Journal of Chromatography, 366 (1986) 335–341
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 782

REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF STEROIDS WITH THE β-CROTONATE SIDE CHAIN

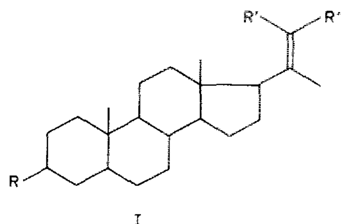
PAVEL DRAŠAR*, VLADIMÍR POUZAR, IVAN ČERNÝ and MIROSLAV HAVEL
Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Flemingovo 2, 166 10 Prague (Czechoslovakia)
(First received March 28th, 1986; revised manuscript received May 6th, 1986)

SUMMARY

An isocratic high-performance liquid chromatographic separation of some ethyl 3-stereoidyl crotonates [ethyl-24-nor-20(22)-cholen-23-oate derivatives] was developed. The separations were achieved by reversed-phase chromatography (Separon Si C_{18}) using methanol, methanol–water, methanol–0.1 *M* formic acid and ethanol–0.01 *M* aqueous phosphoric acid mixtures as mobile phases. The steroidal crotonates were detected at 230 and 240 nm.

INTRODUCTION

Steroidal crotonates of the (20*E*)-24-nor-20(22)-cholen-23-oate type (I) are biologically and pharmaceutically[1–4] as well as synthetically[1–5] important derivatives. Members of this group show activities such as inhibition of $Na^{+}K^{+}$-ATPase or positive inotropic activity.



R = OH, OCH_2CH_3, $OOC(CH_2)_2COOH$, $OOC(CH_2)_2COOCH_2CCl_3$
R' and R'' = H or $COOC_2H_5$

High-performance liquid chromatographic (HPLC) separations of similar types of steroids have been reviewed several times[6,7]; this communication describes a continuation of work on some related separations published previously[8–11]. In order to determine the residues of untransformed steroidal crotonates in biological material, as well as to simplify checking of the synthetic procedures and establishment of the chemical purity of the materials, we developed an isocratic HPLC method. This method allows the separation of members of series having the same steroidal skeleton, or in some cases also members of series with different steroidal skeletons, but with

0021-9673/86/$03.50 © 1986 Elsevier Science Publishers B.V.

the same substituent at position $C_{(3)}$; it also allows the simple determination of these steroidal crotonates. It employs UV-monitored C_{18} reversed-phase chromatography with a polar eluent.

EXPERIMENTAL

Apparatus

The liquid chromatograph consisted of a Milton Roy/LDC pump system support unit I, a stainless-steel column (250 mm × 4 mm I.D.) packed with Separon Si C_{18} (10 μm) (Laboratory Instruments, Prague, Czechoslovakia), a variable-wavelength UV detector UVM-4 (Development Workshop of the Czechoslovak Academy of Sciences, Prague, Czechoslovakia) and a Knauer No. 63.00.00 loop injection valve.

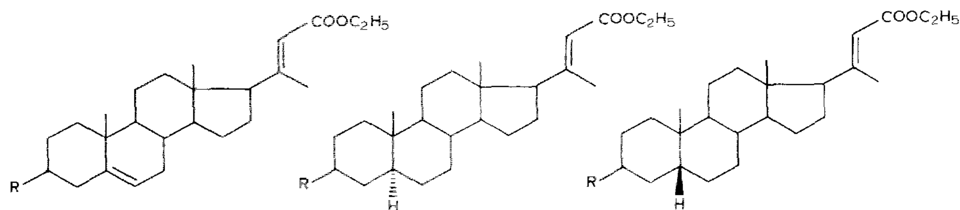
Chromatography

Samples of 10 mg of steroidal crotonates were dissolved in 1 ml of dichloromethane–methanol (1:1). The column was eluted by methanol, methanol–water, methanol–0.1 *M* aqueous formic acid, ethanol–0.01 *M* aqueous phosphoric acid mixtures at flow-rates from 1.25 to 2.25 ml/min at 25°C. Based on the individual UV spectra of the steroidal crotonates, a value of 230 nm (240 nm in one specific case) was chosen as a suitable wavelength for the detection of these compounds. In the determination of the capacity factors, k', the hold-up time, t_0, was evaluated as the retention time of the first inflection point on the chromatogram, which was considered to represent the front of the chromatographic zone.

