result of structural modification relying on the "inactive metabolite" of soft drug concept (Bodor 1984a; 1984b; 1988). It was developed for use in various clinical fields, with indications such as asthma, allergic rhinitis, Crohn's disease and ulcerative colitis.

Etiprednol dicloacetate (BNP-166) has been prepared in a 3-step synthesis from prednisolone as starting material (see Scheme).

As several closely related 1,4-androstadiene-3-one derivatives are also formed in the process of the BNP-166 synthesis shown below, a systematic study had to be made of the relative merits of normal-phase and reversed-phase HPLC methods to achieve adequate separation.

The aim of the present work was to develop an HPLC method capable of a baseline separation, in less than 30 min, of all or almost all the actual and potential impurities most likely to arise during the synthesis, purification and/or storage of BNP-166.

2. Investigations, results and discussion

2.1. Isolation and identification of the impurities

After crystallization of BNP-166, synthetic contaminants (some of them derived from prednisolone and its contaminant hydrocortisone as well as other commercial reagents) were isolated from the mother liquor using conventional silica gel column chromatography for the contaminants well separated by thin layer chromatography (TLC) on silica plates, and semi-preparative reversed-phase HPLC for the rest.

Scheme

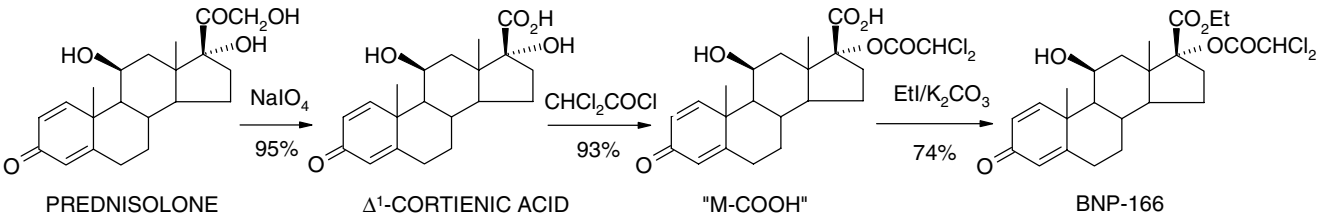
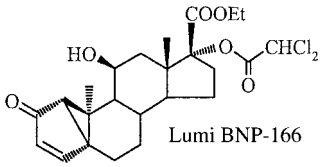


Table: Chromatographic characteristics of BNP-166 and its potential contaminants

BNP-166 and its potential contaminants	RRT values obtained in some HPLC systems				
	V01	V02	01/02/a	01/02/b	02/01
Δ^1-Cortienic acid	0.17	0.17	0.14	0.22	0.12*
M−COOH	0.19	0.22	n.a.	0.29	0.13*
11-Oxo- Δ^1-cortienic acid ethyl ester	0.46*	0.40	0.33	0.45	0.31
17α-Dimethyloxamate, BNP analogue	0.46*	0.42*	0.36*	0.48*	0.33
Δ^1-Cortienic acid ethyl ester (M−OH)	0.46*	0.42*	0.36*	0.50*	0.28
17α-Formate, BNP analogue	0.59	0.49	0.43	0.56*	0.44
11-epi BNP	0.67	0.58	0.50	0.56*	0.60
11-Deoxy M−OH	0.75	0.66	0.60	0.69	0.49
BNP-Methyl ester analogue	0.81	0.76	0.74	0.80	0.74
11-Deoxy-cortienic acid ethyl ester	1.00*	0.94*	0.93	1.01*	0.67
BNP-166 (ret. time: 6, 9, 21, 8,5 and 8 min, resp.)	1.00*	1.00	1.00	1.00*	1.00
Lumi BNP-166	1.00*	1.31	n.a.	n.a.	1.22
11-Oxo BNP analogue	1.04*	0.86	0.83	0.84	1.10*
17α-Chloroiodoacetoxy BNP analogue	1.04*	0.94*	n.a.	0.90	1.07
1,2-Dihydro BNP analogue	1.11	1.12	1.14	1.11	1.13*
11-Deoxy BNP analogue	1.74*	1.65	1.69	1.45	1.89*
17α-Trichloroacetoxy BNP analogue	1.74*	2.27	2.46	2.10	1.90*
1,2-Dihydro-11-deoxy BNP analogue	2.44	2.51	2.76	2.20	2.77

