Analysis of Steroids by Capillary Supercritical Fluid Chromatography with Flame-Ionization and Electron-Capture Detectors

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Abstract

The separation and determination of several steroidal substances are achieved with capillary supercritical fluid chromatography by using supercritical CO_2 as mobile phase. Different stationary phases are used to compare chromatographic results, optimize the separation, and obtain information about the chromatographic behavior of the substances examined. Unexpected elution orders and problems of poor sensitivity of the flame-ionization detector towards some compounds are discussed. Alternative chromatographic conditions are explored: an electron-capture detector is used with suitably optimized pressure gradients in an attempt to overcome these limitations.

Introduction

Steroids constitute a broad class of substances that include compounds of different chemical and physiological properties ranging from anabolic agents to sexual hormones and adrenal cortex hormones. They possess widely distributed chemical and spectroscopic properties, which makes the chromatographic separation and detection of a complex mixture difficult to achieve with a single set of experimental conditions. In addition, selective and sensitive detectors are often required for their determination in real samples. As a consequence, many different sample clean-up procedures, chromatographic separation conditions, and detection techniques (often after suitable derivatization reactions) have been used; each one is applicable to a restricted number of compounds or often to a single one.

Both high-performance liquid chromatography (HPLC) and gas chromatography (GC) have been used in the analysis of these substances. In most instances, derivatization techniques were necessary either to enhance the sensitivity and specificity of detection in HPLC or to enhance volatility in GC (1–6).

In a previously published paper (7), we discussed general HPLC chromatographic conditions for the separation and identification of several anabolic substances. However, no more than six substances were separated in a single chromatographic run as a result of the compromise between the versatility and selectivity of HPLC and the restrictions imposed by ultraviolet detection. In that study, it was necessary to program three different wavelengths during the run.

In recent years, supercritical fluid chromatography (SFC) has been demonstrated to be an effective separation method for nonvolatile and thermally labile compounds. In fact, capillary SFC offers a great chromatographic versatility because of its GC-like separation efficiency and HPLC-like mobile phase solvent strength. It may be a viable alternative for drug analysis.

There are a limited number of capillary SFC applications to steroid separation. Separation of simple mixtures has been performed using a CO_2 mobile phase and a flame-ionization detector (FID) (8). Derivatization with dimethylthiophosphine chloride to produce steroidal thiophosphinic esters has been adopted to exploit the selectivity and sensitivity of phosphorous thermoionic detector (9), and on-line Fourier-transform infrared detection has been used with model mixtures of steroids to gain structural information (10). Supercritical CO_2 was the mobile phase used in these cases. Freon 22 has also been used as a supercritical extractant and a mobile phase to better solubilize the more polar species (11).

In this study, we analyzed several steroids (Figure 1) and one nonsteroidal anabolic agent (diethylstilbestrol) with capillary SFC using supercritical CO_2 as the mobile phase and flame-ionization and electron-capture detectors.

The design of the study reflects a double purpose: to optimize the separation and to try to define the underlying separation mechanism. Hence we chose steroidal substances with correlated structural properties (with the addition of a substance like diethylstilbestrol, which has two aromatic rings and two free hydroxy groups), and we used diverse stationary phases to compare chromatographic results and to optimize resolution.

The application of capillary SFC to the analysis of these compounds is advantageous for the following reasons (in addition to those already stated): (*a*) supercritical CO_2 is miscibile with the solvents more frequently used for the extraction of these compounds from real samples, (*b*) derivatization reac-

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tions are unnecessary, (*c*) a relatively low analysis temperature and a universal detector (FID) are used, and (*d*) the procedure can be applied to routine analysis because column conditioning between runs is unnecessary.

Some of the substances had long retention times and were poorly detected. Because such behavior may be attributable to the slight solubility of the compounds in supercritical CO_2, more specific chromatographic conditions and a more sensitive detection system (ECD), which exploited the presence of several keto and free hydroxy groups, were used in an attempt to improve the quality of the results.