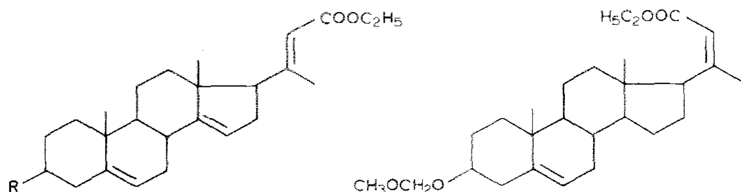
Materials and chemicals

Methanol was *pro analysi* grade (Lachema, Brno, Czechoslovakia), water and ethanol were special HPLC grade (Service Laboratory of the Institute), formic and phosphoric acids were *pro analysi* grade, 99.7 and 84%, respectively (Lachema).

Ethyl (20*E*)-3β-methoxymethoxy-24-nor-5,20(22)-choladien-23-oate (II), ethyl (20*E*)-3β-methoxymethoxy-24-nor-5α-chol-20(22)-en-23-oate (III), ethyl (20*E*)-3β-methoxymethoxy-24-nor-5β-chol-20(22)-en-23-oate (IV), ethyl (20*E*)-3β-methoxymethoxy-24-nor-5,14,20(22)-cholatrien-23-oate (V), ethyl (20*E*)-3β-hydroxy-24-nor-5,20(22)-choladien-23-oate (VI), ethyl (20*E*)-3β-hydroxy-24-nor-5α-chol-20(22)-en-23-oate (VII), ethyl (20*E*)-3β-hydroxy-24-nor-5β-chol-20(22)-en-23-oate (VIII), ethyl (20*E*)-3β-hydroxy-24-nor-chola-5,14,20(22)-trien-23-oate (IX), ethyl (20*E*)-3β-hydroxy-24-nor-5,20(22)-choladien-23-oate 3-[4-(2,2,2-trichloroethoxy)-4-oxobutanoate] (X) and its 3-(3-carboxypropanoate) (XIV), ethyl (20*E*)-3β-hydroxy-24-nor-5α-chol-20(22)-en-23-oate 3-[4-(2,2,2-trichloroethoxy)-4-oxobutanoate] (XI), and its 3-(3-carboxypropanoate) (XV), ethyl (20*E*)-3β-hydroxy-24-nor-5β-chol-20(22)-en-23-oate 3-[4-(2,2,2-trichloroethoxy)-4-oxobutanoate] (XII), and its 3-(3-carboxypropanoate) (XVI), ethyl (20*E*)-3β-hydroxy-24-norchola-5,14,20(22)-trien-23-oate 3-[4-(2,2,2-trichloroethoxy)-4-oxobutanoate] (XIII) and its 3-(3-carboxypropanoate) (XVII) and the only *Z* isomer ethyl (20*Z*)-3β-methoxymethoxy-24-nor-5,20(22)-choladien-23-oate (XVIII) were prepared according to the literature[4].



II, R = OCH_2OCH_3	III, R = OCH_2OCH_3	IV, R = OCH_2OCH_3
VI, R = OH	VII, R = OH	VIII, R = OH
X, R = $OOC(CH_2)_2COOCH_2CCl_3$	XI, R = $OOC(CH_2)_2COOCH_2CCl_3$	XII, R = $OOC(CH_2)_2COOCH_2CCl_3$
XIV, R = $OOC(CH_2)_2COOH$	XV, R = $OOC(CH_2)_2COOH$	XVI, R = $OOC(CH_2)_2COOH$



V, R = OCH_2OCH_3
IX, R = OH
XIII, R = $OOC(CH_2)_2COOCH_2CCl_3$
XVII, R = $OOC(CH_2)_2COOH$

XVIII

RESULTS AND DISCUSSION

The described systems allowed the analysis of all seventeen steroidal crotonates (II–XVIII). The chromatograms obtained typically showed well resolved peaks, except for the free hemisuccinates XIV–XVII with mobile phases without any acid. Over a period of several weeks the retention times of the steroidal crotonates varied slightly within about $\pm 4\%$. The retention times of hemisuccinates XIV–XVII with a non-acidified mobile phase varied more, and sometimes were greater with lower amounts of steroidal crotonate analysed. This behaviour could be explained tentatively by the detergent-like properties of the steroid acid derivatives XIV–XVII.