System 01/02/a Column: Eurospher-100, C18, 5 µm (125 × 4 mm I.D. with a 5 mm integrated pre-column); Eluent: methanol-aqueous buffer = 60 : 40, v/v; Flow rate: 0.8 ml/min; Column temperature: 40 °C. For the rest of the systems in the Table see the figure legends.



Lumi BNP-166

Structure elucidation of the compounds isolated was performed by NMR and MS. Structures were confirmed by synthesis and subsequent comparison of the NMR and MS spectra of the synthetically produced compounds with those of the isolated contaminants.

The short, in-house names of the 17 isolated and identified contaminants are shown in the Table.

2.2. HPLC systems for resolving BNP-166 and related impurities

As was pointed out in earlier studies on the separation of steroids, normal-phase and reversed-phase HPLC systems can complement each other (Lin and Heftmann 1982): compounds inseparable by adsorption HPLC on silica columns can often be separated by reversed-phase HPLC and *vica versa*. In preliminary separation experiments, therefore, both normal-phase and reversed-phase HPLC systems were tested in our laboratory for the separation of the compounds in the Table.

In order to show the separation characteristics of the normal-phase systems, a representative chromatogram obtained for a BNP-166 sample spiked with all the potential impurities is presented in Fig. 1.

Although the separation power of the silica system in Fig. 1 appears to be good, the separation pattern suggests some disadvantages: the separation takes almost one hour, besides, the retention of BNP-166 is such that its peak is eluted into a major group of potential contaminants with similar retentions, meaning that a faster separation by gradient elution or by using an isocratic eluent with an increased elution strength would not result in baseline separations. Also, further difficulties can be anticipated with such systems in developing a sensitive and reliable HPLC method for the quantitation of the contaminants eluting well after the BNP-166 peak, such as the intermediates M-COOH and Δ^1-cortienic acid. Therefore, subsequent ef-

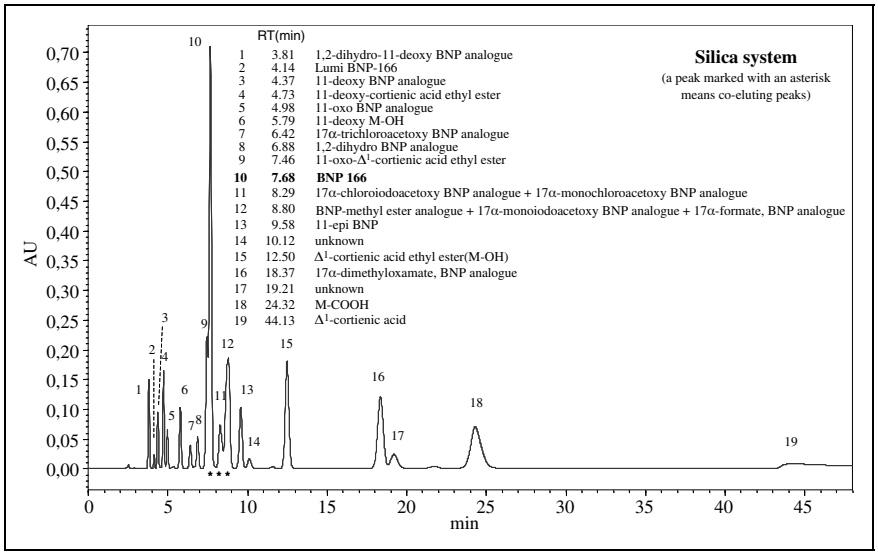


Fig. 1:
Separation of BNP-166 and its potential contaminants in a normal-phase system. Column: Nucleosil 100 Si, 5 µm, (250 × 4 mm I.D.); Eluent: n-hexane-dichloromethane-methanol-glac. acetic acid = 80 : 16 : 4 : 0.1, v/v; Flow rate: 1 ml/min; Column temperature: 25 °C

