Journal of Chromatography, 489 (1989) 139–155
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4602

ON-LINE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DIODE ARRAY SPECTROPHOTOMETRIC ANALYSIS OF STEROIDAL HORMONES IN ILLEGAL PREPARATIONS

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SUMMARY

An on-line high-performance liquid chromatographic diode array spectroscopic analytical method for the identification of more than 60 different steroidal compounds is described. For the chromatographic separation, a gradient elution that could distinguish the esterified and non-esterified steroids in the same run on a reversed-phase C_{18} column, using methanol as modifier, was developed. For both types of compound an internal standard was chosen to establish reproducible relative retention times that could be used as one element of the identification; the second element is the UV spectrum, which is recorded on-line during the separation. The combination of chromatographic and UV spectroscopic recordings selects only a few probable steroids, which could be the unknown. This approach has been applied to forensic analysis of illicit preparations used in cattle-breeding, some examples of which are shown. For those steroids that are very difficult to distinguish using this procedure, because of their chromatographic and spectroscopic similarity on this system, other solvent mixtures are used in place of methanol as modifier, namely acetonitrile or tetrahydrofuran, or both, with the same solvent strength, as proposed by Snyder. In this way totally different elution patterns and separations are obtained, providing complementary information for identification, as shown by the systematic analysis on two other isoeluotropic solvent systems.

INTRODUCTION

Several studies of the separation of particular steroids by high-performance liquid chromatography (HPLC) have been reported by Roos for estrogens [1,2], Sokolova et al. for gestagens and impurities [3] and Williams and Biehl for corticosteroids [4]. Tymes [5] described a reversed-phase HPLC method for the determination of corticoids and related analogues. Rapp and Meyer [6] used HPLC–UV and radioimmunoassay (RIA) for quantitation of nortestosterone in preparations after hydrolysis. Jansen and co-workers [7,8] combined HPLC with

0378-4347/89/$03.50

diode array detection and radioreceptor methods to identify and quantify different hormones.

In this paper, the reversed-phase HPLC properties of about 60 pure steroidal compounds, as well anabolics, estrogens, gestagens and corticosteroids, are systematically described. A gradient elution method using methanol as modifier is combined with an on-line coupled diode-array detector to record the UV spectra of eluting peaks. From the results obtained, a reliable and reproducible standardized identification method is developed for free and esterified steroidal compounds that are often mixed in illegal cocktails [9,10]. Complete chromatographic coincidence of the unknown peaks in the cocktails with the corresponding standard steroids and accurate matching of their UV spectra have to be achieved so as to confirm peak identity.

By means of a simple BASIC search program, consulting a database with the retention parameters of all the steroids examined on this initial elution system, those steroids that have relative retention times close to the measured ones are selected. The corresponding probable steroids then are injected on the HPLC system together with the unknown samples to check for complete co-elution.

The steroids with similar UV spectra and identical retention times on this initial gradient system are further analysed in isoeluotropic solvent systems, resulting in totally different separation patterns. The necessary complementary information to identify unequivocally the steroids of interest, is thus obtained.

An advantage of this method over other published methods is that it scans within the different classes of steroids as gestagens, estrogens, anabolics and corticoids, and permits mostly direct identification of many steroids esters. In addition, isoeluotropic solvent systems that provide different chromatographic selectivity are available for identity confirmation.

EXPERIMENTAL

Apparatus and reagents

We used a Varian 5000 gradient elution HPLC instrument (Varian, Palo Alto, CA, U.S.A.) coupled on-line to a Hewlett-Packard 8450A UV–VIS diode array spectrophotometer (Hewlett-Packard, Palo Alto, CA, U.S.A.) ranging from 200 to 800 nm. This enabled us to record UV spectra of eluting compounds after HPLC separation, through adapted flow-cells (178-32, Helma, Oss, The Netherlands) with stainless-steel tubes suitable for directional bending. A manual injector with a 20-μl loop from Altex (Beckman, Berkeley, CA, U.S.A.) was used.

The samples were diluted with methanol (Baker Analyzed HPLC reagent, Baker Chemicals, Deventer, The Netherlands), which was also used as an HPLC solvent. The other HPLC solvents used were acetonitrile (Burdick & Jackson Labs., Muskegon, MI, U.S.A.) and HPLC-quality tetrahydrofuran (THF) (Merck, Darmstadt, F.R.G.) and high-purity water (Alltech Assoc., Deerfield, IL, U.S.A.). The HPLC column was the Nova Pak C_{18} column (15 cm $\times$ 4.6 mm I.D.) supplied by Millipore-Waters, (Milford, MA, U.S.A.).

Methods

To study the reversed-phase HPLC properties of more than 60 substances for identification purposes the chromatographic system must be reliable, so one of the most important steps is the selection of a high-quality column. The method of Verzele and Dewaele [11] for the evaluation of reversed-phase HPLC chromatographic materials provides information about the column efficiency, the degree of C_{18} coating, the deactivation by endcapping and the degree of demineralization of the silica. Following this evaluation procedure, we selected the Nova Pak C_{18} column.

The second important requirement is the simultaneous separation in one chromatographic run of both free and long-chain fatty acid esterified steroids. Therefore a suitable gradient elution programme was developed, starting from 55% water and 45% solvent B (methanol), rising to 100% solvent B in 12 min and keeping solvent B at 100% for the remainder of the analysis. In the initial HPLC elution system solvent B is pure methanol as organic modifier. In the two other complementary elution systems, methanol is substituted by isoeluotropic acetonitrile (82%, v/v, in water) as solvent C and by an isoeluotropic mixture of acetonitrile–THF–water (41:29:30, v/v) as solvent D. These recommended volume ratios are calculated from the solvent strength weighting factors described by Snyder [12,13]. The flow-rate is kept constant at 2 ml/min, except for the third elution system with solvent D, where a flow-rate of 1.7 ml/min is maintained.

Three internal standards were selected to establish more useful relative retention times on the initial gradient elution system with solvent B:

(1) Fluorometholone: this is suitable for steroids with a UV maximum near 240 nm and eluting within 500 s.

(2) Testosterone valerate: this is suitable for steroids with a UV maximum near 240 nm and eluting from 500 to 900 s.

(3) Chlormadinone acetate: this is suitable for steroids with a UV maximum near 280 nm.

