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Reversed-phase high-performance liquid chromatographic screening method for serum steroids using retention index and diode-array detection

Pirjo Kuronen*, Pirkko Volin, Tarja Laitalainen

Department of Chemistry, Laboratory of Organic Chemistry, P.O. Box 55, University of Helsinki FIN-00014, Finland

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Abstract

A multisteroid screening method has been developed based on the use of 1-[4-(2,3-dihydroxypropoxy)phenyl]-1-alkanones as retention index standards and UV absorbance spectra recorded on-line with a diode-array detector using reversed-phase high-performance liquid chromatographic gradient elution with acetonitrile and water. The effect of chromatographic conditions on retention indices of steroids were studied. The method was tentatively applied to profiling of steroids in serum samples. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Steroids

1. Introduction

The determination of steroids in biological specimens is clinically important. The need for simultaneous monitoring of several steroids has led to the development of a number of techniques for their determination in a variety of matrices. These methods include immunoassays: radioimmunoassay [1–6], enzyme immunoassay [7–9] and fluoroimmunoassay [10,11]. However, a common problem with these techniques is specific and non-specific interference with antibody-analyte binding which may yield false positives.

Among the chromatographic techniques both GC [12,13] and HPLC [12–17] methodologies have been developed for profiling of steroids. However, the low levels of steroids normally encountered in biological samples combined with only moderate detector sensitivity have been a major problem in HPLC of steroids. If the resolving power of HPLC is followed by radioimmunoassay, the determination of structurally very similar steroids, e.g. pair of epimers has been successfully carried out [18,19].

The HPLC method for the identification and quantitation of steroids can be improved by using simultaneously two complementary detectors. For example, diode-array detector (DAD) and MS or DAD and electrochemical detection are powerful combinations. Suzuki et al. [20] devised an automated direct assay for measuring serum estradiol, estrone, progesterone, 17α-hydroxyprogesterone, 20α-hydroxyprogesterone, testosterone and androstenedione using HPLC with a combined UV absorbance and electrochemical detection. Later, Noma et al. [21] described an automated direct assay for

*Corresponding author.

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measuring unconjugated estetrol, estriol, cortisone and cortisol in serum and amniotic fluid.

MS as an on-line detector for LC has been applied to the analysis of natural and synthetic steroids, their sulfates and other conjugates [22]. The combination of HPLC separations with specific mass spectrometric detection has become a practical technique for steroid analyses since the introduction of thermospray (TSP) LC–MS [23], ion-spray (IS) LC–MS [24] and electrospray (ES) LC–MS [14]. Weidolf et al. [25] have shown that picogram quantities of natural and synthetic steroids and their sulfates could be determined using negative-ion IS–LC–MS.

The fast screening and tentative identification of steroids can be carried out by using retention index method, developed originally for GC [26–28]. Steroid-specific standards, such as androstane and cholestane, have been adopted to calculate steroid number in steroid analysis by GC [29–31]. The incorporation of the standards in HPLC analyses eliminates the influence of slight changes in chromatographic conditions and results in the increase of reliability compared to the use of only retention times. Therefore, retention indices have been widely adopted as a method for recording and comparing retentions, for identifications and as the basis of prediction methods [32]. The first retention index standards in HPLC were 2-alkanones reported in 1979 by Baker and Ma [33]. The RP-HPLC retention indices based upon 2-alkanones were used for the first time in the identification of steroids by Baker and Fifer [34] and subsequently in the optimization of resolution both under isocratic and gradient elution [35,36].

In 1982 Smith [37] and Kuronen [38] introduced 1-phenyl-1-alkanones for reversed-phase (RP)-HPLC in an isocratic solvent system and under gradient elution conditions, respectively. The two aforementioned homologous series have been the most commonly used index standards in HPLC. Subsequently, 1-[4-(2,3-dihydroxypropoxy)phenyl]-1-alkanones (D-standards) [39] and 1-nitroalkanes [40] have been used in RP-HPLC, especially due to their wider range of polarities. Three databases of HPLC indices of drugs in the 1-nitroalkane scale have been established [41] and a number of different applications of retention indices have been reviewed [32,42]. The reliability of the identification of trichothecenes using TSP and dynamic fast atom bombardment (FAB) LC–MS has been assisted by the use of retention indices in the 1-[4-(2,3-dihydroxypropoxy)-phenyl]-1-alkanone scale [43].

The aim of the present study was to develop a multisteroid screening method based on the use of 1-[4-(2,3-dihydroxypropoxy)phenyl]-1-alkanones (D-standards) as retention index standards and UV absorbance spectra produced by diode-array detector (DAD) under RP-HPLC gradient elution conditions and to apply the method tentatively to the profiling of steroids in serum samples.

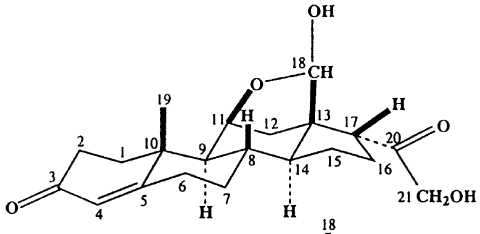
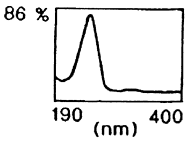
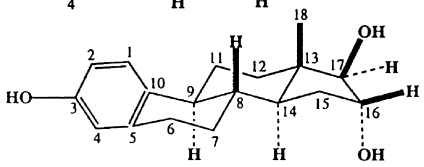
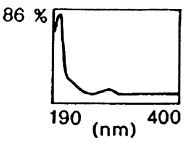
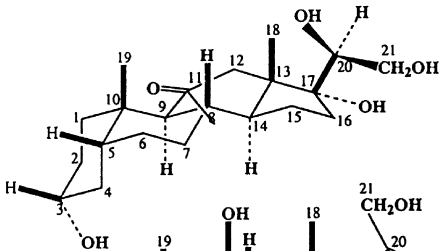
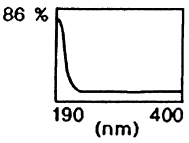
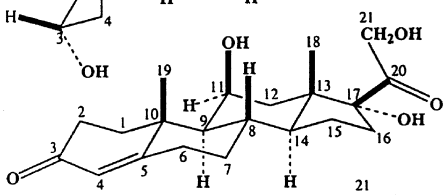
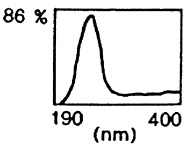
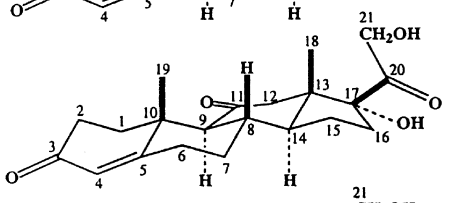
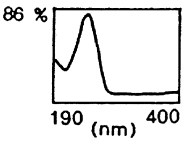
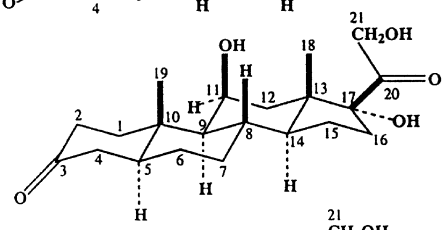
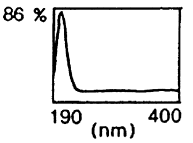
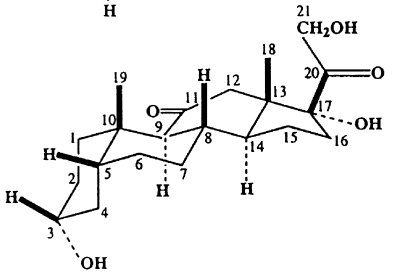
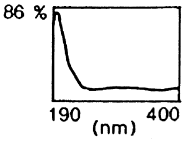
2. Experimental