The wavelength chosen (230 nm) was characteristic of steroidal derivatives the type I and corresponds to the very broad maximum having log ε about 4.2 at wavelengths between 215 and 230 nm due to the n–π^* transition of the conjugated ester moiety in the side chain[4]. It enabled us to analyse mixtures of steroidal crotonates in the presence of other types of steroidal derivatives with low extinction in this region. However, with methanol–aqueous formic acid as eluent we chose a wavelength of 240 nm because of the absorption cut-off of the mobile phase at about 230 nm. It is necessary to work with a freshly prepared mixture only, however, since after several hours the cut-off moves to higher wavelengths due to the formation of traces of methyl formate.

However, the analysis of all the seventeen steroidal crotonates II–XVIII in one chromatographic experiment was not the exclusive aim of this work, as there is no

interest in applying them together in biological material or in studying the retention of mixtures containing all crotonates. There is a good possibility to analyse mixtures of derivatives of one parent steroid skeleton with different substituents at position $C_{(3)}$, *i.e.*, the groups II, VI, X, XIV; III, VII, XI, XV; IV, VIII, XII, XVI; V, IX, XIII, XVII in most of the mobile phases. Moreover, groups of derivatives having the same substitution at position $C_{(3)}$, *i.e.*, II–V, VI–IX, X–XIII and XIV–XVII could be analysed in several mobile phase systems.

In particular, the separation of isomer II and XVIII was studied, in the mobile phases used, with the exception of methanol–water (8:2) where the peaks were too broad and tailing.

As the basic solvent system, methanol–water was chosen, the content of water increasing from 0 to 30% (pure methanol to methanol–water, 7:3). Mobile phases with water contents higher than 20% appeared to be unsuitable, as the steroidal crotonates are not sufficiently soluble in such systems and the chromatograms show broad, tailing peaks with very long retention times (Table I).

These mobile phases are not generally suitable for analyses of the hemisuccinates XIV–XVII due to very broad tailing and often split peaks with bad reproducibility (see above). Nevertheless, these compounds could be analysed by addition of acid to the methanol–water mixture. A suitable mixture for analysis of all seventeen crotonates including the hemisuccinates was methanol–0.1 *M* aqueous formic acid (9:1). In spite of the fact that ethanol itself is not suitable for analyses of compounds II–XVIII because of its lower polarity in comparison with methanol, the mixture of ethanol and aqueous 0.01 *M* phosphoric acid (9:1) was one of the best mobile phase systems. This mixture also avoids the insufficient UV transparency of the mixture containing formic acid. It could also be used for a long time, without any change (contrary to the above mixture with formic acid).

The retention times, capacity factors and relative retentions of compounds II–XVIII are summarized in Tables II–VI. The best results were obtained with ethanol–0.01 *M* aqueous phosphoric acid (9:1), all the steroidal crotonates II–XVIII being successfully separated in an isocratic experiment (Table VI). The separation

TABLE I

RETENTION TIMES *VERSUS* WATER CONTENT IN THE METHANOL MOBILE PHASE FOR ISOMERIC CROTONATES II AND XVIII

Solvent flow-rate: 2.25 ml/min. Pressure from 5.25 MPa (1050 p.s.i.) through 12.67 MPa (1850 p.s.i.). Column packing: Separon Si C_{18} (10 μm). Samples were applied in dichloromethane–methanol (1:1) solution. Detection at 230 nm.