The relative retention times of all the steroids examined are measured versus their appropriate internal standards. At least five different measurements on different occasions, e.g. at column replacements or at day-to-day room temperature fluctuations, are used to calculate mean values. These means were then stored in the database, which is consulted by the BASIC search program after experimental retention times of unknown peaks have been obtained. A time-window corresponding to a deviation of $\pm 5\%$ of the stored values is included in the search program, so several standard steroids eluting near the unknown peak may be selected initially.

The chromatographic behaviour of the analysed sample is verified with the other two solvent systems C and D, provided there is no unambiguous information in the retention data obtained with elution solvent B or in the UV spectrum.

Before analysis the cocktail samples, which may be suspensions, emulsions or oily solutions, are diluted 1:25 or 1:50 with methanol, vigorously shaken and allowed to stand for a few minutes until immiscible phases separate.

Cow implants may also be used for analysis. These are extracted by shaking with five to ten times the volume of methanol, which is collected and stored for

TABLE I

STEROIDS UNDER STUDY AND THEIR RELATIVE RETENTION TIMES (RRT) VERSUS A SUITABLE INTERNAL STANDARD

HPLC solvent B; the internal standard is indicated in parentheses after each hormone name: F = fluorometholone; T = testosterone valerate; C = chlormadinone acetate.

No.	Steroid hormone	RRT
1	Androst-4-ene-3,17-dione (F)	0.8847
2	Betamethasone (F)	1.1125
3	Boldenone (F)	0.9755
4	Chlormadinone acetate (F)	0.6600
5	Chlormadinone acetate (T)	1.4512
6	Chlorotrianisene (T)	1.0788
7	Cortisone (F)	1.4909
8	Dexamethasone (F)	1.0854
9	Dexamethasone acetate (F)	0.8877
10	Dexamethasone phenyl propionate (T)	1.2345
11	Dienestrol (F)	0.8367
12	Dienestrol diacetate (C)	0.8511
13	Dienestrol diacetate (F)	0.5638
14	Diethylstilboestrol (F)	0.8820
15	Epiestriol (C)	3.1788
16	Estradiene (1,3,5,10) – 2,3,17-β-triol-2-methyl ether (F)	0.8350
17	Estradiene (1,3,5,10) – 2,3,17-β-triol-2-methyl ether (T)	1.7700
18	Estrone (C)	1.3636
19	Estrone (F)	0.8865
20	Ethinylestradiol (C)	1.3636
21	Ethisterone (C)	1.2121
22	Ethisterone (F)	0.8235
23	Ethisterone (T)	1.7451
24	Fluorohydrocortisone acetate (F)	1.0662
25	Fluoxymesterone (F)	0.9625
26	Hexestrol (C)	1.2600
27	Hydrocortisone (F)	1.3583
28	Hydrocortisone acetate (F)	1.0694
29	Hydroxyprogesterone-17-α-caproate (T)	1.1195
30	Hydroxyprogesterone-17-α-heptylate (T)	1.0565
31	Hydroxyprogesterone-17-α (F)	0.8000
32	Medroxyprogesterone acetate (C)	1.0000
33	Medroxyprogesterone acetate (F)	0.6405
34	Medroxyprogesterone acetate (T)	1.4167
35	Megestrol acetate (C)	1.0000
36	Megestrol acetate (F)	0.6613
37	Megestrol acetate (T)	1.4274
38	Mestranol (C)	0.8255
39	Metenolone acetate (T)	1.1508
40	Methandrostenolone (F)	0.8824
41	Methylprednisolone (F)	1.0845
42	Methylprednisolone acetate (F)	0.8953
43	Methyltestosterone (17-α) (F)	0.7309

TABLE I (continued)

No.	Steroid hormone	RRT
44	Methyltestosterone (17-α) (T)	1.5826
45	Nandrolone decanoate (T)	0.8541
46	Nandrolone hexyloxyphenyl propionate (T)	0.8571
47	Nandrolone laurate (T)	0.8087
48	Nandrolone phenyl propionate (T)	1.0202
49	Norethisterone (norethindrone) (F)	0.9158
50	Norgestrel (α) (F)	0.7560
51	Nortestosterone-19 (nandrolone) (F)	0.9121
52	Nortestosterone-19 (nandrolone) (T)	2.0057
53	Nortestosterone benzoate (T)	1.0324
54	Oestradiol (α) (C)	1.3333
55	Oestradiol (β) (C)	1.4328
56	Oestradiol benzoacetate (T)	0.9175
57	Oestradiol cypionate (C)	0.6356
58	Oestradiol cypionate (T)	0.9224
59	Oestradiol diacetate (C)	0.7576
60	Oestradiol diacetate (T)	1.0414
61	Oestradiol diacetate (T)	1.0833
62	Oestradiol dipropionate (C)	0.6666
63	Oestradiol dipropionate (T)	0.9674
64	Oestradiol monobenzoate (T)	1.0287
65	Oestradiol valerate (C)	0.7101
66	Oestradiol valerate (T)	1.0305
67	Oestriol (C)	3.0189
68	Prednisolone (F)	1.3475
69	Prednisone (F)	1.6200
70	Progesterone (F)	0.6293
71	Progesterone (T)	1.3700
72	Stanozolol (T)	1.2464
73	Stilboestrol dipropionate (T)	1.0576
74	Testosterone acetate (T)	1.2297
75	Testosterone (F)	0.8119
76	Testosterone benzoate (T)	1.0000
77	Testosterone cyclohexylpropionate (F)	0.4113
78	Testosterone cyclohexylpropionate (T)	0.8768
79	Testosterone cypionate (T)	0.9025
80	Testosterone isobutyrate (T)	1.0482
81	Testosterone enanthate (T)	0.9115
82	Testosterone propionate (T)	1.1266
83	Testosterone undecylenate (T)	0.8233
84	Trenbolone (F)	1.0031
85	Trenbolone acetate (F)	0.6694
86	Triamcinolone (F)	2.5056

at least 3 h in a freezer to allow the fatty material to precipitate. The clear supernatant is used for chromatographic analysis.