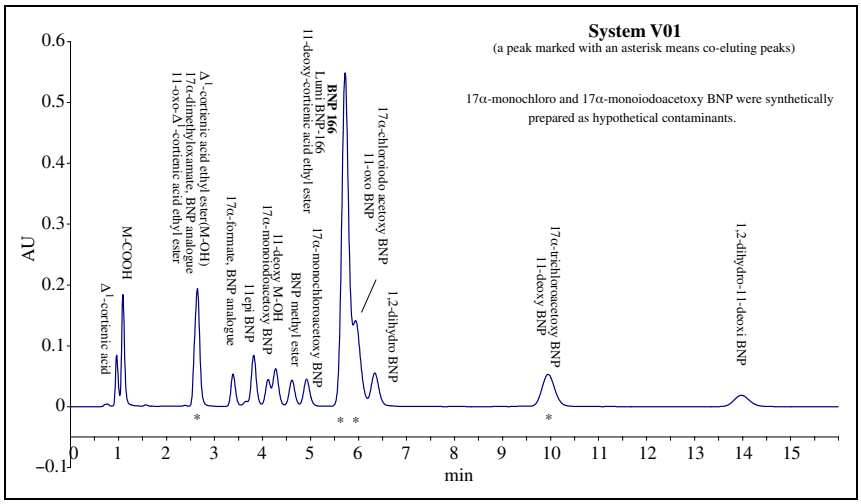


Fig. 2:
Separation of BNP-166 and its potential contaminants in a reversed-phase system. Column: Eurospher-100, C18, 5 µm (125 × 4 mm I.D. with a 5 mm integrated pre-column); Eluent: acetonitrile-aqueous buffer = 60 : 40, v/v; Flow rate: 1 ml/min; Column temperature: ambient

forts to optimize the separation concentrated on reversed-phase HPLC systems.

Two different types of reversed-phase column packing, a thoroughly end-capped packing material with a specific surface area of 350 m^2/g (Eurospher-100, C18, 5 µm) and a packing material with no special end-capping and a specific surface area of 175 m^2/g (Hypersil ODS, 3 µm) were tested.

Because a change from a protic organic modifier to an aprotic eluent additive can offer a further separation alternative for steroids in reversed-phase systems (Francis and Kinsella 1984), protic and aprotic mobile phase additives, such as methanol and acetonitrile, respectively, were used in the eluent optimization studies.

As there are no ionic groups in the molecules of BNP-166 and most of the related potential contaminants, the pH of the aqueous part of the eluent was kept constant at 4.5, a safe compromise allowing satisfactory stability for the ester moieties in the BNP-166 molecule.

Column temperature, as another factor influencing column performance, was varied between ambient and 40 °C.

A selection of chromatograms from the reversed-phase separation and optimization experiments is shown in Figs. 2–5, with the respective relative retention time (RRT) values in the Table. System V01 (with a packing of large surface area, almost fully end-capped, and an aprotic eluent additive) and System V02 (with a packing of much smaller surface area, no special end-capping, and a protic eluent additive) were the main alternative versions, whereas all the other systems tested, e.g. Systems 01/02/a, 01/02/b and 02/01 can be regarded as mixtures of the basic versions.

An inspection of these chromatograms reveals that System V01 (Fig. 2) resulted in a less than satisfactory separation of the main peak, with several co-elutions involving 10 compounds, as indicated by the asterisks (*) at the base of the peaks concerned. The run time, however, would be advantageous, less than 15 min.