Experimental

A Model SFC 3000 (Carlo Erba Strumentazione, Milan, Italy) was used. It was equipped with a split injector, an FID, and an ECD. SFC-grade carbon dioxide (SIAD, Milan, Italy) was used as the mobile phase. Injection was accomplished via a pneumatically actuated valve with an internal sample loop volume of 1.0 µL. Split injection was used for all analyses (split ratio, 40:1). The following capillary columns were used: a bonded polydimethylsiloxane column (10 m × 50-µm i.d., 0.25-µm film thickness) (OV-1; Carlo Erba Strumentazione), a bonded polyethyleneglycol (10 m × 50-µm i.d., 0.25-µm film thickness) (Carbowax; Lee Scientific, Salt Lake City, UT) and a bonded 14% cyanopropylphenyl 86% polydimethylsiloxane column (10 m × 50-µm i.d., 0.25-µm film thickness) (OV-1701; Carlo Erba Strumentazione). Integral restrictors (12) formed directly at the end of the columns were used to assure the pressure restriction required for the detectors. Reference chemicals were obtained in the highest purity available from commercial sources (Sigma, Rochester, NY).

The following compounds were used: dexamethasone, 17-α-CH_3-5-androsten-3β,17β-diol, diethylstilbestrol, 19-nortestosterone, ethisterone, testosterone, 16-OH-progesterone, progesterone, 1-dehydrotestosterone, trenbolone, corticosterone, cortisone-21-acetate, prednisolone, and prednisolone-21-acetate. Stock solutions of these standards were prepared in methanol (200 ppm).

Chromatographic conditions that were used for all separations illustrated include an injection valve temperature of 50°C and a split ratio of 40:1. The temperature of the FID was 300°C. The ECD operating conditions were as follows: temperature, 300°C; pulse voltage, 50 mV; pulse width, 1.0 µs; reference current, 1.2 mA; make-up gas, argon–5% methane.

The elution conditions for the separation in Figure 2 were as follows: initial pressure, 10 MPa; ramp time, 1.5 MPa/min; final pressure, 35 MPa; oven temperature, 180°C; detector, FID. The elution conditions for the separation in Figure 3 were as follows: stationary phase, OV-1701; oven temperature, 90°C; initial pressure, 10 MPa; ramp time, 0.5 MPa/min; final pressure, 35 MPa; detector, FID. The elution conditions for the separation in Figure 4 were as follows: stationary phase, OV-1701; oven temperature, 90°C; initial pressure, 15 MPa; ramp time, 0.9 MPa/min; final pressure, 35 MPa; detector, ECD.

Results and Discussion

The SFC separation of steroidal compounds on a polar packed column (silica) has been reported (13). In this study, it was necessary to use a fraction of organic modifier in the supercritical CO_2 to elute the solutes with reasonable retention times and good peak profiles.

Irreversible retention or strong peak tailing are not substantial problems with the use of capillary columns with medium polarity stationary phases like cyanopropylphenylmethylsilicone (OV-1701) or polyethyleneglycol (Carbowax). In fact, on these types of stationary phases and on poly-