2.1. Chemicals and reagents

Steroids were obtained from Sigma (St. Louis, MO, USA). Stock solutions of 1.0–5.0 mg/ml were prepared in ethanol (99.5%, ETAX AaS, Primalco Oy, Rajamäki, Finland). These solutions were used to make steroid mixtures ranging from 50 to 1000 nmol/l of each steroid in water. HPLC-grade methanol and acetonitrile were from Lab-Scan Analytical Sciences (Labscan, Dublin, Ireland). Distilled water was further purified with Gelman's Water I apparatus (Gelman Sciences, Ann Arbor, MI, USA). The HPLC solvents were filtered through a 0.45-μm membrane filter (Millipore, Milford, MA, USA) and thoroughly degassed by application of ultrasound. Millex LCR 0.45-μm cartridges (Millipore) were used for the filtration of the HPLC samples. *o*-Phosphoric acid (85%, p.a.) was from Merck (Darmstadt, Germany). Solid-phase extraction (SPE) cartridges were Bond Elut C18-bonded silica columns (100 mg, Analytichem International, Harbor City, CA, USA).

A mixture of six 1-[4-(2,3-dihydroxypropoxy)-phenyl]-1-alkanones (D-standards, Table 1) in amounts ranging from 0.04 to 0.05 mg/ml each was used as reference for retention index determinations. The steroid mixtures were generally measured with simultaneous injection of the D-standard mixture (the internal standard method). In some chromatographic experiments the external standard method was also used. The D-standards were synthesized

Table 1
The numbering system, the trivial and systematic names, the retention times, the structures and UV absorbance spectra of the steroids 1–24 and retention index standards

Steroid Number	Systematic Name [Trivial Name]	Retention Time [min][a]	Structure	UV absorbance spectrum
1	11β,21-Dihydroxy-4,17α-pregnen-18-al-3,20-dione [17-Isoaldosterone]	4.58		
2	3,16α,17β-Trihydroxy-1,3,5[10]-estratriene [Estriol]	5.05		
3	3α,17α,20α,21-Tetrahydroxy-5β-pregnan-11-one [α-Cortolone]	5.71		
4	11β,17α,21-Trihydroxy-4-pregnene-3,20-dione [Cortisol]	6.14		
5	17α,21-Dihydroxy-4-pregnene-3,11,20-trione [Cortisone]	6.55		
6	11β,17α,21-Trihydroxy-5α-pregnane-3,20-dione [5α-Dihydrocortisol]	7.47		
7	3α,17α,21-Trihydroxy-5β-pregnane-11,20-dione [Tetrahydrocortisone]	7.64		

(Cont.)

