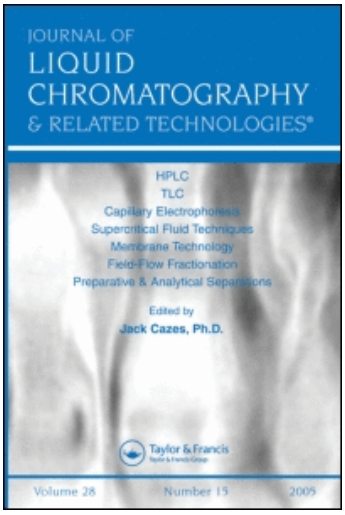


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# Isolation by Means of Preparative Reversed-Phase Liquid Chromatography of Epimeric Alcohols Formed Upon Reduction of Pregnenolone and Progesterone

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# ISOLATION BY MEANS OF PREPARATIVE REVERSED-PHASE LIQUID CHROMATOGRAPHY OF EPIMERIC ALCOHOLS FORMED UPON REDUCTION OF PREGNENOLONE AND PROGESTERONE

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## ABSTRACT

A method is described which permits complete separation on a preparatory scale of the 20R and 20S epimeric alcohols obtained from lithium aluminium hydride and sodium borohydride reduction of pregnenolone and progesterone, respectively. The retention behaviour and resolution obtained on chromatography of the epimers on C-18 bonded phase material and elution with different acetonitrile/water and methanol/water mobile phases were studied. The order of retention is in both cases in accordance with $^1$H-NMR chemical shift data which indicate a stable conformation with a more exposed 20-OH group in the 20S (=20$\alpha$) epimer. Deviations from the elution order expected for true reversed-phase retention mechanisms were found on elution with mobile phase systems of reduced water content.

## INTRODUCTION

Studies on certain enzymatic conversions (1) of pregnenolone (I), prompted us to synthesize tritium labelled pregn-5-ene-3$\beta$,20$\alpha$-diol (=pregn-5-ene-3S,20S-diol) (IIa) by lithium aluminium hydride (LAH) reduction of labelled I. This, in turn, necessitated a method for the separation of the epimeric 3S,20S and 3S,20R (IIb) -diols formed in this reaction. Attempts to change the epimer ratio in favour of IIa by LAH/$AlCl_3$-reduction under conditions favouring

equilibration resulted in a rather complex mixture of steroids (2). For identification purposes pregn-4-ene-20α-ol-3-one (pregn-4-ene-20S-ol-3-one) (IVa) and its 20b (20R) epimer (IVb) were prepared by sodium borohydride reduction of progesterone (III). The conditions for optimal preparative separation, by means of reversed phase high performance liquid chromatography (HPLC), of the epimeric pairs of alcohols formed in these reactions were therefore the primary object for this study.

## EXPERIMENTAL

### Reduction of pregnenolone and progesterone

A detailed description of the reaction conditions used for metal hydride reduction of these ketones has recently been reported elsewhere (2).

### HPLC

Columns (analytical 4.6x200 mm and preparative 10.0x250 mm) were prepared by upward slurry packing with Nucleosil C-18 5μ (Macherey and Nagel, Düren, G.F.R.). The liquid chromatograph was composed of an Altex mod. 100 constant flow solvent pump, a Rheodyne mod. 2710 injector valve, an LDC Spectromonitor III variable wavelength UV-VIS detector and a Hitachi mod. 561 potentiometric recorder.

For the analytical work a 20 μl loop volume was used which was changed to 50 μl in combination with preparative columns. With the latter system amounts of the order 1-2 mg were loaded onto the column. Collection of the compounds eluted during preparative runs was effected manually during observation of the UV-absorbancy registered on the recorder. 10-20 repeated injections were sufficient to permit isolation of the amounts necessary for identification by means of melting point, NMR, GC/MS and optical rotation (2).