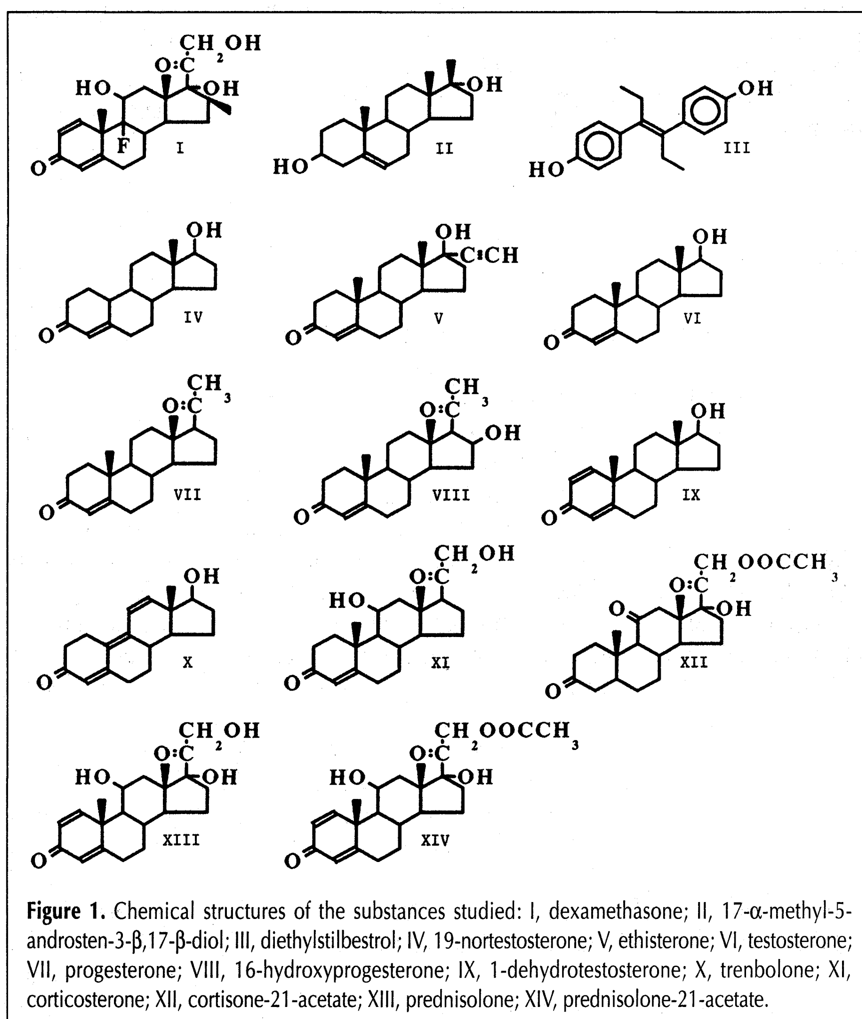

Figure 1. Chemical structures of the substances studied: I, dexamethasone; II, 17-α-methyl-5-androsten-3-β,17-β-diol; III, diethylstilbestrol; IV, 19-nortestosterone; V, ethisterone; VI, testosterone; VII, progesterone; VIII, 16-hydroxyprogesterone; IX, 1-dehydrotestosterone; X, trenbolone; XI, corticosterone; XII, cortisone-21-acetate; XIII, prednisolone; XIV, prednisolone-21-acetate.

methylsilicone, we achieved separations with satisfactory peak profiles if the retention times were not excessively long (Figure 2). Only pressure programming (which influenced the solvent strength of mobile phase) and temperature (which affected both the mobile phase strength and the separation mechanism) were important in determining the quality of the separation. On the other hand, any attempt to improve resolution by tuning the flow rate of the mobile phase by means of varied restrictor conditions was unsuccessful. This result was not unexpected; the van Deemter equation is rigorously applicable only in the absence of gradient elution conditions because of the overwhelming effect of mobile phase programming on any separation. An analogous scale of importance of experimental variables was found in a previous work (14).

Table I shows the retention times and density values at elution of the various compounds for all the columns used. All the substances eluted at pressure (or density) values that corresponded to the maximum value of the gradient program (there were a few exceptions, as in the case of the Carbowax column). There were some coelution problems, but any attempt to obtain a separation under isobaric conditions at the highest density value (0.478 g/cc) failed.

The optimal temperature and pressure gradient conditions for the three separations were identical, so to rationalize the results obtained, we made the simplifying assumption that the different chromatographic behaviors of the compounds eluted on the diverse columns are a direct consequence of solute–stationary phase interactions only.

However, according to the data in Table I, there are several anomalies in the elution orders that were expected on the basis of the assumption that only solute–stationary phase interactions prevail. For instance, corticosterone and cortisone-21-acetate are the last substances to elute both on the apolar OV-1 column and on the polar Carbowax column.