Water in methanol (%)	*Retention time (min)*	
	II	*XVIII*
0	4.8	4.5
5	9.0	7.16
10	21.67	16.33
15	28.83	23.3
20	78.62	64.0

TABLE II

RETENTION TIMES, t_R, CAPACITY FACTORS, k', AND RELATIVE RETENTIONS WITH METHANOL

Solvent flow-rate: 1.25 ml/min. Pressure: 4.65 MPa (675 p.s.i.). Other details as in Table I.

Compound	t_R *(min)*	k'	*Relative retention*	*Compound*	t_R *(min)*	k'	*Relative retention*
II	7.97	3.55	2.03	XI	6.25	2.57	1.60
III	8.58	3.90	2.19	XII	5.17	1.95	1.32
IV	7.45	3.26	1.90	XIII	5.30	2.03	1.35
V	7.47	3.27	1.90	XIV*	20.67	10.81	5.27
VI	4.00	1.29	1.02	XV*	32.00	17.29	8.16
VII	4.42	1.53	1.13	XVI*	18.83	9.76	4.80
VIII	3.92	1.24	1.00	XVII*	38.00	20.71	9.69
IX	3.95	1.26	1.01	XVIII	6.81	2.89	1.74
X	5.60	2.20	1.43				

* Broad tailing peak.

TABLE III

RETENTION TIMES, t_R, CAPACITY FACTORS, k', AND RELATIVE RETENTIONS WITH METHANOL–WATER (9:1)

Solvent flow rate: 2.25 ml/min. Pressure: 10.69 MPa (1550 p.s.i.). Other details as in Table I.

Compound	t_R *(min)*	k'	*Relative retention*	*Compound*	t_R *(min)*	k'	*Relative retention*
II	21.67	20.67	3.02	XI	27.67	26.67	3.86
III	24.23	23.23	3.38	XII	19.33	18.33	2.70
IV	21.66	20.66	3.02	XIII	20.43	19.43	2.85
V	22.50	21.50	3.14	XIV	10.67	9.67	1.49
VI	7.23	6.23	1.01	XV	12.50	11.50	1.74
VII	8.60	7.60	1.20	XVI	7.17	6.17	1.00
VIII	7.23	6.23	1.01	XVII	10.67	9.67	1.49
IX	7.23	6.23	1.01	XVIII	16.33	15.33	2.28
X	24.17	23.17	3.37				

TABLE IV

RETENTION TIMES, t_R, CAPACITY FACTORS, k', AND RELATIVE RETENTIONS WITH METHANOL–WATER (8:2)

Solvent flow-rate: 2.25 ml/min. Pressure: 12.76 MPa (1850 p.s.i.). Other details as in Table I. The chromatographic conditions used are not suitable for the separation of the less polar compounds and steroidal acids II–V and X–XVIII.

Compound	t_R *(min)*	k'	*Relative retention*
VI	32.00	31.43	1.13
VII	38.90	38.43	1.37
VIII	31.50	30.93	1.11
IX	28.33	27.71	1.00

TABLE V

RETENTION TIMES, t_R, CAPACITY FACTORS, k', AND RELATIVE RETENTIONS WITH METHANOL–0.1 *M* AQUEOUS FORMIC ACID (9:1)

Solvent flow-rate: 1.25 ml/min. Pressure: 6.48 MPa (940 p.s.i.). Other details as in Table I, except detection at 240 nm.

Compound	t_R *(min)*	k'	*Relative retention*	*Compound*	t_R *(min)*	k'	*Relative retention*
II	12.73	19.10	3.64	XI	14.62	22.08	4.18
III	13.93	20.99	3.98	XII	10.75	15.97	3.07
IV	12.40	18.58	3.54	XIII	11.48	17.13	3.28
V	11.73	17.52	3.35	XIV	4.19	5.62	1.20
VI	4.58	6.23	1.31	XV	4.95	6.82	1.41
VII	5.35	7.45	1.53	XVI	3.50	4.53	1.00
VIII	4.47	6.06	1.28	XVII	3.97	5.27	1.13
IX	4.29	5.77	1.23	XVIII	9.55	14.09	2.73
X	12.87	19.32	3.68				

obtained with methanol–0.1 *M* aqueous formic acid (9:1) was similar, but with the disadvantage of aging of the mobile phase and a higher cut-off.