Table 1. Continued

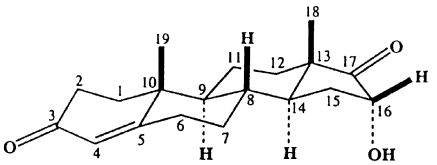
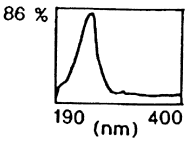
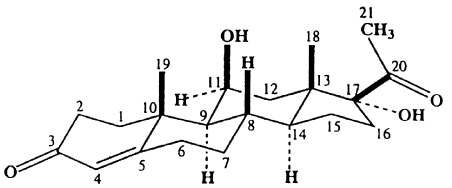
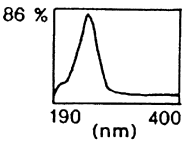
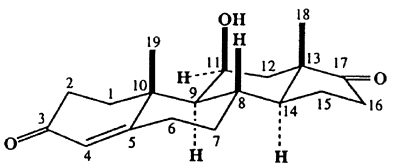
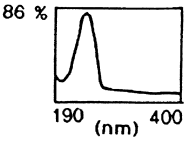
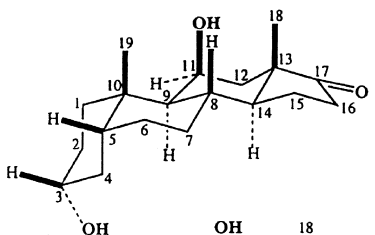
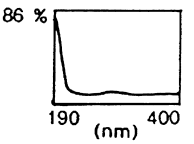
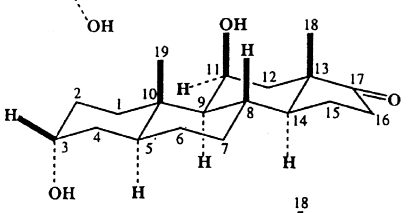
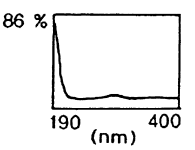
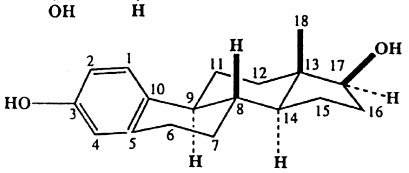
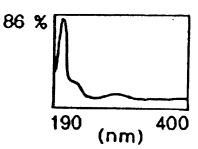
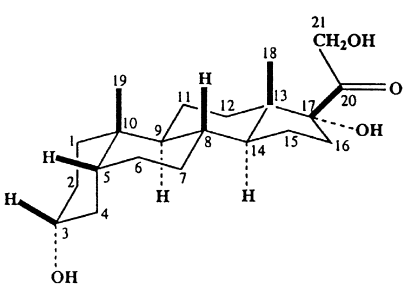
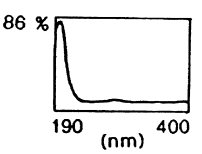
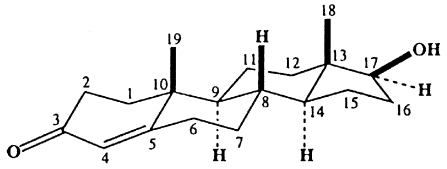
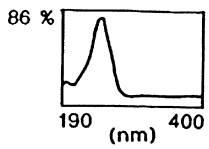
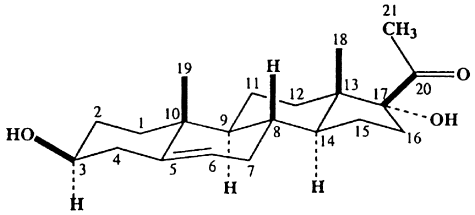
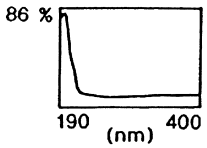
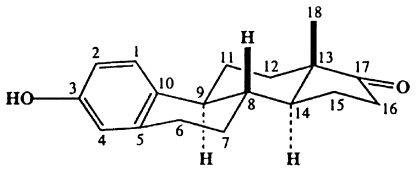
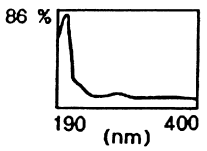
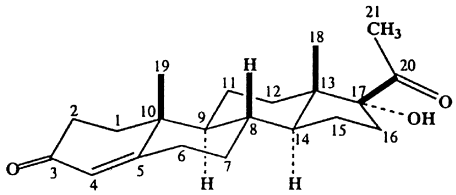
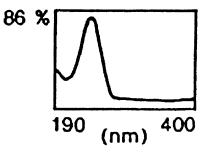
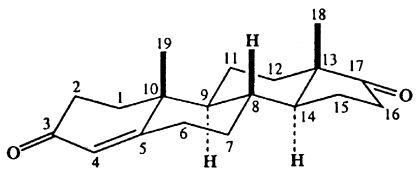
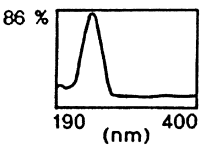
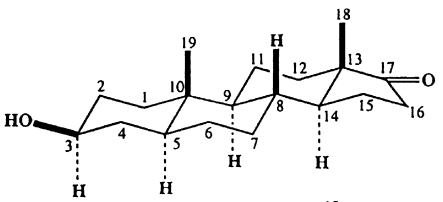
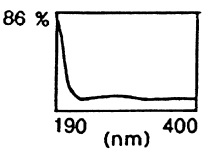
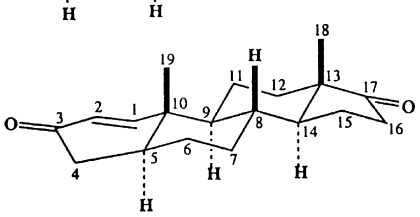
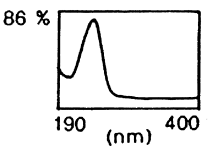
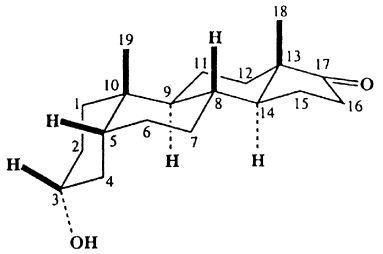
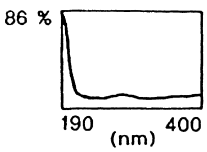
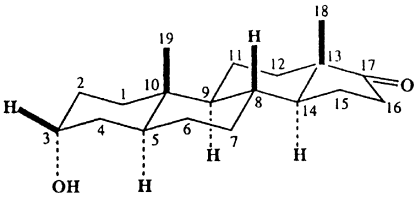
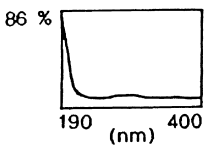
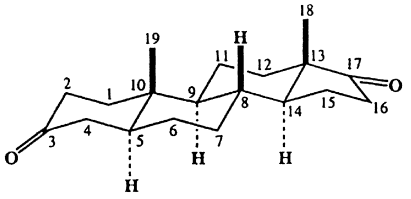
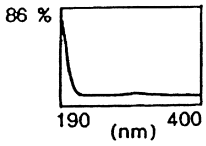
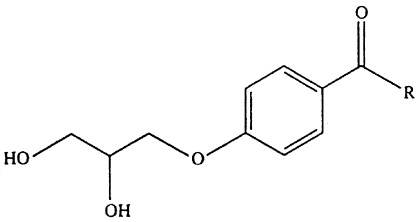
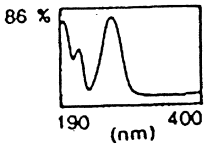
Steroid Number	Systematic Name [Trivial Name]	Retention Time [min][a]	Structure	UV absorbance spectrum
8	16α-Hydroxy-4-androstene-3,17-dione [16α-Hydroxy-androstenedione]	8.09		
9	11β,17α-Dihydroxy-4-pregnene-3,20-dione [21-Deoxycortisol]	8.88		
10	11β-Hydroxy-4-androstene-3,17-dione [11β-Hydroxy-androstenedione]	9.50		
11	3α,11β-Dihydroxy-5β-androstan-17-one [11β-Hydroxy-etiocholanolone]	10.03		
12	3α,11β-Dihydroxy-5α-androstan-17-one [11β-Hydroxy-androsterone]	10.42		
13	3,17β-Dihydroxy-1,3,5[10]-estratriene [β-Estradiol]	11.34		
14	3α,17α,21-Trihydroxy-5β-pregnan-20-one [11-Deoxytetra-hydrocortisol]	11.81		