Next, we characterized the polarity and selectivity of the stationary phases by using the McReynolds constants (15) in an attempt to find a correlation with the elution orders obtained. We were unsuccessful. In fact, identical mobile phase conditions do not necessarily imply that the chromatographic differences depend only on the type of stationary phase because mobile phase–stationary phase interactions must be taken into account in SFC.

In addition, the variation of solubility in supercritical CO_2 of compounds that differ in the number of polar moieties (keto and hydroxy groups) present in their structures may further complicate the chromatographic behavior in an unpredictable manner.

A similar situation was found in a previous work that dealt with the SFC separation of triacylglycerols in vegetable oils (14).

Lower solubility may also affect the detection sensitivity. In fact, more polar substances like cortisone acetate and corticosterone, when eluted within reasonable retention times, are detected with poor sensitivity at the scale expansion typical of detection of more apolar solutes (for example, Figure 2A) or are not detected (Figure 2B). To obtain an acceptable detection of these two steroids under the same chromatographic conditions used for Figure 2B, it is necessary to use concentrations on the order of 1500–2000 ppm (80 ppm is used in the separation shown in Figure 2B). In one of his studies that dealt with the solvation power of dense gases, Giddings (16) determined parameters like threshold pressure, which is the lowest pressure that yields a detectable signal. That work contained some useful information about the solubility in supercritical CO_2 of solutes that have a structure similar to those considered in this

