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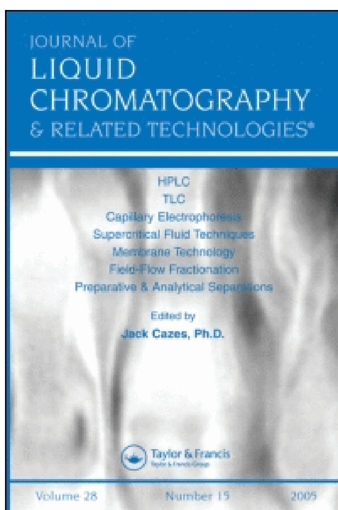

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RESOLUTION AND IDENTIFICATION OF STEROID OXIME SYN AND ANTI ISOMERS BY HPLC

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ABSTRACT

Oxime derivatives of a number of steroidal 4-en-3-one 17-alcohols and 17-esters have been separated into syn and anti isomers by HPLC under normal or

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reverse-phase conditions. The elution order of the isomeric pairs was found to vary with the nature of the steroid and the type of stationary phase: a peak ratio method at two wavelengths provides a very convenient method for unambiguously identifying the geometries of the isomeric pairs.

INTRODUCTION

Oxime derivatives of 3-keto-17-hydroxysteroids and of the corresponding steroid esters are of interest as hormonal agents for fertility regulation [1-7]. The analysis of these compounds and of their oxime-containing metabolites is complicated by the presence of two geometric isomers, each potentially having its own distinct biological properties. (In oximes of the steroidal 4-en-3-ones, shown in Figure 1, the terms syn and anti refer to the N-hydroxyl group orientation relative to the 4,5-double bond: the stereochemistries of the syn and anti isomers are alternatively described as Z and E, respectively).

A reverse phase HPLC method has been described for the analysis of oral contraceptive tablets containing levonorgestrel acetate oxime (norgestimate) and ethynylestradiol [8]. We have briefly reported [9] a reverse-phase HPLC method for the analysis of the long-acting injectable progestogen, levonorgestrel cyclopentylcarboxylate oxime (2) and possible metabolites, including levonorgestrel oxime (1). It was found [9] that the ester oximes (2) could be resolved on ODS-silica, but not Phenyl-silica, whereas the opposite was true for the 17-hydroxy-steroid oximes (1), a complete analysis of mixtures containing both sets of geometric isomers therefore requiring a combination of the two phases, either sequentially or in a mixed-bed column. Furthermore, the elution order was found to reverse for these two pairs of isomers in a mixed-bed column, syn levonorgestrel cyclopentylcarboxylate oxime eluting before the anti isomer, but anti levonorgestrel eluting before syn. We have

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therefore investigated a series of oxime derivatives of steroids (1-14) in order to establish general conditions for their separation and we have also found a very rapid and convenient HPLC procedure to identify each isomer in a mixture.

MATERIALS AND METHODS

HPLC analyses were performed on 25 x 4.5 cm i.d columns packed with 5 um spherical particles of ODS-Hypersil, Octyl-Hypersil, Phenyl-Hypersil or Hypersil SiO_2, eluted with ultrasonically degassed, HPLC-grade solvents. Injections of steroids in MeOH or MeCN were made via a Rheodyne 7125 injector fitted with a 20 ul loop. Detection was effected with a Cecil 2112 or Cecil 1220 UV monitor. The preparation of the steroid esters and their oximes has been described elsewhere [1-7].

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RESULTS AND DISCUSSION

Oximes of esters of levonorgestrel (2-4), northethisterone (6-11) and testosterone (13,14) were all found to be resolvable on ODS-Hypersil, using MeOH-H_2O as mobile phase. By contrast, the oxime isomers (5) of norethisterone itself, like those of levonorgestrel (1), did not resolve on this stationary phase, but could be separated either under reverse phase conditions on Phenyl-Hypersil, or on under normal phase conditions on Hypersil.