RESULTS

Table I lists the examined steroids and their relative retention times versus a suitable internal standard, using solvent B. For some steroids relative retention times versus two or three internal standards are recorded, so that their selection by the search program is not dependent on one internal standard. The proposed gradient system with solvent B initially was tested for analysis of a cow implant that still contained the oily liquid from a recent steroid injection (Fig. 1). Some of the peaks were identified as ethynylestradiol, medroxyprogesterone acetate, testosterone propionate, estradiol monobenzoate, testosterone cypionate and nandrolone decanoate. The unknown peak at the end of the chromatogram was eventually identified as testosterone decanoate. These identities were confirmed by co-chromatography with the probable standard steroids.

Together with the peaks giving a UV spectrum with a sharp maximum at 240 nm, a peak with an estrogen spectrum and the same retention time as ethynylestradiol was found. An other one was present, which yielded the UV spectrum of estradiol monobenzoate and its retention time. Both on-line recorded UV spectra are given in Fig. 2.

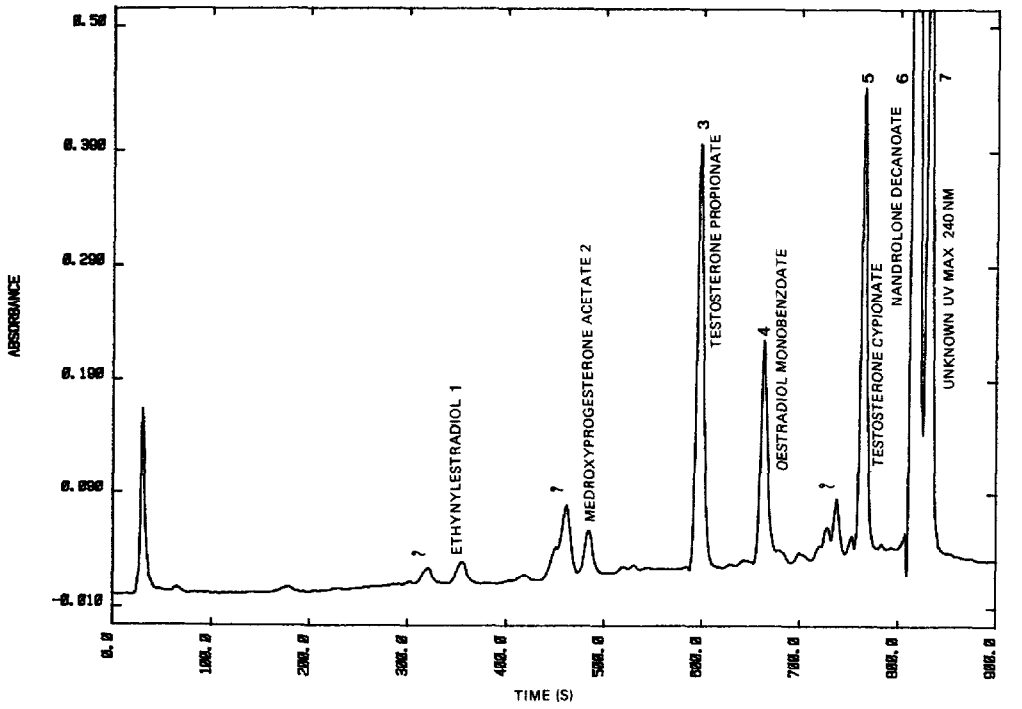

Fig. 1. Gradient elution chromatogram of the methanolic extract of an implant, sampled from a cow. The organic modifier solvent B is pure methanol, detection at 240 mm.