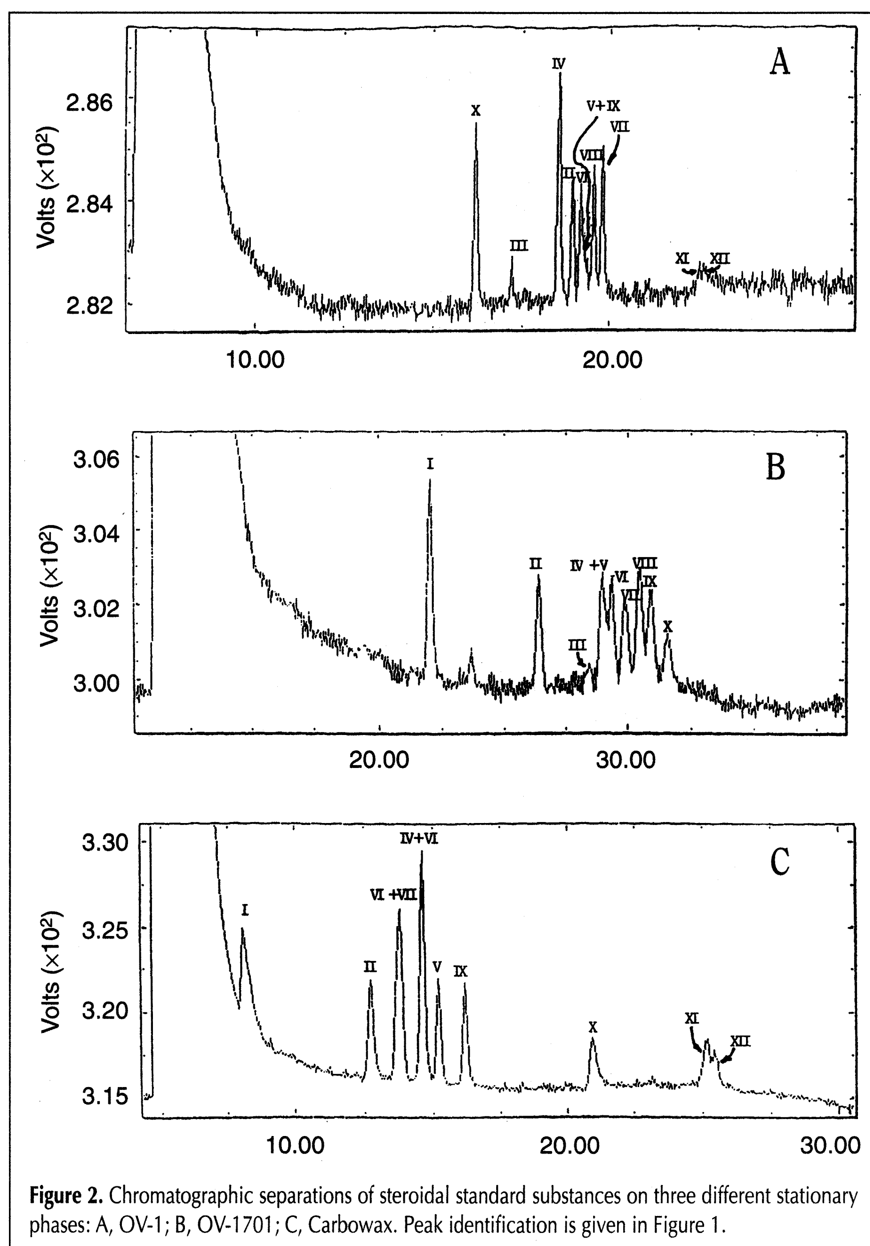

Figure 2. Chromatographic separations of steroidal standard substances on three different stationary phases: A, OV-1; B, OV-1701; C, Carbowax. Peak identification is given in Figure 1.

study. In particular, hydrocortisone and cortisone were shown to exhibit poor migration properties at a CO_2 pressure of 1300 atm and a temperature of 40°C; this is in agreement with our findings relative to cortisone-21-acetate and corticosterone.

To better elucidate the influence of solubility on detection sensitivity, we used an OV-1701 column to separate a model mixture of steroidal substances with different sensitivities to FID detection (Figure 3). The components of the mixture included two compounds that eluted early with good sensitivity (dexamethasone and ethisterone), two that eluted late with low sensitivity (corticosterone and cortisone-21-acetate), and a pair not taken into account before (prednisolone and prednisolone-21-acetate, which were used to evidentiate the effect of the substituent group).

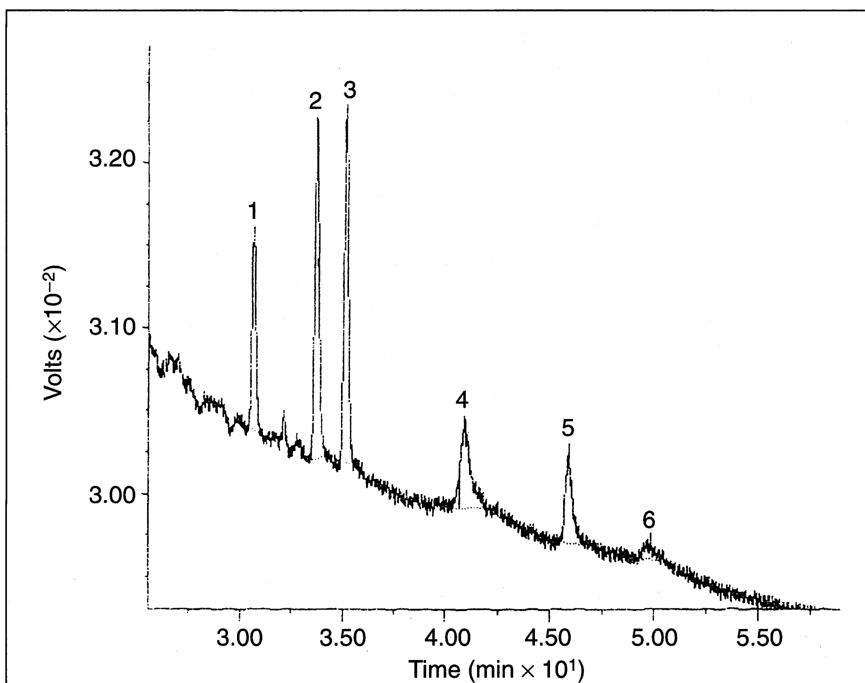

Figure 3. Chromatographic separation of six steroids on an OV-1701 stationary phase. Peaks (retention times): 1, dexamethasone (30.67); 2, ethisterone (33.79); 3, prednisolone (35.17); 4, corticosterone (41.18); 5, cortisone acetate (45.88); and 6, prednisolone-21-acetate (49.82).