Table 1. Continued

Steroid Number	Systematic Name [Trivial Name]	Retention Time [min][a]	Structure	UV absorbance spectrum
15	17β-Hydroxy-4-androsten-3-one [Testosterone]	12.61		
16	3β,17α-Dihydroxy-5-pregnen-20-one [17α-Hydroxy-pregnenolone]	12.96		
17	3-Hydroxy-1,3,5[10]-estratrien-17-one [Estrone]	13.45		
18	17α-Hydroxy-4-pregnene-3,20-dione [17α-Hydroxy-progesterone]	14.19		
19	4-Androstene-3,17-dione [Androstenedione]	14.60		
20	3β-Hydroxy-5α-androstan-17-one [Epiandrosterone]	15.42		
21	1-[5α]Androstene-3,17-dione [5α-Androst-1-ene-3,17-dione]	16.11		

(Cont.)

Table 1. Continued

Steroid Number	Systematic Name [Trivial Name]	Retention Time [min][a]	Structure	UV absorbance spectrum
22	3α-Hydroxy-5β-androstan-17-one [Etiocholanolone]	16.81		
23	3α-Hydroxy-5α-androstan-17-one [Androsterone]	17.69		
24	5α-Androstane-3,17-dione [Androstanedione]	18.01		
D-Standards				
1-[4-(2,3-Dihydroxy-propoxy)phenyl]-1-alkanones	D_1	3.27		
	D_3	5.96		
	D_5	10.42		
	D_7	15.10		
	D_9	20.79		
	D_{11}	26.05		

R = $CH_3(CH_2)_{n-1}$; n = 1,3,5,7,9,11

[a] Chromatographic conditions: HP 1090; column, Zorbax Rx-C_{18} (250×4.6 mm I.D.), 5 μm; column temperature, ambient; flow-rate, 1.0 ml/min; stepwise acetonitrile–water gradient, 30, 35, 35, 42, 42, 50, 60, 70 and 100% acetonitrile at 0, 3.00, 3.20, 5.00, 6.90, 8.00, 15.00, 18.00 and 30.00 min, respectively; UV absorbance detection at 205 nm.

according to the method reported by Kuronen [39,44].

2.2. HPLC instrumentation and separation conditions

HPLC experiments were performed with a Hewlett-Packard HP 1090A (Waldbronn, Germany) with autoinjector, a built-in diode-array detector, HP 85B computer control, HP 3392A integrator, and HP 9121D disk memory. Another apparatus consisted of a Waters 600 HPLC pump, a Rheodyne 7725i injector with a 20-μl loop (Cotati, CA, USA), a Waters column heater module, a Waters 996 diode-array detector and Millennium 2010 Chromatography Manager (Waters Assoc., Milford, MA, USA).

The analytical RP columns were Zorbax Rx-C18 (250×4.6 mm I.D.) of 5-μm particle size (Rockland

Technologies, Newport, DE, USA, column 1) and Inertsil ODS-3 (250×4.6 mm I.D.) of 5-μm particle size (Gl Sciences, Tokyo, Japan, column 2). The columns were operated at ambient temperature (22±4°C) and at constant temperatures of 25.0 and 35.0°C at a flow-rate of 1.0 ml/min. The linear acetonitrile–water gradient was 20–100% acetonitrile in 40.00 min and the multistep acetonitrile–water gradient consisted of 30, 35, 35, 42, 42, 50, 60, 70 and 100% acetonitrile at time 0.00, 3.00, 3.20, 5.00, 6.90, 8.00, 15.00, 18.00 and 30.00 min, respectively. DAD monitors all spectral wavelengths and collects data for future processing. The on-line UV spectra of steroids were recorded from 190 to 400 nm with the DAD at the eluent composition at which they eluted in the chromatographic run (Table 1).

Retention index values of steroids under different chromatographic conditions were calculated by a cubic spline interpolation method [45] as the mean value of five determinations (Table 2).

2.3. Preparation of serum samples

To 1 ml of centrifuged serum sample 50 μl of phosphoric acid was added. The sample was heated at 40°C for 10 min. SPE cartridge column was conditioned with 5 ml of methanol and then 10 ml of water. Excess conditioning solvent was removed and

Table 2
Retention times (t_R, min) and index values (RI_D) of steroids (1–24)[a] using different column temperatures, gradient profiles, columns and instruments

Compound No	HP 1090												Waters	
	Column 1										Column 2		Column 1	
	Amb. temp.		25°C		35°C						35°C		35°C	
	Step grad.		Lin. grad.		Step grad.		Lin. grad.		Step grad.		Step grad.		Step grad.	
	t_R	RI_D	t_R	RI_D	t_R	RI_D	t_R	RI_D	t_R	RI_D	t_R	RI_D	t_R	RI_D
1	4.57	203	8.04	242	4.32	203	7.79	245	4.30	211	6.92	227	4.45	200
2	5.02	238	8.59	262	4.73	237	8.06	256	4.56	230	7.63	262	4.72	218
3	5.69	284	10.05	319	5.68	296	10.09	332	5.78	314	8.45	300	6.48	325
4	6.10	311	10.16	321	5.94	315	9.90	325	5.87	318	8.88	320	6.57	325
5	6.52	335	10.59	337	6.33	337	10.17	334	6.12	332	9.14	332	6.85	337
6	7.45	382	11.77	378	7.35	386	11.49	380	7.22	386	10.61	397	8.47	397
7	7.61	389	12.24	394	7.60	396	12.07	400	7.55	400	10.75	403	9.14	416
8	8.07	409	–	–	–	–	–	–	–	–	–	–	–	–
9	8.85	440	13.49	437	8.72	439	13.10	434	8.49	436	12.10	458	10.23	445
10	9.46	464	14.02	455	9.26	459	13.54	449	8.92	452	12.44	471	10.66	456
11	10.00	484	–	–	–	–	–	–	–	–	–	–	–	–
12	10.40	500	15.06	490	10.25	497	14.90	492	10.04	496	13.65	518	12.05	493
13	11.34	538	16.55	542	11.28	538	10.86	528	10.86	528	14.64	555	12.92	525
14	11.80	558	17.22	565	11.77	559	16.97	566	11.62	561	15.24	577	14.00	566
15	12.59	593	17.98	592	12.44	589	17.24	575	11.94	576	15.35	581	14.16	573
16	12.85	609	18.54	612	12.83	606	17.86	598	12.33	594	16.26	613	14.59	593
17	13.44	630	19.12	633	13.30	628	18.18	609	12.59	606	16.84	635	14.85	601
18	14.19	662	19.85	659	14.01	659	19.02	639	13.33	640	17.46	657	15.63	634
19	14.55	679	19.85	659	14.10	663	18.93	637	13.22	638	17.25	650	15.52	633
20	15.39	713	20.19	671	15.22	710	20.17	681	14.37	686	18.26	686	16.77	685
21	16.10	739	21.15	704	15.86	735	20.67	700	14.90	709	19.18	722	17.22	700
22	16.81	764	21.80	728	16.51	759	21.73	737	15.82	745	20.17	760	18.24	739
23	17.68	795	23.32	781	17.42	791	22.47	766	16.47	771	21.03	795	18.93	766
24	18.00	806	22.50	752	17.72	801	22.49	765	16.58	773	20.82	786	18.90	763