The HPLC solvents used were acetonitrile HPLC grade S from Rathburn Chemicals (Walkerburn, Peeblesshire, Scotland) and methanol of p.a. quality from Merck (Darmstadt, G.F.R.). Glass distilled water was used to prepare the various mobile phases.

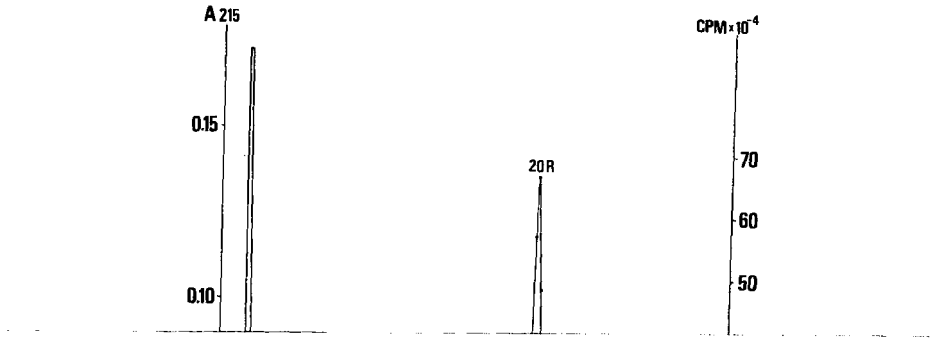
Radiochromatograms from analytical HPLC of the product mixture obtained on reduction of tritiated pregnenolone were obtained by continuous fraction collection using 15 sec/fraction, except for the first and last 10 min of the chromatogram, where 1 min/fraction was used The collected fractions were then mixed with Aquasol (NEN Chemicals, Dreieich, G.F.R.) and the tritium activity determined by liquid scintillation counting.

## RESULTS AND DISCUSSION

The reduction of I by LAH results in a mixture of IIa and IIb in an approximate ratio of 1:4. This is shown by Fig. 1 which originates from an experiment where tritium labelled I was used. The complete separation of the epimeric diols obtained, encouraged attempts to scale up the method to suit preparative purposes. A 10x250 mm steel column packed with 5 $\mu$ C-18 bonded phase material was run with 70% acetonitrile as the mobile phase and permitted injected amounts up to ca. 2 mg without any significant signs of overload. The separation result after repeated injections and eluate collection is shown by Fig. 2, which demonstrates the purity obtained as monitored by analytical HPLC. If a certain sacrifice in yield is permitted, the purity of the 20S-epimer can be further increased.

The complex reaction mixture from reduction of I by LAH/$AlCl_3$ under conditions of equilibration analyzed by HPLC at two different wavelengths is shown by Fig. 3. The peaks remaining at 235 nm were suggested to originate from reaction products having a conjugated carbonyl chromophore, most likely the pregn-4-ene-3-one system.

A sodium borohydride reduction of III gave, besides a large amount of unreacted III, the two epimeric alcohols IVa and IVb. These were readily separated on a preparatory scale, Fig. 4. When the reaction mixture was analyzed by HPLC using 60 % methanol as the mobile phase, IVa was completely overlapped by III. The superimposition of peaks from UV absorption at 247 nm for identification purposes is well illustrated by Fig. 5.