Although the chromatogram obtained with System V02 (Fig. 3) takes 24 minutes to run, the main peak is well separated, and the number of co-eluting contaminants has been reduced to 4.

Systems 01/02/a and 01/02/b (and several other "mixed" systems tested) offer no special advantages over System V02. System 02/01, however, with 7 co-eluting contaminants though, can still complement System V02 if resolution of M−OH and the BNP-166 analogue, 17α-dimethyloxamate should be needed (the silica system in Fig. 1 can also be considered here).

Of the reversed-phase systems tested, System V02 was chosen as the best compromise for the various GXP studies in this Institute. All the more so as the main problem here, the co-eluting diastereomer pair of the 17α-chloroiodoacetoxy BNP-166 analogue (one isomer co-elutes with the potential contaminant 11-deoxy-cortienic acid ethyl ester, and the other, not shown, with the main peak) has been eliminated by replacing ethyl iodide with diethyl sulfate in the last step of the synthesis of the drug candidate (see Scheme).

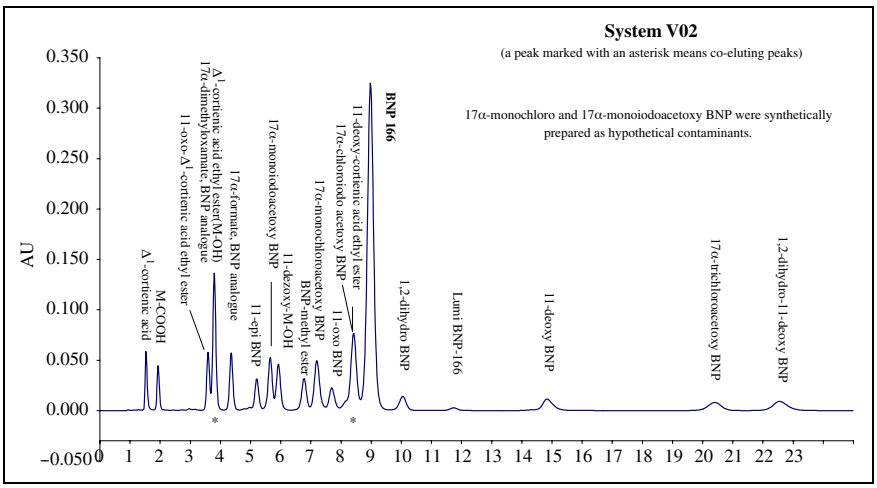


Fig. 3:
Separation of BNP-166 and its potential contaminants in a reversed-phase system. Column: Hypersil ODS, 3 µm (100 × 4 mm, I.D.); Eluent: methanol-aqueous buffer = 60 : 40, v/v; Flow rate: 0.8 ml/min; Column temperature: 40 °C