Column 1, Zorbax Rx-C_{18} (250×4.6 mm I.D.), 5 μm; column 2, Inertsil ODS-3 (250×4.6 mm I.D.), 5 μm; flow-rate 1.0 ml/min; linear acetonitrile–water gradient 20–100% acetonitrile over 40 min, stepwise acetonitrile–water gradient 30, 35, 35, 42, 42, 50, 60, 70 and 100% acetonitrile at 0, 3.00, 3.20, 5.00, 6.90, 8.00, 15.00, 18.00 and 30.00 min, respectively; detection at 205 nm.

[a] Mean values of five determinations.

the sample was loaded onto a SPE column. The column was washed with 5 ml of water and 3 ml of 20% methanol–water. The analytes were eluted with 2 ml of methanol. The eluent was evaporated to dryness under a gentle stream of nitrogen at 40°C. The residue was reconstituted in 50 ml of ethanol (99.5%) for the analysis by HPLC.

3. Results and discussion

3.1. Molecular structures and chromatographic separation

In Table 1 are presented the names and structures and on-line recorded UV absorbance spectra of the steroids (1–24) and index standards (D-standards) relevant to this study. The steroids are arranged in the order of increasing retention time under stepwise gradient elution conditions at ambient temperature. Under these conditions good separation of the 24 closely related steroid structures was obtained in 18 min (Fig. 1). The elution order for 3, 4; 18, 19 and 23, 24 was reversed or they were co-eluted with changes in the chromatographic conditions (Table 2).

In this work an RP gradient technique was found to be the most suitable method for analysing the steroids 1–24, which show both different stereochemical features and polarities. The steroids were analysed on Zorbax Rx-C18 and Inertsil ODS-3 columns which are densely bonded and are based on highly pure and low acidity silica. Acetonitrile was