It was observed that, for each sample containing a syn/anti mixture, the minor ester oxime isomer generally, but not always, eluted before the major isomer, whereas for the 17-hydroxysteroid oximes, the major isomer eluted first on both Phenyl-Hypersil and Hypersil. The crystallized oximes obtained from reactions of steroidal 4-en-3-ones with hydroxylamine

generally contain a ratio >1 of anti/syn isomers, but this alone cannot be used as an infallible guide to identity for any particular sample presented for analysis, since factors such as recrystalization, chromatography and metabolism, as well as isomerization in solution, may affect the ratio.

Preparative TLC of levonorgestrel cyclopentylcarboxylate oxime (2) afforded pure samples of the syn and anti isomers, which were identified by their NMR spectra. In agreement with the published data [10-12] for steroid oximes, the anti isomer shows a singlet at 5.86 ppm for the olefinic hydrogen H-4, whereas the syn isomer shows 6.54 ppm. The UV spectra of the two isomers both exhibit a maximum at 241 nm (MeOH), but are significantly different in other respects: the anti isomer has a more intense but narrower absortion band than the syn isomer. Quantitative HPLC analysis of solutions containing each of these two isomers were then performed on an ODS-Hypersil column, varying the wavelength of the UV detector over the range 220-270 nm. The results, plotted in Figure 2, indicate two important points: firstly, that when quantitative analysis of steroidal 4-en-3-one oxime isomer mixtures is being carried out by HPLC, UV detection at 240-245 nm will give maximum sensitivity, whereas detection at 255 nm will ensure that equal responses are obtained for both isomers, making calibration easier when only a mixed standard is available; secondly, that if a mixture of the isomers is analysed first at 240 nm and then at 265 nm, there will be a change in relative response, with the peak area ratio for syn/anti increasing. This peak ratio method was found to be generally applicable to the steroidal oximes, identity being checked by correlation of the NMR chemical shifts of the major and minor isomers with their relative elution orders.

The HPLC results are shown in Table 1. For all cases of the oximes of esters of levonorgestrel, northethisterone and testosterone which were examined

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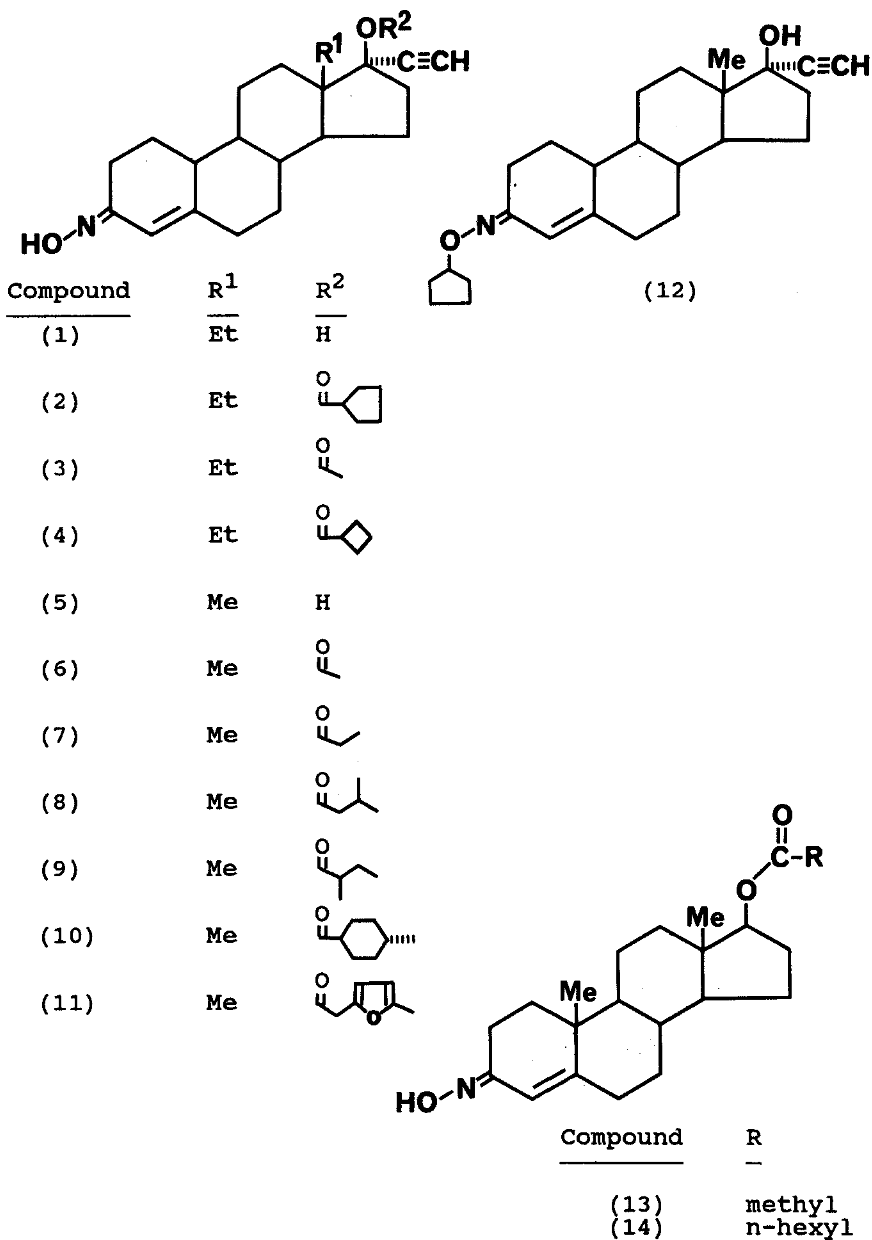