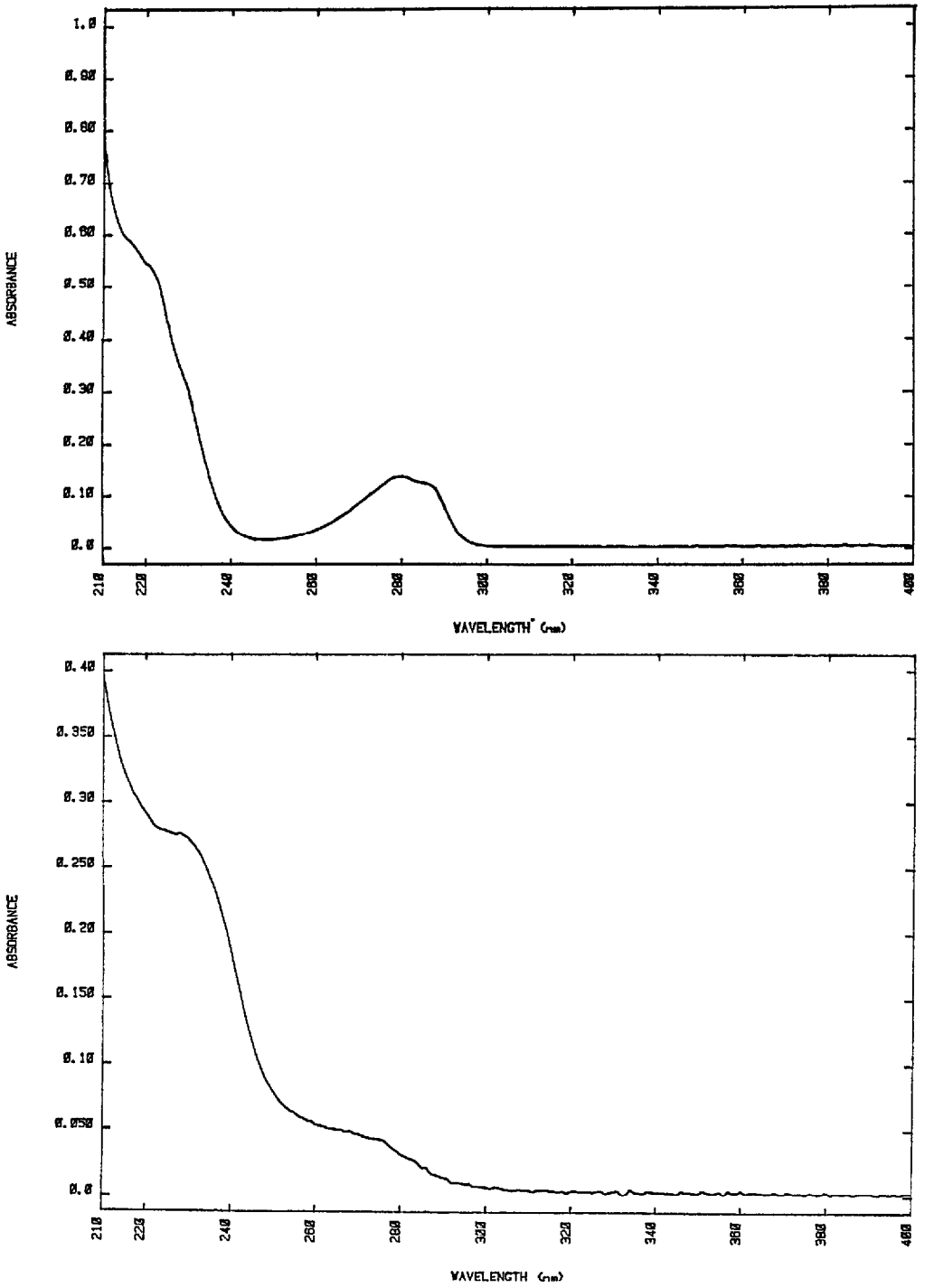

Fig. 2. On-line recorded UV spectra of peak 1 (ethynylestradiol) (upper) and of peak 4 (estradiol monobenzoate) (lower), during HPLC analysis of steroid implant. For peak positions see Fig. 1.

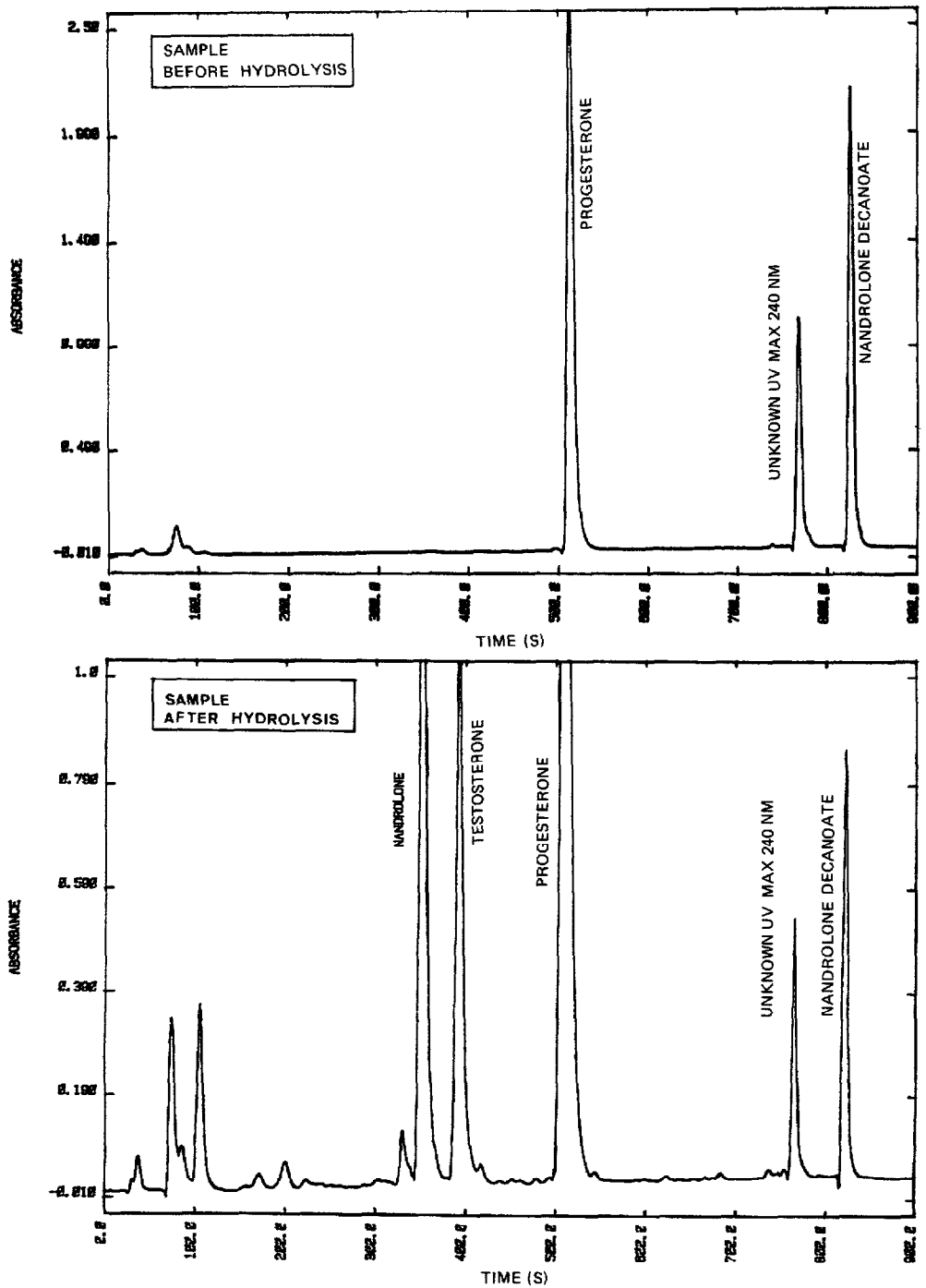

Fig. 3. Gradient elution chromatogram using solvent B of methanolic extract of illegal preparation, detected at 240 nm, before (upper) and after (lower) mild acid hydrolysis.

TABLE II

RESULTS OF A COMPUTER SEARCH FOLLOWING INPUT OF RETENTION TIMES

Give the *FIRST letter* of the used *INTERNAL STANDARD* (F or C or T)[a]
T
Give the *RETENTION TIME* of the *INTERNAL STANDARD*
690
Give the *RETENTION TIME* of the *UNKNOWN STEROID*
760

The STEROIDS with RELATIVE RETENTION TIME 0.907895 are:
1 0.9175 oestradiol benzoacetate (T)
2 0.8768 testosterone cyclohexylpropionate (T)
3 0.9025 testosterone cypionate (T)
4 0.9115 testosterone enanthate (T)
5 0.9224 oestradiol cypionate (T)

1 GIVEN: the relative retention time → WHICH STEROID?
2 GIVEN: a steroidal substance → WHICH RELATIVE RETENTION TIME?
3 ALPHABETICAL listing of SUBSTANCES and RELATIVE TIMES
4 EXIT

Select 1, 2, 3 or 4

[a]For internal standard abbreviations, see Table I.