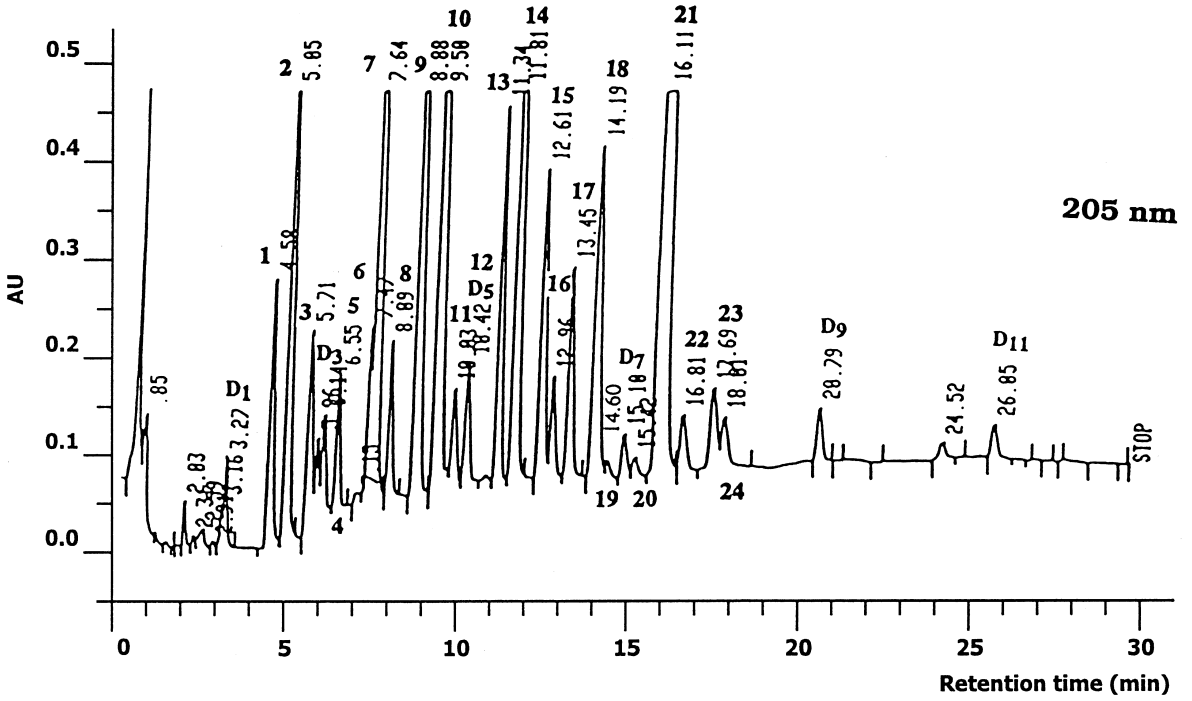


Fig. 1. Representative chromatogram of the steroids (1–24) and D-standards (D_1, D_3, D_5, D_7, D_9, D_{11}) with their retention times (min): 1=17-isoaldosterone (4.58), 2=estriol (5.05), 3=α-cortolone (5.71), 4=cortisol (6.14), 5=cortisone (6.55), 6=5α-dihydrocortisol (7.47), 7=tetrahydrocortisone (7.64), 8=16α-hydroxyandrostenedione (8.09), 9=21-deoxycortisol (8.88), 10=11β-hydroxyandrostenedione (9.50), 11=11β-hydroxyetiocholanolone (10.03), 12=11β-hydroxyandrosterone (10.42), 13=β-estradiol (11.34), 14=11-deoxytetrahydrocortisol (11.81), 15=testosterone (12.61), 16=17α-hydroxypregnenolone (12.96), 17=estrone (13.45), 18=17α-hydroxyprogesterone (14.19), 19=androstenedione (14.60), 20=epiandrosterone (15.42), 21=5α-androst-1-ene-3,17-dione (16.11), 22=etiocholanolone (16.81), 23=androsterone (17.69) and 24=androstanedione (18.01). D_1 (3.27), D_3 (5.96), D_5 (10.42), D_7 (15.10), D_9 (20.79) and D_{11} (26.05 min). Chromatographic conditions: HP 1090; column, Zorbax Rx-C18 (250×4.6 mm I.D.), 5 μm; ambient temperature; flow-rate, 1.0 ml/min; UV absorbance detection at 205 nm; stepwise acetonitrile–water gradient, 30, 35, 35, 42, 42, 50, 60, 70 and 100% acetonitrile at 0.00, 3.00, 3.20, 5.00, 6.90, 8.00, 15.00, 18.00 and 30.00 min, respectively.

used as the basic organic modifier of the mobile phase because of its low UV cut-off which allows the use of 205 nm as one of the detection wavelengths. The use of a multiwavelength detection (205, 220, 245 and 280 nm) during a single chromatographic run made it possible to detect the steroids selectively and sensitively.

Epimeric steroids (11, 12; 22, 23 and 20, 23) could be well resolved at column temperatures of ambient, 25 and 35°C used in this study. According to Sheikh and Touchstone [46–48] the use of subambient temperatures (−20°C and even −50°C) was necessary for the separation of epimeric 17α- and 17β-estradiols and 5-androstene-3β,17α-diol and 5-androstene-3β,17β-diol. Watson et al. [49] separated testosterone (17β-OH) from its 17α-OH epimer, epitestosterone, using a normal-phase silica column, whereas their reduced 5α-metabolites were separated on an RP system.

Bush [50] has widely described, in his book, correlations between the structure and mobility in the chromatography of steroids. Later, Lin and Heftmann [51] have investigated the relationship between the structure and the chromatographic mobility of androstane derivatives in adsorption chromatography and in RP-HPLC. Both in the steroids 11, 12 and 22, 23 and among those studied by Lin and Heftmann, the majority of the stereoisomeric pairs, which differ only in the A/B-junction, the *cis*-A/B-isomer is more polar than the *trans* A/B-isomer. Further, the inspection of ketoalcohols 20 and 23 or diols [51] shows that the 3β- or 17β-isomers are always more polar than their α-epimers (the β-trend). The β-trend is very significant in the case of the 3,17-diols. It is to be seen from the data of Lin and Heftmann [51] that the retention times of androstane-3α,17α-diols are as much as 2.5–3.0 times as high as those measured for the androstane-3β,17β-diols. Stereochemistry and molecular interactions seem to be very significant in determining the retention. The attempts to quantitate the various interactions affecting the retention of steroids have shown the complexity of the problem [52–56].

3.2. Retention indices

The reporting of liquid chromatographic retention data in a meaningful form has been a difficult problem. The retention of a particular compound in RP-HPLC system depends on the structure of the compound, the composition of the mobile phase, the temperature and the chemistry of the stationary phase. Retention indices, unlike other retention parameters, are decisively more precise, because they are determined by interpolation between the retention factors or retention times of a series of retention index standards.

A number of different homologous series have been used as retention index standards in RP-HPLC [42,57]. The 1-phenyl-1-alkanones, introduced by Smith [37] and Kuronen [38] as suitable retention index standards for RP-HPLC, have subsequently found widespread application for the identification of a wide range of polar and nonpolar analytes [32]. In our hands the most polar steroids, however, could not be analysed as precisely with 1-phenyl-1-alkanones as with 1-[4-(2,3-dihydroxypropoxy)-phenyl]-1-alkanones (D-standards). D-standards cover more polar compounds than 1-phenyl-1-alkanones and they have been successfully applied before in the case of chemical warfare agents, their degradation products and precursors [39,44,58–60] and mycotoxins [43,61–63]. The retention times of all steroids examined were within the range of D-compounds.

In Fig. 2 a plot of the absolute retention time against the number of carbon atoms in the alkyl group of the six D-standards (D_1, D_3, D_5, D_7, D_9, D_{11}) under linear and stepwise gradient elution conditions used in this study is shown. Non-linear chromatographic behaviour is demonstrated. Therefore, retention indices (RI_D) of steroids were calculated by a cubic spline interpolation [45] between the absolute retention times of D-standards, which were added to the sample or used as external standards.

The reproducibility of retention indices is essential for a reliable identification of steroids. Using the same chromatographic conditions the indices were highly reproducible with standard deviations (SD) typically ±0.5 index units (i.u.). The absolute retention times of D-compounds and steroids were also reproducible under nominally unchanged chromatographic conditions. All the RSD% values were smaller than 1%, 0.37% on an average. However changes in chromatographic conditions affected the reproducibility of retention times much more than