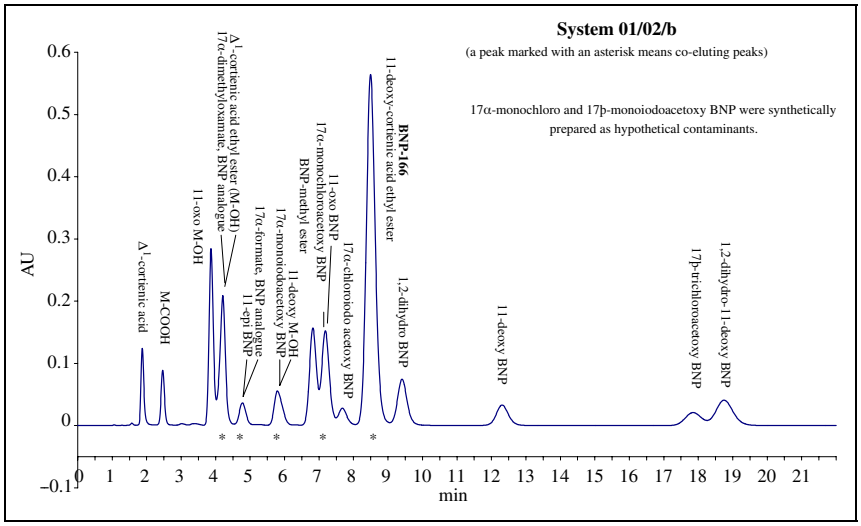


Fig. 4:
Separation of BNP-166 and its potential contaminants in a reversed-phase system. Column: Eurospher-100, C18, 5 µm (125 × 4 mm I.D. with a 5 mm integrated pre-column); Eluent: methanol-aqueous buffer = 70 : 30, v/v; Flow rate: 0.8 ml/min; Column temperature: ambient

System V02 has been thoroughly tested for precision and accuracy of BNP-166 determinations in various analytical assignments. Detection was performed at 243 nm, the strong UV absorption maximum of the 4-en-3-one conjugated ketone structure [E_{max}: ∼ 15000, Kelce 1988; typical RSD values: well below ± 1.0%]. A more complete validation study of the method is under way.

2.3. Some physico-chemical factors affecting the separation

Our findings conform to some generalizations concerning retention in normal- and reversed-phase HPLC systems.

In this group of steroids, the least polar compound was 1,2-dihydro-11-deoxy BNP (eluting first in normal-phase systems and last in reversed-phase ones), showing the importance of the 11-hydroxy or 11-oxo group in increasing the polarity (H-bonding capability) of such molecules. The contribution of the 11α-hydroxy group to polarity is apparently stronger than that of the 11β-hydroxy group (see the retentions of 11-epi BNP and BNP-166). As expected, in normal-phase systems, 11β-hydroxy derivatives are remarkably more polar than the respective 11-oxo derivatives (thus BNP-166 and 11-oxo BNP as well as Δ^1-cortienic acid ethyl ester and 11-oxo-Δ^1-cortienic acid ethyl ester can be separated easily, see Fig. 1), but in reversed-phase systems the polarity difference between these pairs of compounds is much smaller (see the acetonitrile-containing systems in Fig. 2 and Fig. 5), or can even be reversed (see the elution orders of BNP-166 and 11-oxo BNP in the methanol-containing systems in Fig. 3 and Fig. 4).

The 1,2-dihydro derivatives (coming mostly from the hydrocortisone contaminant of the starting material prednisolone) appeared to be less polar than the respective 1,4-diene derivatives (coming mostly from prednisolone; compare the retentions of 1,2-dihydro BNP and 1,2-dihydro-11-deoxy BNP with those of BNP-166 and 11-deoxy BNP, respectively, in Figs. 1–5).

The most powerful enhancer of molecule polarity, however, was the 17β-carboxylate moiety (if present and solvated; see the retentions of M−COOH and Δ^1-cortienic acid in Figs. 1–5).

It is also worth noting that whereas the resolving power of most of the reversed-phase systems for Δ^1-cortienic acid ethyl ester and the 17α-dimethyloxamate BNP-166 analogue approached zero, their resolution was the greatest of all in the normal-phase system.

The effect of elevated temperature was beneficial from the point of view of system pressure in methanol-containing reversed-phase systems, but it had no real effect on elution orders or resolution (compare the RRT values obtained with systems 01/02/a and 01/02/b in the Table).