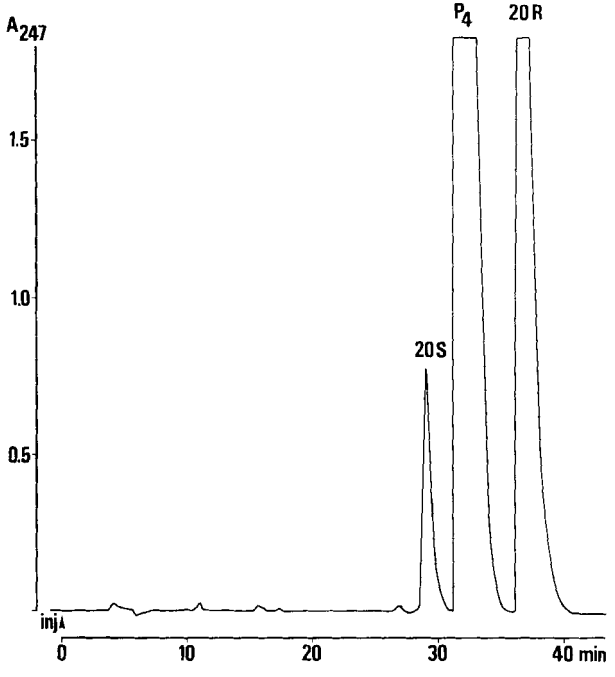




FIGURE 4. Resolution of pregn-4-ene-20S-ol-3-one and pregn-4-ene-20R-ol-3-one obtained on sodium borohydride reduction of progesterone. Preparative column, 70% acetonitrile, flow rate 2.4 ml/min, ca. 1 mg injected.

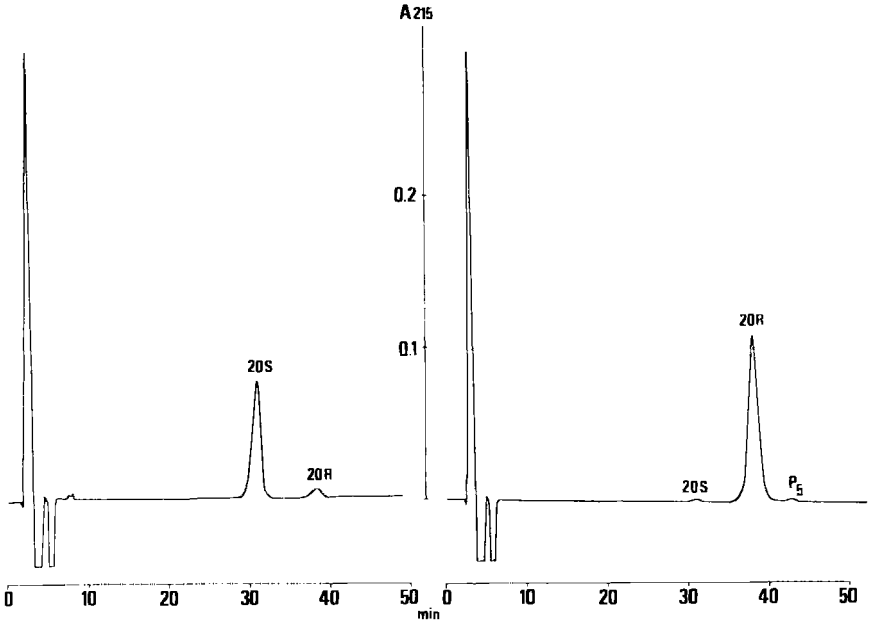



FIGURE 2. Results from a preparative HPLC separation of pregn-5-ene-3S, 20S-diol and pregn-5-ene-3S,20R-diol, as shown by analysis of purity. Analytical column, 50% acetonitrile, flow rate 1.0 ml/min.

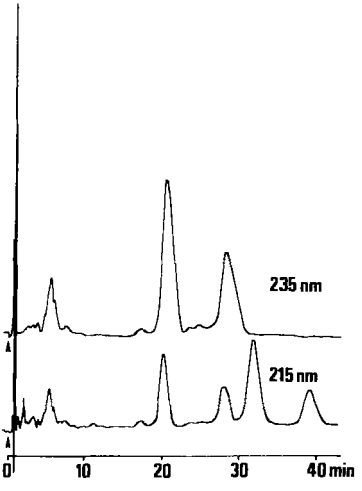



FIGURE 3. Illustration of the use of different UV-detector wavelengths to probe the presence of compounds with conjugated carbonyl group chromophores. Product mixture obtained on LAH/$AlCl_3$-reduction of pregnenolone.