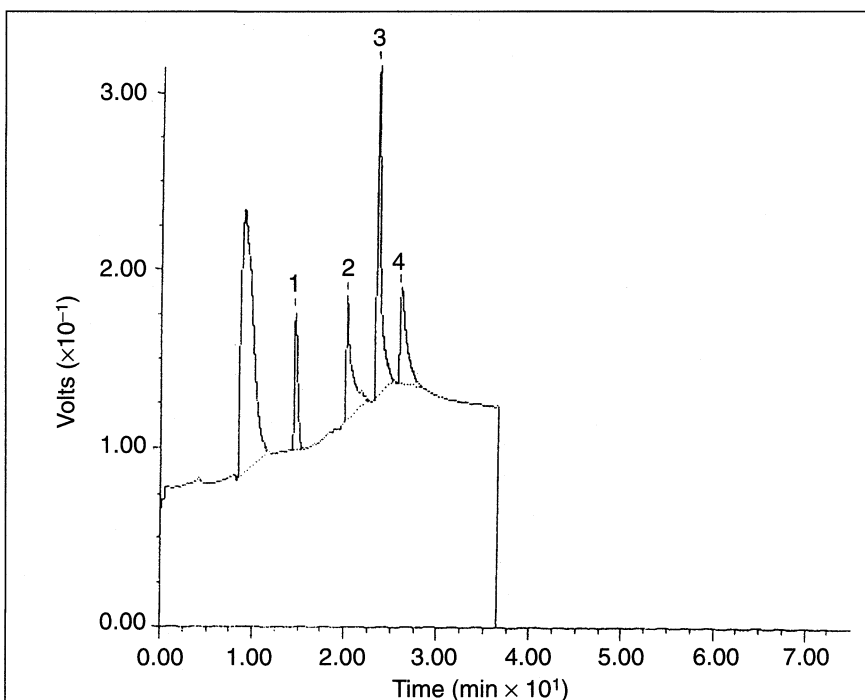

Figure 4. Chromatographic separation with ECD detection on an OV-1701 stationary phase. Peaks (retention times): 1, trenbolone (14.47); 2, corticosterone (20.09); 3, cortisone-21-acetate (23.33); 4, prednisolone-21-acetate (25.90).

The difference in detection sensitivity resulted from the effect of the presence of more polar groups after ionization of carbon atoms in the detection system and the diminished solubility in the mobile phase. Therefore, the solute concentration was higher (150 ppm) and the separation was performed at a temperature much lower (90°C) than the values used in the separation shown in Figure 2. This was done chiefly to enhance the solvent ability of the mobile phase and to improve detectability. The pressure gradient also was changed to achieve an optimized separation (see Experimental).

As seen in Figure 3, an increase in the polarity of the compounds corresponded to a diminution of sensitivity that is particularly dramatic for prednisolone-21-acetate (peak 6) with respect to prednisolone (peak 3) even if for the last ones the only difference is the presence of the acetate group that does not imply a great difference in polarity.

Although it was complex to provide a persuasive rationalization of the chromatographic results, we tried to find experimental conditions that improved the determination of the substances that have posed problems of separation and detection.