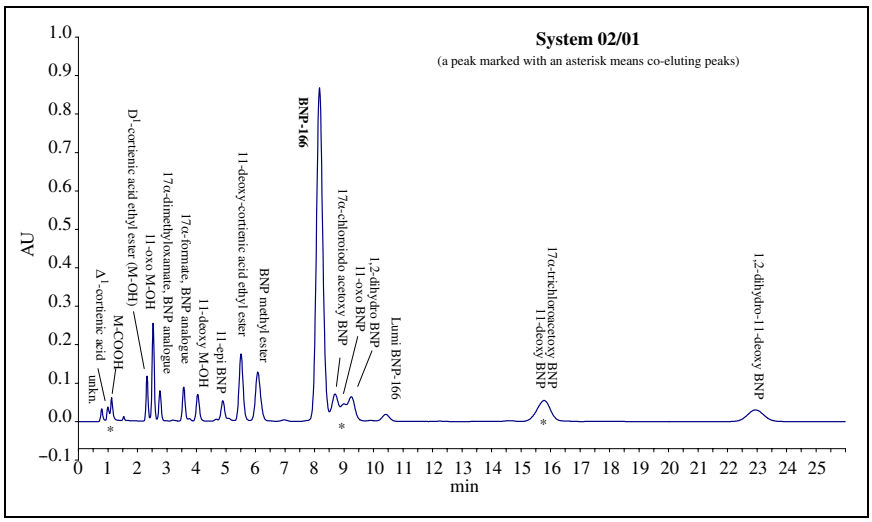


Fig. 5:
Separation of BNP-166 and its potential contaminants in a reversed-phase system. Column: Hypersil ODS, 3 µm (100 × 4 mm, I.D.); Eluent: acetonitrile-aqueous buffer = 48 : 52, v/v; Flow rate: 1 ml/min; Column temperature: ambient

3. Experimental

3.1. Materials

All the major eluent components (distilled water included) were of HPLC grade. Acetonitrile and distilled water were purchased from Riedel-deHaën (Seelze, Germany), methanol, ethyl-acetate and dichloro-methane were the product of Merck (Darmstadt, Germany), n-hexane was from Carlo Erba (Milano, Italy) and glacial acetic acid was obtained from Reanal (Budapest, Hungary). All other chemicals, such as sodium hydroxide, were commercially available products of special reagent grade. The Hypersil ODS and Nucleosil 100 Si chromatographic columns were purchased from GROM (Herrenberg-Kayh, Germany), while the Eurospher-C18 column was from Knauer (Berlin, Germany).

BNP-166 was synthetized in-house, and all the impurities concerned were isolated and/or synthetized in our laboratories. Lumi BNP-166 (ethyl 17α-dichloroacetoxy-11β-hydroxy-1α,5β-cycloandrost-3-ene-2-one-17β-carboxylate), also prepared here (Csanádi et al. 2004), was made by analogy with the known photo-rearrangement of prednisolone into lumiprednisolone (Williams et al. 1980).

3.2 Analytical method

The HPLC equipment used in the separation studies and for the BNP-166 assay and purity tests consisted of a Waters 1525 binary solvent pump, a Waters 2487 dual wavelength absorbance detector (operated at 243 nm), a Rheodyne 7725i manual injector with a 20 µl sample loop, a Waters column thermostat (an attachment to the pump) and a Waters Breeze software for system control and data handling/processing.

Two reversed-phase HPLC columns were used (as shown in the legends of Figs. 2–5): either a Hypersil ODS, 3 µm column (100 × 4 mm, I.D.) or a Eurospher-100, C18, 5 µm column (125 × 4 mm I.D. with a 5 mm integrated pre-column).

Reversed-phase HPLC elution solvents consisted of an aqueous buffer to which either methanol or acetonitrile was admixed at varying volume ratios.

Preparation of the aqueous buffer (1 liter): to 990 ml distilled water 1.0 ml of glacial acetic acid was added and the pH of the solution was adjusted to 4.5 ± 0.05 using 2 N NaOH and a pH meter equipped with a glass electrode. The buffer was then diluted to volume with distilled water. The eluent (aqueous buffer + organic modifier) was filtered and degassed before or during use.

The retention time of BNP-166 in the respective reversed-phase system, the eluent flow rate, column temperature, as well as all the system parameters of a representative normal-phase chromatogram are specified in the respective figure or figure legend.

BNP-166 sample concentrations (from ∼ 200 µg/ml solutions in the eluent or, preferably, acetonitrile – aqueous buffer mixtures of 1 : 1, v/v to avoid even the slightest ester decomposition) were determined by comparing the sample peak area with calibration standard peak areas.

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