For samples showing various peaks on the chromatogram between 500 and 900 s, the presence of long-chain fatty acid steroid esters is suspected. It can be helpful to generate the free steroids by mild acid hydrolysis in an ethanolic medium and to re-examine the hydrolysate for the presence of the free steroidal part of the components.

This is of particular interest for samples containing testosterone, nandrolone or estradiol esters. If free steroids are concerned, e.g. progesterone, no peak shift is observed (Fig. 3).

Table II presents the results of a computer search following the input of the retention times of an unknown peak and its internal standard. The standard steroids selected are further compared with the unknown peak with respect to chromatographic and UV spectroscopic similarity. Using this method, some steroids, e.g. boldenone and fluoxymesterone or testosterone benzoate and valerate or megestrol acetate and chlormadinone acetate, are faintly discernible. Here the selectivity of the C_{18} column may be influenced by other eluents. As illustrated in Figs. 4–7, substitution of solvent B for solvent C or D in the gradient elution HPLC analysis changes the separation and peak resolution.

Table III summarizes for all the steroids studied their modified elution patterns on the proposed gradient elution HPLC system using solvents B, C and D, respectively, as modifier.

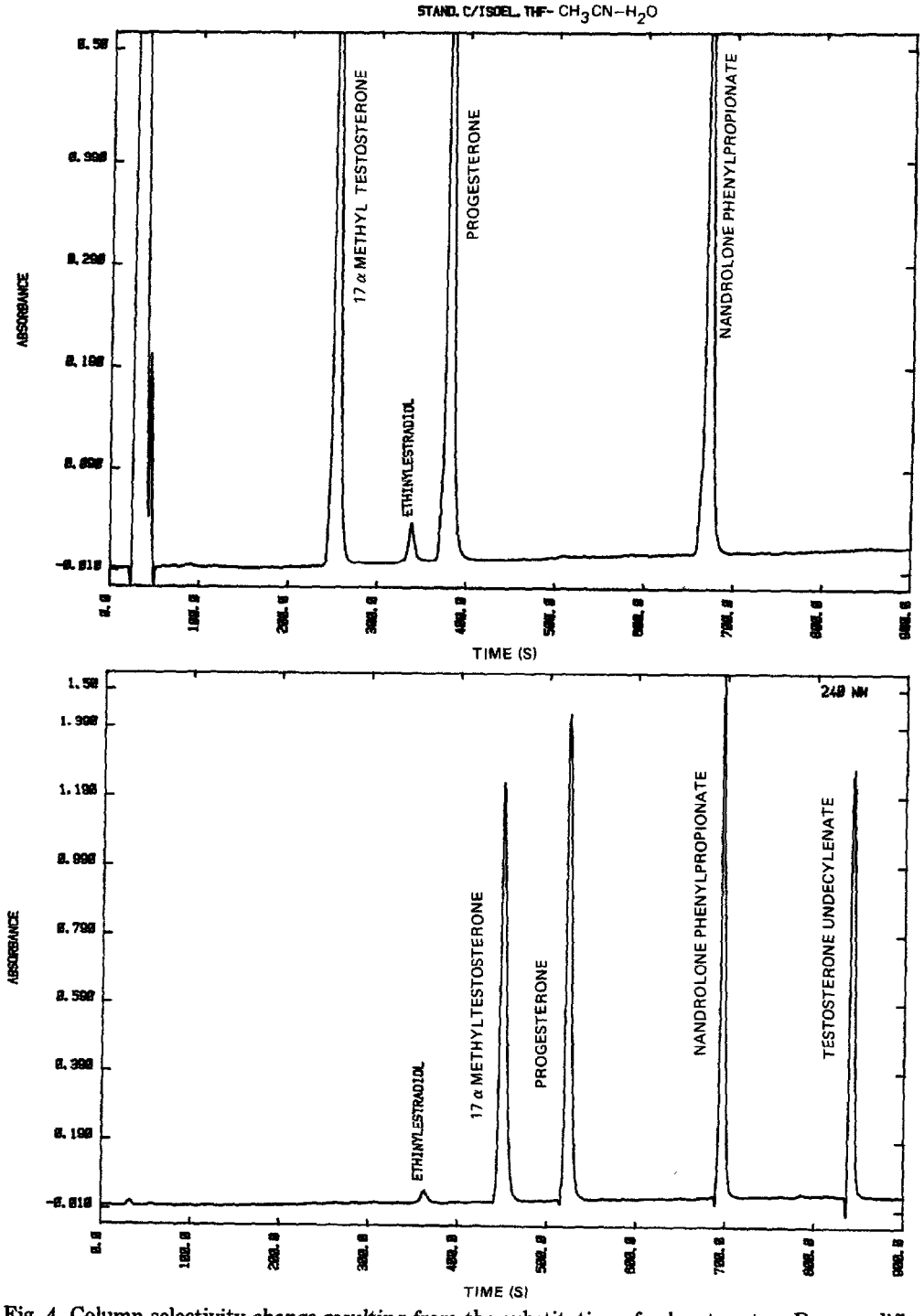

Fig. 4. Column selectivity change resulting from the substitution of solvent system D as modifier (upper) to solvent B (lower).