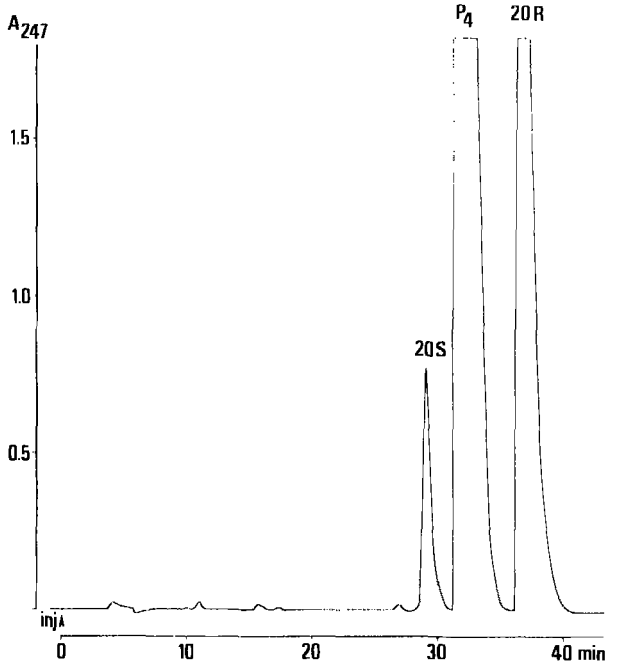



FIGURE 4. Resolution of pregn-4-ene-20S-ol-3-one and pregn-4-ene-20R-ol-3-one obtained on sodium borohydride reduction of progesterone. Preparative column, 70% acetonitrile, flow rate 2.4 ml/min, ca. 1 mg injected.



It is interesting to note that the chromatographic results correlate well with $^1$H-NMR chemical shift data of the epimeric pairs. The downfield shift of 0.09 ± 0.01 ppm for the H-21 resonance signal and the same upfield shift for the H-18 signal found for the 20S relative to the 20R epimers can be taken as strong evidence for a stable conformation with a less exposed 20-OH group in the 20R epimer (5), which accordingly should be the more hydrophobic species. It can also be found from Table 1 and Fig. 4 that the elution order predicted for true reversed-phase chromatography, where the compounds are eluted in order of hydrophobicity, i.e. in this case IIa, IIb, I and IVa, IVb, III, is only fulfilled when mobile phase systems of sufficient water content are used. Obviously, the deviation from this order of

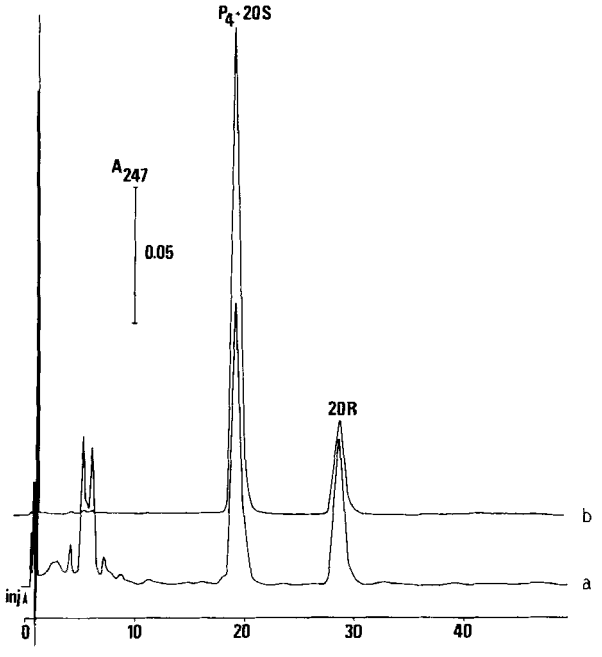



FIGURE 5. Chromatographic identification of the pregn-4-ene-3-one system by UV-monitoring at 247 nm. Analytical column, 60% methanol, flow rate 2.25 ml/min. a) Product mixture obtained on LAH/$AlCl_3$-reduction of pregnenolone. b) Products resulting from sodium borohydride reduction of progesterone.