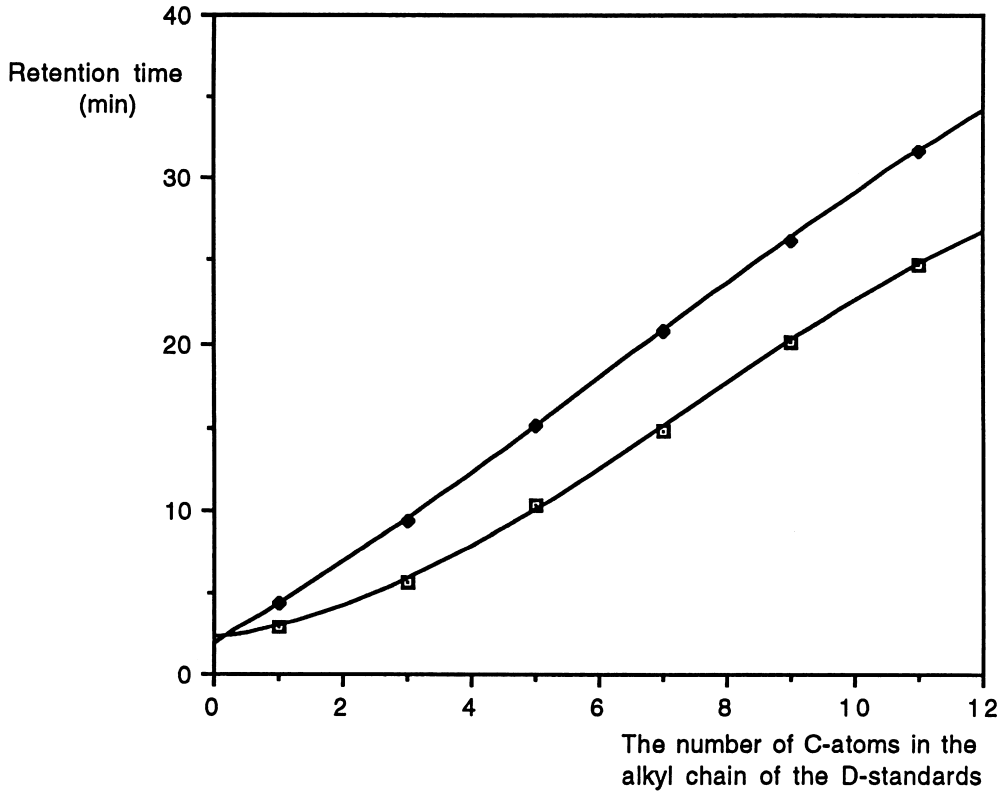


Fig. 2. Plot of unadjusted retention times against number of C-atoms in the alkyl chain of the D-standards under linear (♦) and stepwise (□) gradient elution conditions. HP 1090; column, Zorbax Rx-C18 (250×4.6 mm I.D.), 5-μm; column temperature 35°C; flow-rate 1.0 ml/min; UV absorbance detection at 220 nm; linear gradient program from 20% acetonitrile–water to 100% acetonitrile in 40 min and the multistep acetonitrile–water gradient consisted of 30, 35, 35, 42, 42, 50, 60, 70 and 100% acetonitrile at time 0.00, 3.00, 3.20, 5.00, 6.90, 8.00, 15.00, 18.00 and 30.00. min, respectively.

that of retention indices (Table 2). In this study the following parameters on retention times and retention indices of steroids were studied: column temperature, gradient profile, intercolumn reproducibility and make of instrument. In Table 2 are reported the absolute retention times (t_R) and RI values for the steroids (1–24) under different chromatographic conditions.

The results of Table 2 indicate that over a small temperature range (ambient and 25°C) the effect on retention indices is small (<5 i.u.), except for the compound 3 (12 i.u.). Changing the temperature from 25.0 to 35.0°C the index variations are 11 i.u. on average (range 0-34 i.u.). The retention times of steroids decreased 0.5–7% with increase in temperature.

Two gradient profiles, linear and multistep, were used in the operating procedure for RP separations of steroids. The results of Table 2 indicate that index variations are 0–49 i.u. (14 i.u. on an average) and 0–34 i.u. (7 i.u. on an average) between two gradient profiles at two temperatures of 25.0 and 35.0°C, respectively, whereas change in the gradient profile from linear to stepwise decreased the retention times of steroids as much as 20–45%.

The retention indices of steroids were compared on two C18 columns from different manufacturers. Retention times varied again as much as 20–40% between the columns, but retention indices showed variations only 0–32 i.u. (15 i.u. on average).

Comparison was also made of two instruments from different manufacturers. Retention times varied 0.15–1.59 min (13–17%) between two instruments, being shorter on the HP instrument. The retention

index values showed variations of only 1–12 i.u. (6 i.u. on average) in spite of a complex solvent gradient.

The mean SD for all the steroids and all changes in chromatographic conditions together was 10.8 i.u.

The internal standard method is generally preferred in retention index monitoring, because of the insensitivity to small variations in chromatographic conditions. One advantage of external standardization is that with samples of unknown composition, there is no possibility of a sample component being masked by the peak for the standard. We tested as an alternative to the external retention index standard method, where the retention times of index standards were measured in a separate calibration run. The average variation in RI value of steroids was 1.3 i.u. between internal and external standard methods.

Previously we have carefully studied and noted the chromatographic parameters with the greatest effect on the reliability of the gradient indices of chemical warfare agents, their degradation products and precursors [44,58,59]. The most critical parameters were the source of the RP columns, column temperature, the organic modifier of the eluent, the pH of the