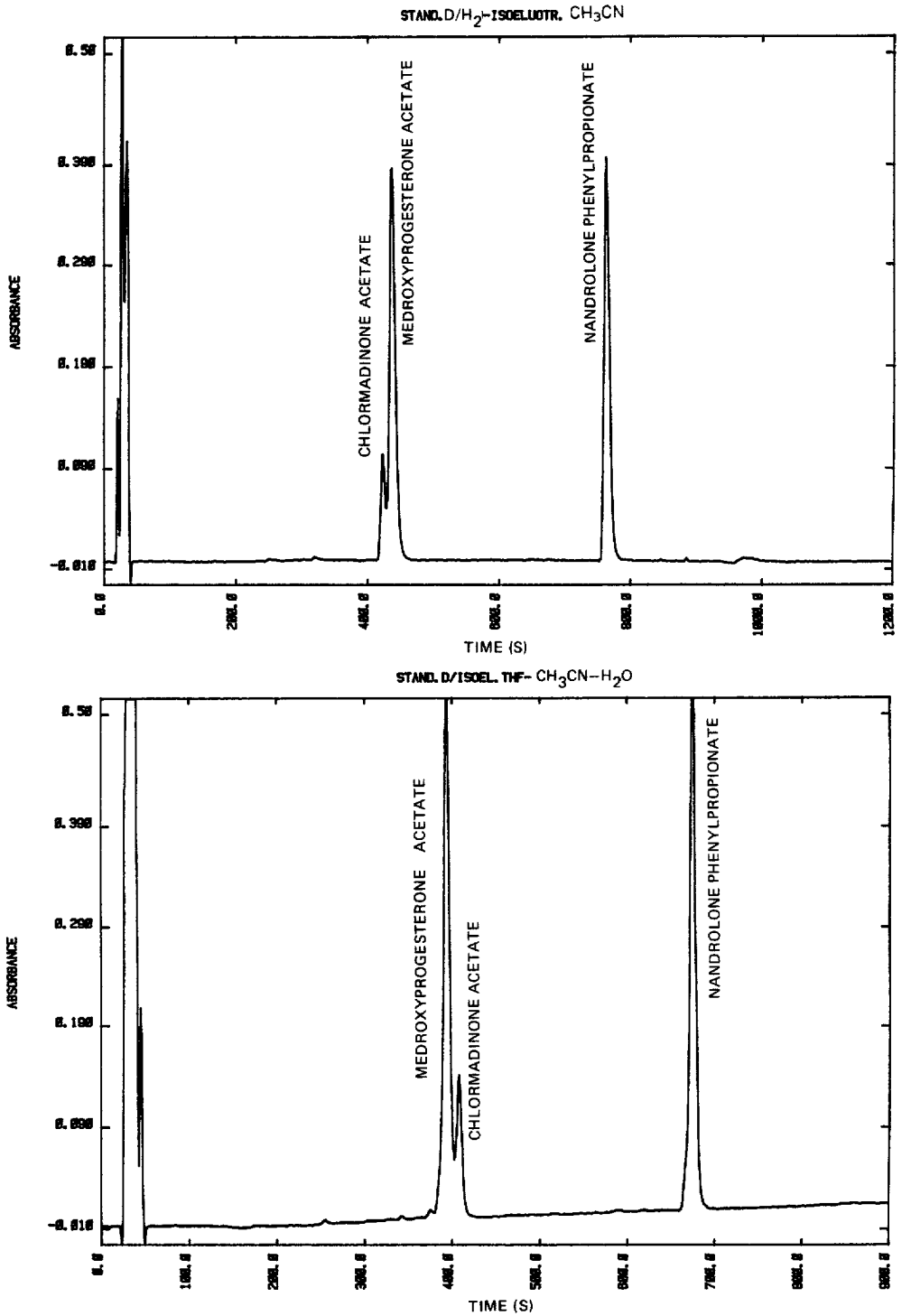

Fig. 5. Elution order change of chlormadinone acetate and medroxyprogesterone acetate on changing the organic modifier from solvent C (upper) to solvent D (lower).