When comparing the separations with methanol or methanol–water as mobile phase it can be concluded that the separation is in most cases sufficient, with the exception of several pairs, *e.g.*, IV, V or VIII, IX and hemisuccinates XIV–XVII with methanol, groups II, IV or VI, VIII, IX and XIV–XVII with methanol–water (9:1). However, with methanol–water (8:2) we could perform useful separations only in the case of alcohols VI–IX. The use of systems with a water content higher than 20% resulted in broad tailing peaks and very high retention times due to the low solubility of the steoidal derivatives, as could be anticipated. In several cases, such systems also resulted in strong retention of the compounds analysed.

With the exception of the chromatographic behaviour of the hemisuccinates

TABLE VI

RETENTION TIMES, t_R, CAPACITY FACTORS, k', AND RELATIVE RETENTIONS WITH ETHANOL–0.01 *M* AQUEOUS PHOSPHORIC ACID (9:1)

Solvent flow-rate: 1.25 ml/min. Pressure: 11.38 MPa (1650 p.s.i.). Other details as in Table I.

Compound	t_R *(min)*	k'	*Relative retention*	*Compound*	t_R *(min)*	k'	*Relative retention*
II	9.00	4.40	2.55	XI	8.92	4.35	2.53
III	9.67	4.80	2.74	XII	7.17	3.30	2.03
IV	8.50	4.10	2.41	XIII	7.42	3.45	2.10
V	8.25	3.95	2.34	XIV	4.05	1.53	1.15
VI	4.23	1.64	1.20	XV	4.15	1.59	1.18
VII	4.61	1.88	1.31	XVI	3.53	1.21	1.00
VIII	4.27	1.67	1.21	XVII	3.62	1.26	1.03
IX	4.09	1.56	1.16	XVIII	7.33	3.58	2.07
X	8.25	3.95	2.34				

XIV–XVII in methanol–water mixtures and methanol, the retention times reflect to the polarities of the molecules, as expected.

This chromatographic separation method is a useful tool for the identification of compounds and the establishment of their purity in many cases where thin-layer chromatography is not suitable. A study of the behaviour of other analogues of steroidal cardiotonics and related derivatives is now in progress.

ACKNOWLEDGEMENTS

The authors thank Drs. Ivan Rosenberg, Tomáš Vaněk and Miroslav Ledvina of this Institute for valuable discussions.

REFERENCES

1 F. Theil, C. Lindig and K. Repke, *J. Prakt. Chem.*, 322 (1980) 1012.
2 R. Thomas, J. Boutagy and A. Gelbart, *J. Pharm. Sci.*, 63 (1974) 1649.
3 R. Thomas, J. Boutagy and A. Gelbart, *J. Pharmacol. Exp. Ther.*, 191 (1974) 219.
4 I. Černý, V. Pouzar, P. Drašar, F. Tureček and M. Havel, *Collect. Czech. Chem. Commun.*, 51 (1986) 128.
5 J. Boutagy and R. Thomas, *Chem. Rev.*, 74 (1974) 87.
6 Sj. van der Wal and J. F. K. Huber, *J. Chromatogr.*, 251 (1982) 289.
7 E. Heftmann and I. R. Hunter, *J. Chromatogr.*, 165 (1979) 283.
8 P. Drašar, V. Pouzar, I. Černý, M. Havel, F. Tureček, D. Schmiedová and K. Vereš, *Collect. Czech. Chem. Commun.*, 50 (1985) 2760.
9 P. Drašar, V. Pouzar, I. Černý and M. Havel, *Collect. Czech. Chem. Commun.*, 49 (1984) 1051.
10 P. Drašar, V. Pouzar, I. Černý, J. Smolíková and M. Havel, *Collect. Czech. Chem. Commun.*, 49 (1984) 1039.
11 P. Drašar, V. Pouzar, I. Černý and M. Havel, *J. Chromatogr.*, 283 (1984) 396.