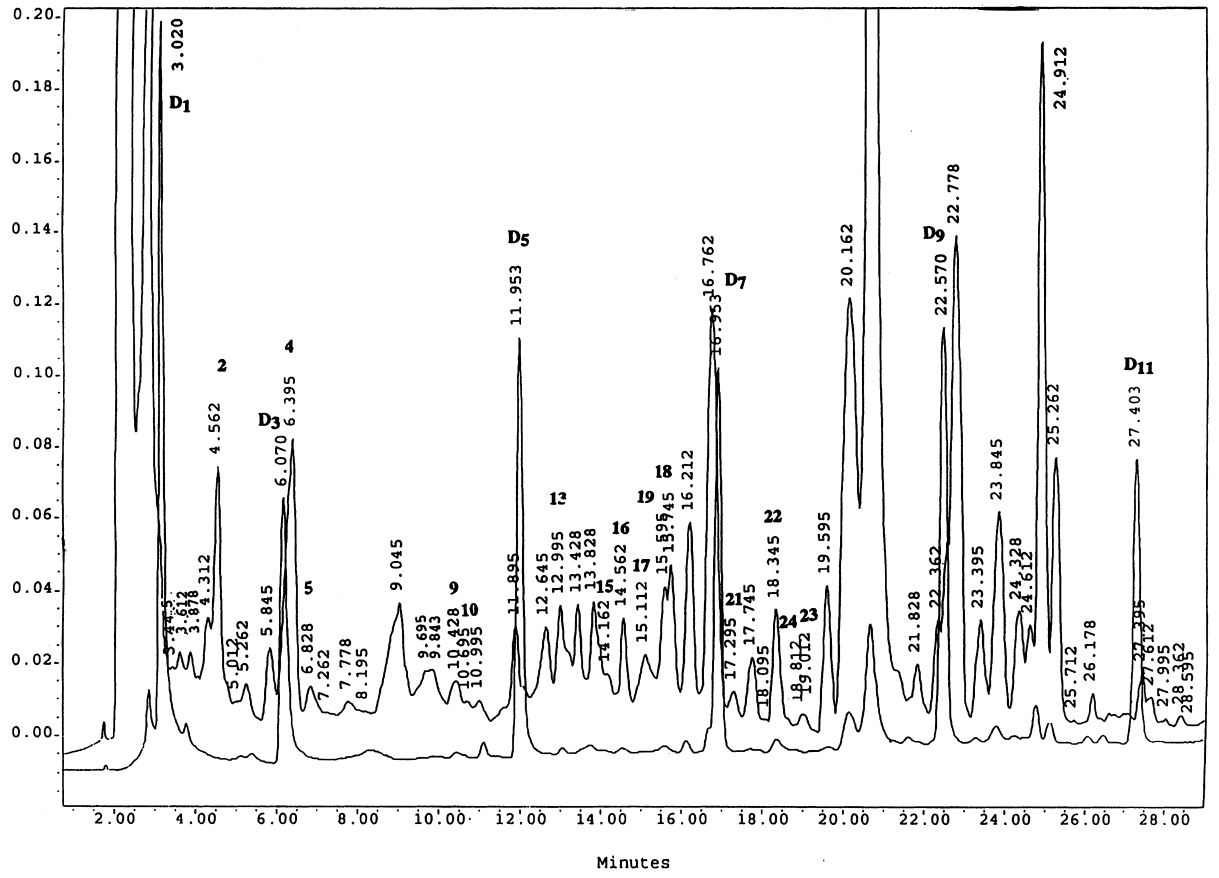


Fig. 3. Steroid profiling of a female serum sample. Chromatographic conditions: Waters; column, Zorbax Rx-C18 (250×4.6 mm I.D.), 5 μm; column temperature, 35°C; flow-rate, 1.0 ml/min; stepwise acetonitrile–water gradient, 30, 35, 35, 42, 42, 50, 60, 70 and 100% acetonitrile at 0.00, 3.00, 3.20, 5.00, 6.90, 8.00, 15.00, 18.00 and 30.00 min, respectively; UV absorbance detection, 205 nm. Peaks: 2=estriol (4.562 min), 4=cortisol (6.395 min), 5=cortisone (6.828 min), 9=21-deoxycortisol (10.428 min), 10=11β-hydroxyandrostenedione (10.695 min), 13=β-estradiol (12.995 min), 15=testosterone (14.162 min), 16=17α-hydroxypregnenolone (14.562 min), 17=estrone (15.112 min), 19=androstenedione (15.595 min), 18=17α-hydroxyprogesterone (15.745 min), 21=5α-androst-1-ene-3,17-dione (17.295 min), 22=etiocholanolone (18.345 min), 24=androstanedione (18.812 min) and 23=androsterone (19.012 min). D_1 (3.020 min), D_3 (6.070 min), D_5 (11.953 min), D_7 (16.762 min), D_9 (22.570 min) and D_{11} (27.403 min).

eluent with ionizable compounds and the exclusion of those members of the index series strongly determining the shape of the interpolation curve. Further, the change in the gradient program caused the index reliability to deteriorate a little.

3.3. UV spectra

The DAD provides a great amount of multiwavelength chromatographic and spectroscopic data for the identification and confirmation of the analytes. The on-line UV absorbance spectra which have now been recorded in gradient elution conditions for each steroid (Table 1) provide a useful identification parameter in addition to retention indices. The UV absorbance spectra shown in Table 1 are well-known in the literature [64], but it is also well known that the spectra taken in different solvent conditions are not fully comparable [65]. The UV absorbance spectrum is functional group-specific in its maxima and overall shape. Estriol (2), β-estradiol (13) and estrone (17) have a conjugated system formed by the aromatic ring and the hydroxyl group and therefore they produce UV spectra with strong absorption at 200 nm and two weaker ones at 220 and 280 nm. The compounds in which the double bond is conjugated to a carbonyl group (1, 4, 5, 8, 9, 10, 15, 18, 19 and 21) generate a characteristic UV absorption at 240–250 nm region. The steroids having isolated carbonyl groups (3, 6, 7, 11, 12, 14, 20, 22, 23, 24) or that with the unconjugated double bond–carbonyl group function (16) exhibit an end absorbance near 200 nm and a very low intensity and broad absorption at 280–290 nm. Thus UV absorbance spectra are useful for group identification of compounds from different groups of structural analogs. Even though the D-standards were co-eluted in some experiments with the sample steroid, they could easily be distinguished by their UV absorbance spectra (Table 1).

3.4. Biological applications

A steroid profile is a valuable screening test in the cases where dysfunction of steroid-forming organs is suspected.The applicability of the RI method was tentatively tested by screening a series of serum samples from adult patients.The profiles looked very similar. In Fig. 3 is illustrated an example of the separation of estrogenic, cortico- and androgenic steroids in a serum sample. The steroids were identified using external retention indices and UV absorbance spectral profiles. In Table 3 are summarized the retention indices of the steroids in the serum sample, determined using Waters HPLC apparatus, a Zorbax Rx-C18 column and multistep gradient profile with acetonitrile–water at the temperature of 35°C. The steroids could be identified on average within 11 i.u. of the values of the calibration run by the external RI method.

Table 3
Retention indices of steroids in a serum sample determined by the external RI standard method

Steroid No	Retention index	
	Serum sample	Calibr. run
1	–	202
2	208	218
3	–	323
4	317	323
5	337	336
6	–	395
7	–	415
9	454	443
10	462	455
12	–	494
13	537	524
14	–	566
15	584	571
16	601	590
17	624	601
18	651	634
19	644	630
20	–	682
21	713	699
22	752	739
23	776	764
24	769	763

Chromatographic conditions, see Fig. 3.

4. Conclusions

The screening method was developed for the identification of serum steroids by using RP-HPLC system. It contains retention index data based on 1-[4-(2,3-dihydroxypropoxy)phenyl]-1-alkanones, obtained with an acetonitrile–water gradient system and UV absorbance spectra produced by DAD.

Stereochemistry and molecular interactions seem to be very significant in determining the retention. Under stable chromatographic conditions both retention times and retention indices are reproducible, but under changing chromatographic conditions the retention indices demonstrate higher reproducibility and transferability between systems than retention times. The RI system provided a simple screening method for the identification of androgens, estrogens and corticosteroids in serum samples.

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