Figure 1 **Structures of steroid oximes examined**

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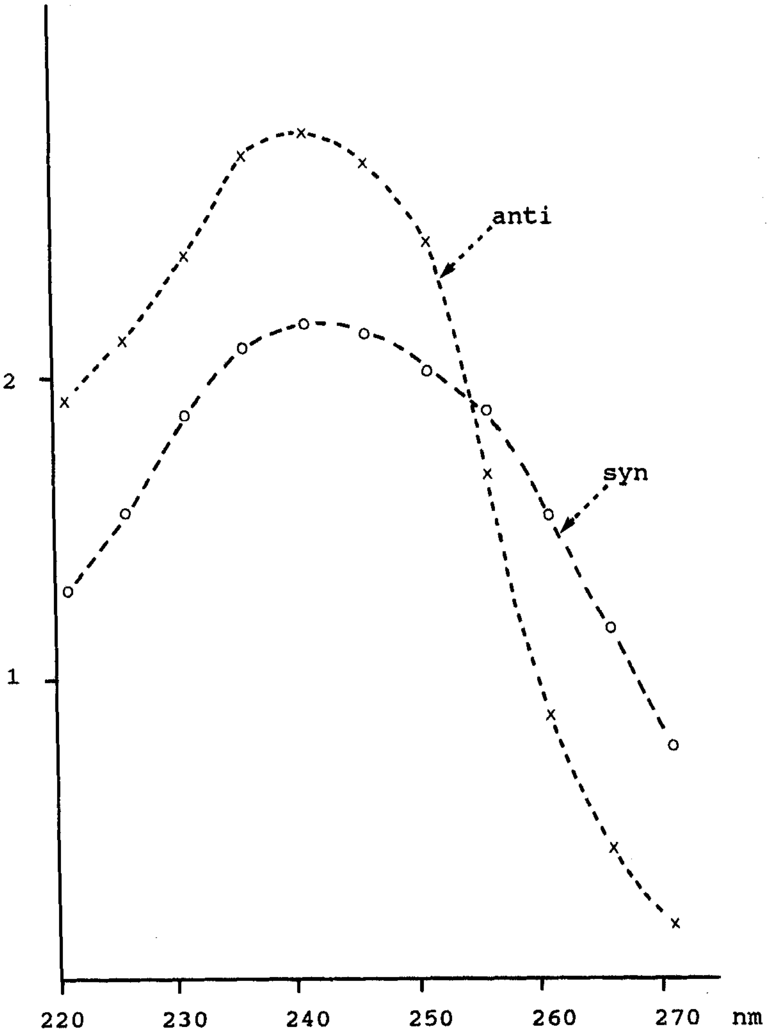