In Figure 4, an OV-1701 capillary column was used to separate a model mixture of trenbolone, corticosterone, cortisone-21-acetate, and prednisolone-21-acetate, which were the substances that eluted late and were poorly detected under the previous conditions. We used an ECD. As a result, the separation conditions were changed because of the operative restrictions imposed by such detector that shows a certain sensitivity towards the eluent. In fact, the baseline drifted with variations in density during the chromatographic run. Under these new conditions, any problems in the separation and detection of the compounds were resolved; in particular, the detection sensi-

tivity was increased (by a factor of approximately 20 as compared with FID). This increase can be seen in the mV scale reported on the vertical axis. Obviously, there is a loss of generality of application of the procedure because the other substances coeluted with the solvent peak under the conditions used to obtain Figure 4. Therefore, two different sets of conditions must be used to analyze all the substances in Figure 1.

Screening conditions are possible for nine compounds even if they have different sensitivity to FID. The reproducibility of retention times was within 1.0%, and the relative standard deviation of the precision in area determination was ±5.0%, which assured the reliability of qualitative and quantitative results. The limit of detection was 50 ppm.

For late eluting compounds, it was possible to obtain better separation conditions with good resolution features and higher detection sensitivity with the ECD system. The reproducibility of qualitative and quantitative determinations was close to those obtained under the other conditions. The detection limit was obviously much better; it ranged from 1.0 to 5.0 ppm.

With this procedure, a good preconcentration technique is needed for the trace analysis of such substances. However, in the case of physiological fluids, preconcentration factors cannot be high, so further improvements in sensitivity must be obtained by adopting other strategies such as the use of more polar supercritical fluids like Freon 22 or the use of a splitless injection system. In the latter case, some instrumental modifications and careful standardization of injection conditions are necessary. Studies in such a direction are in progress in our laboratory.

No definitive rationalization was possible for the chromatographic behavior of the substances. In fact, it was clear that simplified interpretation patterns are useless and that the operative parameters affect the separation in a complex way. In particular, the mobile phase solubility seemed to have important and not easily predictable effects on the separation, essentially because it has a limited range defined by the supercritical fluid adopted. To our knowledge, an analogous situation does not occur in HPLC because it is always possible to choose a mobile phase composition that covers all the solubility properties of the solutes.

With regard to the detection system, ECD is not commonly used in SFC separations, and we had to overcome some problems to exploit its features. To obtain a low background noise, we used flow rates that were as low as possible, consistently with reasonable elution times and relatively low pressure or density ramps because the baseline drift was high. The use of argon–methane (5%) as the makeup gas was suitable.

Table I. Retention Times and Density Values for the Mixture Constituents on the Columns Used

Compound	Retention time (min)	Density (g/mL)
OV-1701		
Dexamethasone	21.97	0.478
17-α-Methyl-5-androsten-3β,17β-diol	26.33	0.478
Diethylstilbestrol	28.35	0.478
19-Nortestosterone + ethisterone	28.94	0.478
Testosterone	29.26	0.478
Progesterone	29.85	0.478
16-OH-Progesterone	30.43	0.478
1-Dehydrotestosterone	30.82	0.478
Trenbolone	31.54	0.478
Corticosterone	ND*	
Cortisone-21-acetate	ND	
OV-1		
Trenbolone	16.15	0.469
Diethylstilbestrol	17.30	0.478
19-Nortestosterone	18.52	0.478
17-α-Methyl-5-androsten-3β,17β-diol	18.93	0.478
Testosterone	19.15	0.478
1-Dehydro-testosterone + ethisterone	19.34	0.478
16-OH-Progesterone	19.52	0.478
Progesterone	19.75	0.478
Corticosterone	22.75	0.478
Cortisone-21-acetate	23.00	0.478
Dexamethasone	ND	
Carbowax		
Dexamethasone	8.01	0.307
17-α-Methyl-5-androsten-3β,17β-diol	12.51	0.402
Progesterone + 16-OH-nortestosterone	13.64	0.424
Testosterone + 19-nortestosterone	14.39	0.438
Ethisterone	14.99	0.449
1-Dehydro-testosterone	15.96	0.466
Trenbolone	20.76	0.478
Corticosterone	24.80	0.478
Cortisone-21-acetate	25.18	0.478

* ND = not determined.

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