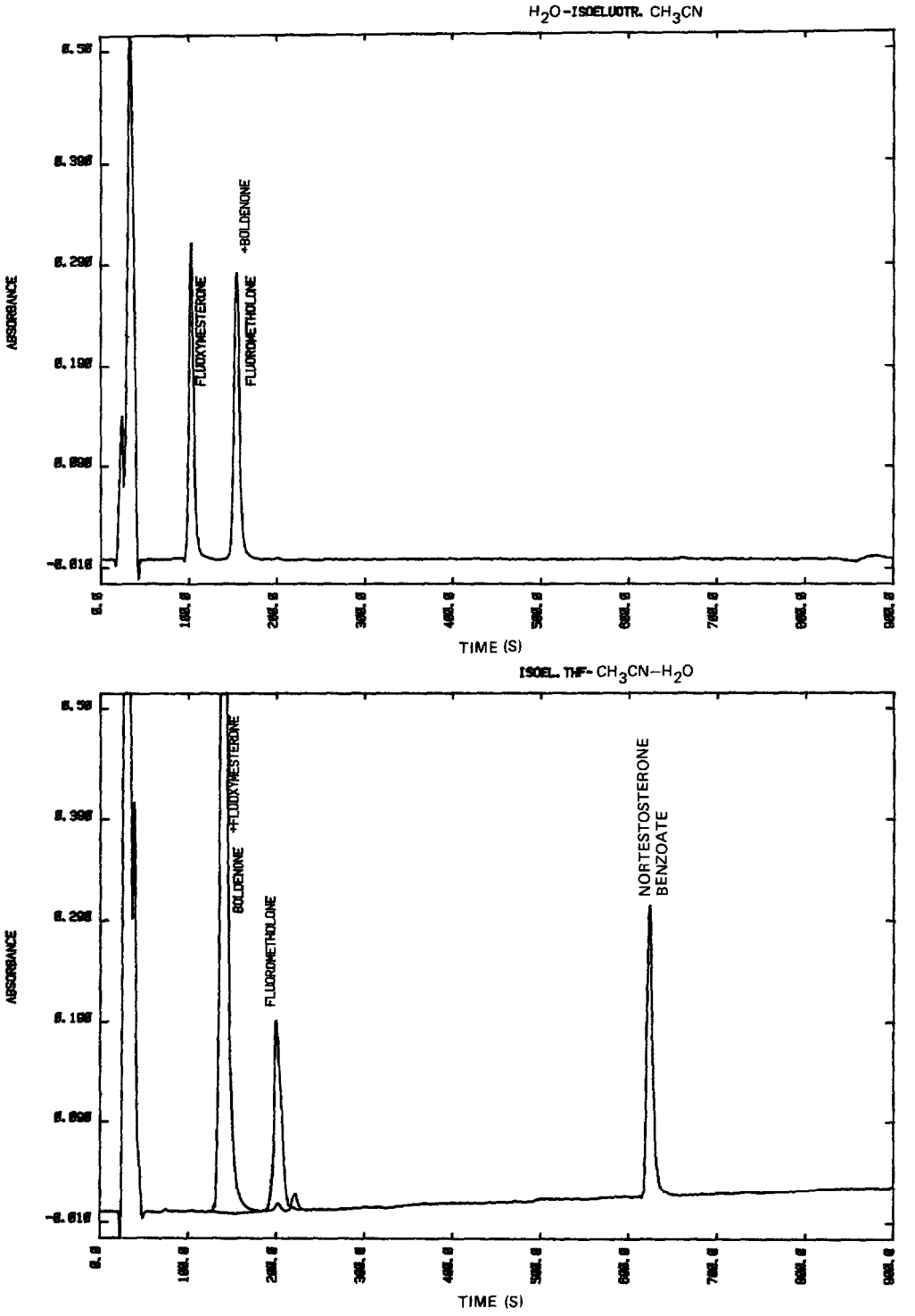

Fig. 6. Boldenone, fluoxymesterone and fluorometholone are indistinguishable on HPLC analysis using solvent B. Boldenone and fluoxymesterone are, however, separated using solvent C (upper), whereas boldenone and fluorometholone are separated using solvent D (lower).

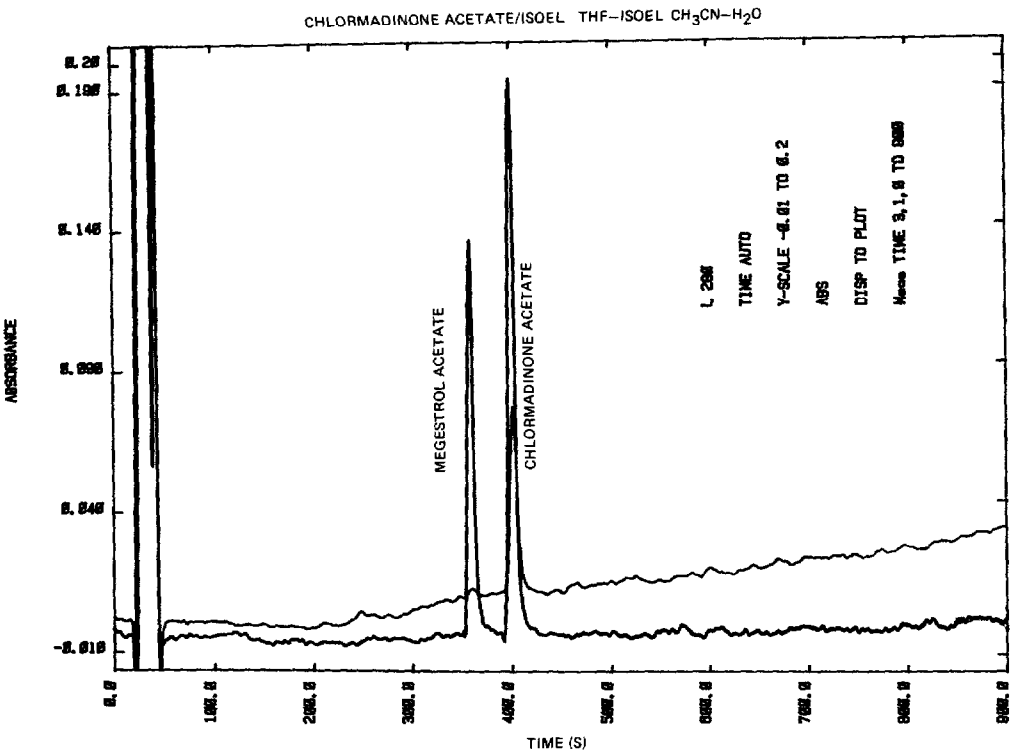

Fig. 7. The structurally related steroids megestrol acetate and chlormadinone acetate are difficult to separate with solvent B or solvent C as modifier. Using the isoeluotropic solvent D, complete resolution is obtained.

DISCUSSION

Table IV explains how acetonitrile–water and THF–water are prepared to be isoeluotropic with pure methanol, starting from the solvent strength weighting factors. This principle is also used in the 'Sentinel' system developed by Glajch et al. [14] and in the isocratic reversed-phase elution on cyanopropyl columns, developed by De Smet et al. [15]. These data also make it clear why a 50:50 (v/v) mixture of isoeluotropic acetonitrile (82%) and isoeluotropic THF (58%), composing solvent D, is isoeluotropic with pure methanol.

As to the UV spectra of the steroids studied, some of them are very diagnostic, e.g. chlorotrianisene, chlormadinone acetate and megestrol acetate, DES, dienestrol, hexestrol, nandrolone hexyloxyphenylpropionate, trenbolone and its acetate, estradiol monobenzoate and benzoacetate and stanozolol [16,17].

Steroids with a sharp UV maximum near 240 nm and no absorbance at all at 280 nm are cortisone, ethisterone, fluoxymesterone, hydrocortisone and hydrocortisone acetate, progesterone, medroxyprogesterone acetate, 17α-methyltestosterone, testosterone and nandrolone and their esters. Another group, however, shows a pronounced UV maximum near 240 nm with weak absorbance at 280 nm: dexamethasone and its esters, prednisone and prednisolone, methylprednisolone and methandrostenolone.