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Figure 2 Quantitative responses at different wavelengths to equimolar injections of syn and anti levonorgestrel cyclopentylcarboxylate oximes

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Table 1 **HPLC results for syn (S) and anti (A) steroid oximes**

Compound	ODS-Hypersil				Phenyl-Hypersil				Hypersil			
	MP	k'_S	k'_A	$\alpha_{A/S}$	MP	k'_S	k'_A	$\alpha_{A/S}$	MP	k'_S	k'_A	$\alpha_{A/S}$
1	a	1.82	1.82	1.00	b	4.55	3.50	0.77	c	2.53	1.73	0.68
	b	11.71	11.43	0.98								
	d,b	11.78	12.44	1.06								
2	e	3.11	3.43	1.10	f	7.95	7.95	1.00				
3	a	5.72	6.28	1.21								
	d,a	3.36	3.64	1.08								
4	e	3.46	3.79	1.10								
5	f	2.50	2.50	1.00					g	4.23	1.54	0.36
6	f	5.53	6.32	1.14					g	6.50	3.17	0.49
	d,f	5.28	5.76	1.09								
7	f	9.53	10.95	1.15					h	9.33	4.00	0.43
8	i	1.88	2.12	1.13					j	5.86	4.93	0.84
9	i	2.75	3.13	1.25					j	5.43	4.71	0.87
10	i	7.53	8.24	1.09					j	5.29	4.64	0.88
11	i	2.88	3.13	1.09					j	5.36	4.57	0.85
12	i	8.31	8.19	0.99	f	9.00	6.82	0.76	k	0.71	0.54	0.76
13									c	2.20	1.47	0.67
14	e	6.22	6.50	1.05					c	1.87	1.27	0.68

a. Mobile Phase: $MeOH-H_2O$, 75:25 v/v
b. Mobile Phase: $MeOH-H_2O$, 60:40 v/v
c. Mobile Phase: CH_2Cl_2-MeCN, 80:20 v/v
d. Octyl-Hypersil instead of ODS-Hypersil
e. Mobile Phase: $MeOH-H_2O$, 85:15 v/v
f. Mobile Phase: $MeOH-H_2O$, 70:30 v/v
g. Mobile Phase: MeCN-i-PrOH, 96:4 v/v
h. Mobile Phase: MeCN-i-PrOH, 99:1 v/v
i. Mobile Phase: $MeOH-H_2O$, 80:20 v/v
j. Mobile Phase: CH_2Cl_2-MeCN, 95:5 v/v
k. Mobile Phase: Hexane-EtOAc, 80:20 v/v

on ODS-Hypersil, the syn isomer always eluted before the anti isomer. The acetate ester oximes of levonorgestrel (3) and norethisterone (6) were also examined on an Octyl-Hypersil column: again, each syn isomer eluted before the corresponding anti oxime, but with poorer separation factors than on the octadecyl phase (Table 1). During analysis of the levonorgestrel acetate oxime, it was observed that a methanol solution freshly prepared from the solid oxime sample had an anti:syn ratio of 65:35, but this changed to 40:60 after the solution had stood for several hours.

The oxime isomers of levonorgestrel (1) and norethisterone (5) and the O-cyclopentyl ether of norethisterone oxime (12) all failed to resolve on an ODS phase, but were well separated either under reverse phase conditions on Phenyl-Hypersil, or under normal phase conditions on Hypersil. They all showed the elution order anti<syn on both these phases and this also proved to be the case for the ester oximes, which in every case examined on silica gave a substantially shorter retention for the anti than for the syn isomer. The shift in intensity ratio of the syn and anti peaks was also observed for the O-cyclopentyl oxime (12) and was also shown to occur when using normal phase conditions, e.g. for the testosterone alkanoate oximes (13) and (14). Thus, this two-wavelength peak ratio method has wide applicability and provides a very convenient and very sensitive alternative to the use of NMR to establish isomer identity for the oximes of steroidal 4-en-3-ones.

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