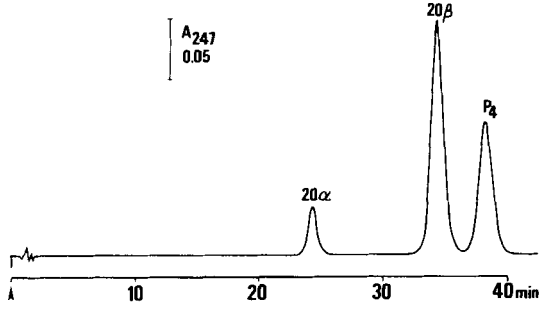



FIGURE 6. Illustration of the C-18 reversed-phase separation efficiency with 40% acetonitrile as the mobile phase. Product mixture obtained on sodium borohydride reduction of progesterone. Analytical column, flow rate 2.0 ml/min, UV 247 nm.

TABLE 1. Capacity Ratios (k'), Separation Factors (α) and Resolution (Rs) Obtained with Some Different Mobile Phases

| Compound no.: | IIa | IIb | III | IVa | IVb | Mobile phase |
|---|---|---|---|---|---|---|
| k' | 17.0 | 23.4 | 26.85 | 16.7 | 24.0 | |
| α | 1.375 | | | 1.434 | | A |
| Rs | 4.56 | | | 5.06 | | |
| k' | 9.0 | 12.1 | 14.1 | 9.0 | 12.7 | |
| α | 1.338 | | | 1.402 | | B |
| Rs | 4.07 | | | 4.64 | | |
| k' | 6.05 | 7.9 | 9.5 | 6.0 | 8.3 | |
| α | 1.308 | | | 1.380 | | C |
| Rs | 3.62 | | | 4.29 | | |
| k' | 20.2 | 26.6 | 13.1 | 13.9 | 19.6 | |
| α | 1.317 | | | 1.407 | | D |
| Rs | 3.92 | | | 4.65 | | |
| k' | 9.4 | 11.9 | 6.2 | 6.2 | 8.7 | |
| α | 1.275 | | | 1.404 | | E |
| Rs | 3.40 | | | 4.43 | | |
| k' | 5.1 | 6.3 | 3.5 | 3.5 | 4.7 | |
| α | 1.227 | | | 1.341 | | F |
| Rs | 2.76 | | | 3.66 | | |

Mobile phase systems: Acetonitrile in water: A = 40%, B = 45%, C = 50%. Methanol in water: D = 65 %, E = 70 %, F = 75 %

elution observed in other cases is the result of a competitive retention mechanism based on adsorption to polar groups of the stationary phase.

## REFERENCES

1. Allenmark, S., Å:son Berg, A., Hammar, M. and Lindström, E., Combined Paper and Reversed-phase High-performance Liquid

Chromatography Method for the Study of Pregnenolone and Progesterone Metabolites, J. Chromatogr. Biomed. Appl., in press.

2. Allenmark, S. and Borén, H., Lithium Aluminium Hydride Reduction of Pregnenolone under Conditions of Assumed Kinetic and Equilibrium Control, Acta Chem. Scand., in press.

3. Heftmann, E., Behaviour of Stereoisomeric Steroids in Liquid Chromatography, J. Liquid Chromatogr. 2, 1137 (1979).

4. Lin, J.-T., Heftmann, E. and Hunter, I., High-performance Liquid Chromatography of the Reduction Products of Progesterone, J. Chromatogr. 190, 169 (1980).

5. Lee, H. and Wolff, M.E., C-18 Functional Steroids and D-Homo Steroids, J. Org. Chem. 32, 192 (1967).