TABLE III

ELUTION ORDERS AND RETENTION TIMES WITH DIFFERENT SOLVENTS

Retention time (s)	Solvent used as organic modifier		
	B	C	D
<50		Estriol	
50–100		Prednisolone	Prednisone
		Prednisone	Cortisone
		Hydrocortisone	Hydrocortisone
		Cortisone	Estriol
		Dexamethasone	Prednisolone
100–150	Triamcinolone	Fluoxymesterone	Methylprednisolone
		Trenbolone	Dexamethasone
		Hydrocortisone acetate	Fluoxymesterone
			Boldenone
			Trenbolone
150–200	Estriol	Fluorometholone	Nandrolone
	Prednisone	Boldenone	Methandrostenolone
		Nandrolone	Hydrocortisone acetate
		17-β-Estradiol	
		Methandrostenolone	
200–250	Cortisone	17-α Estradiol	Fluorometholone
	Hydrocortisone	Testosterone	Testosterone
	Prednisolone	Norethisterone	Androstenedione
		Dexamethasone acetate	Methylprednisolone acetate
		Ethynylestradiol	
		Estrone	Norethisterone
250–300	Methylprednisolone	Ethisterone	17α-Methyltestosterone
	Dexamethasone	17α-Methyltestosterone	Ethisterone
	Hydrocortisone acetate	Androstenedione	17-β-Estradiol
		Hydroxyprogesterone	Dexamethasone acetate
		DES	Hydroxyprogesterone
		Hexestrol	
		Dienestrol	
		α-Norgestrel	
300–350	Trenbolone		Estrone
	Fluorometholone		17-α-Estradiol
	Boldenone		α-Norgestrel
	Fluoxymesterone		Ethynylestradiol
	17-β-Estradiol		
	Methylprednisolone acetate		
	Dexamethasone acetate		
	Ethynylestradiol		
	Nandrolone		
350–400	17-α-estradiol		Trenbolone acetate
	Norethisterone		Megestrol acetate
	Estrone		Progesterone
	DES		Medroxyprogesterone acetate
	Androstenedione		
	Methandrostenolone		
	Dienestrol		
	Hexestrol		
	Ethisterone		
	Testosterone		

TABLE III (continued)

Retention time (s)	Solvent used as organic modifier		
	B	C	D
400–450	α-Norgestrel 17-OH-Progesterone Methyltestosterone	Trenbolone acetate Chlormadinone acetate Megestrol acetate Medroxyprogesterone acetate Progesterone	Dienestrol Chlormadinone acetate DES Hexestrol
450–500	Megestrol acetate Chormadinone acetate Medroxyprogesterone acetate Trenbolone acetate	Dexamethasonephenyl propionate Mestranol	Testosterone acetate
500–550		Testosterone acetate	Metenolone acetate Mestranol
550–600	Stanozolol Dexamethasone phenylpropionate Testosterone acetate Dienestrol diacetate Mestranol	Metenolone acetate Dienestrol diacetate	Testosterone propionate Dexamethasone phenyl-propionate Dienestrol diacetate
600–650	Methenolone acetate Testosterone propionate Hydroxyprogesterone caproate Estradiol diacetate Chlorotrianisene Stilbestrol dipropionate	Testosterone propionate	Hydroxyprogesterone caproate Estradiol monobenzoate Testosterone isobutyrate Nortesterone benzoate Estradiol diacetate
650–700	Hydroxyprogesterone heptylate Testosterone isobutyrate Nortestosterone benzoate Estradiol valerate Estradiol monobenzoate Nandrolone phenylpropionate Testosterone benzoate Testosterone valerate	Estradiol diacetate Estradiol monobenzoate Chlorotrianisene Hydroxyprogesterone caproate	Hydroxyprogesterone heptylate Testosterone benzoate Nandrolone phenylpropionate Chlorotrianisene
700–750	Estradiol dipropionate Estradiol cypionate	Estradiol valerate Testosterone isobutyrate Nortestosterone benzoate	Testosterone valerate Estradiol valerate Stilbestrol dipropionate
750–800	Testosterone enanthate Estradiol benzoacetate Testosterone cypionate Testosterone cyclohexylpropionate	Nandrolone phenylpropionate Hydroxyprogesterone heptylate Stilbestrol dipropionate	Estradiol dipropionate
800–850	Nandrolone hexyloxyphenylpropionate Nandrolone decanoate Testosterone undecylenate Testosterone decanoate Nandrolone laurate	Testosterone benzoate Estradiol dipropionate Testosterone valerate	Estradiol benzoacetate Estradiol cypionate

TABLE IV

SOLVENT STRENGTH WEIGHTING FACTORS OF THE COMPONENTS OF SOLVENTS B, C AND D

Solvent	Strength in reversed phase
Water	0
Methanol	2.6
Acetonitrile	3.2
THF	4.5

Solvent strength of 100% methanol = 2.6
Solvent strength of water–acetonitrile = $(1-x)$ 3.2 = 2.6 $x = 0.18$
Solvent strength of water–THF = $(1-x)$ 4.5 = 2.6 $x = 0.42$
So water–acetonitrile (18:82) and water–THF (42:58) are isoeluotropic with 100% methanol.

A third important group has a rather weak but distinct UV maximum at 280 nm, e.g. mestranol, estradiol and some of its esters, estrone and ethynylestradiol.

All this UV information can be gathered with the diode array detector during HPLC analysis, and is of great importance for identification.

It can be decided from the chromatographic properties of the steroids in three different solvent systems which of these systems is best for solving a particular separation problem. It also can be established how compounds that co-elute with one solvent system can be separated with another isoeluotropic system.

From this study it seems that a better separation of corticosteroids is obtained with methanol than with acetonitrile. Williams and Biehl [4] preferred a mixture of 25% THF and 12.5% methanol in water for the separation of several corticosteroids in one isocratic run. On the other hand, it seems from Table III that unresolved clusters of free and short-chain esterified steroids on the chromatogram with solvent B are separated with THF–acetonitrile.

An interesting point is the isolated steroid group of progesterone, megestrol acetate, medroxyprogesterone acetate and chlormadinone acetate in the chromatogram with the solvent B, between 450 and 510 s, and with the solvent system C, between 400 and 450 s. With solvent D this isolation is lost, but now megestrol acetate and chlormadinone acetate, which have the same UV spectrum, are completely separated.

The chromatographic behaviour of the stilbenes DES, hexestrol and dienestrol in solvent D is remarkable. Their retention time increments are significantly longer than those of testosterone and nandrolone and their esters.

For the HPLC analysis of most long-chain esterified steroids, solvent B allows elution within 900 s. Solvent C and D are less suitable because of the much longer retention time.

Thus the chromatographic strategy we propose for the identification of unknown steroids in mixtures is a preliminary HPLC gradient elution run, using solvent B as modifier, with detection at 240 and 280 nm and with the UV spectra being recorded. Long-chain fatty acid steroids are simultaneously screened in a reasonable time. Next the unknown sample is injected, together with the proper

internal standard to calculate the relative retention times. With the search program the possible corresponding standard steroids are sorted. Finally the selected standard steroids and the unknown peaks of the sample have to match accurately, both chromatographically and spectroscopically. If chromatographic and spectroscopic distinction is insufficient, the more complementary of the other two isoeluotropic solvent systems C or D (or both) is used to complete the identification.

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