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Analysis of steroids Part 50[1]. Derivatization of ketosteroids for their separation and determination by capillary electrophoresis[2]

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Abstract

4-Ene-3-ketosteroids and 17-ketosteroids were quantitatively transformed into the corresponding hydrazones using Girard P and T reagents, respectively. The positively charged derivatives were separated by capillary electrophoresis. The spectrophotometric characteristics of the derivatives permitted their sensitive detection in the 230–280 nm range. The steroids investigated included nortestosterone and its phenylpropionate, norethisterone and its oenanthate, *d,l*-norgestrel, dehydroepiandrosterone, androstenedione and ethisterone.

Keywords: Capillary electrophoresis; Derivatization; 4-Ene-3-ketosteroids; Girard reagents P and T; 17-Ketosteroids

1. Introduction

Native steroid hormones are lipophilic uncharged molecules in acidic, neutral and moderately alkaline media and are therefore not suitable for separation and quantification by capillary electrophoresis (CE). This applies also to most of their synthetic analogues widely used in the therapy of various diseases.

However, micellar electrokinetic chromatography (MEKC), which can be considered to be a mode of capillary electrophoresis, is suitable for the separation and determination of these materials. Here the separation is based on the differential distribution of the analytes between a background electrolyte solution and micelles formed from various surfactants and on the differential migration of the micelles.

The surfactant used in steroid analysis is usually sodium dodecyl sulphate (SDS) [2–6], but the use of others such as bile salts [7], FC-135 and cetyltrimethylammonium bromide [8] has also been described. The separation power of the system involving the use of SDS can greatly be increased by adding urea [2,3], γ-cyclodextrin [3] or methanol [4] to the background electrolyte

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solution. The steroids separated were corticosteroids [2,3,7] and testosterone esters [5]. The solution of a few practical problems has also been described, such as the purity control of fluticasone propionate [4], the simultaneous determination of hydrocortisone and neomycin in ear drops [8] and the stability test of a perfusion solution containing methylprednisolone hemisuccinate, aminophylline and furosemide [6].

The aim of this work was to introduce a different approach to enable steroid hormone analogues to be determined by CE. This was intended to be achieved by a precapillary derivatization reaction by which the uncharged steroids were transformed into ionic derivatives, thus creating the basis for their differential migration under ordinary CE conditions.

1-(Carbazoylmethyl)- pyridinium chloride and carbazoylmethyltrimethylammonium chloride (Girard P and T reagents) were selected as the derivatizing agents. There were three reasons for this choice. First, these reagents form stable hydrazones with ketone groups. Since the majority of steroid hormones and hormone analogues contain ketone group(s), this seemed to be the most likely way of introducing charged moieties into their molecules. Second, the chemistry of the derivatization reactions of various ketosteroids with the Girard reagents is well known [9]. Although the primary use of the reagents is in the separation of mixtures of natural products (mainly steroids) into ketonic and non-ketonic fractions, their analytical applications in the paper [10] and thin-layer chromatographic [11], spectrophotometric and polarographic [12] analysis of ketosteroids is also well documented. Third, although the primary aim of using the derivatization reaction was to introduce a (positive) charge into the molecules of ketosteroids, the spectrophotometric properties of the hydrazones formed seemed to be favourable in respect of the sensitivity and selectivity of detection of the separated ketosteroids.

This paper is a preliminary report on the initial results obtained using the derivatization strategy outlined above for the determination of ketosteroids by CE. Although derivatization aimed at the formation of covalently bound charged derivatives is not widely used in CE analysis, this approach is not unprecedented. For example, reductive amination using 2-aminopyridine as the derivatizing agent [13] and a two-step derivatization of the same type with 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde as the reagent [14] were successfully used in the CE analysis of carbohydrates.

2. Experimental

2.1. Apparatus

An HP ^{3D}CE capillary electrophoresis system equipped with a diode-array detector, HP ^{3D}CE ChemStation software, an HP Vectra 486/66U computer and a LaserJet IV printer (Hewlett-Packard, Palo Alto, CA, USA) was used.

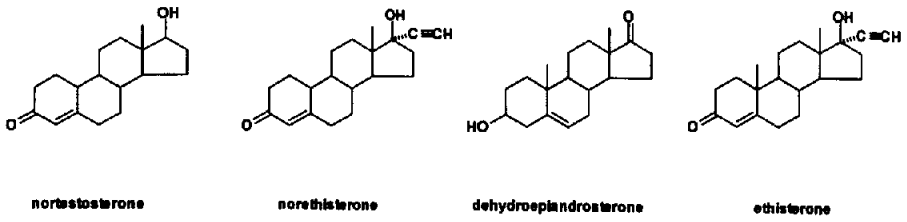
The experiments were carried out using a 64.5 cm (effective length 56 cm from the injector to the detector) by 50 μm i.d. fused-silica capillary with bubble cell from Hewlett-Packard. Detection was by UV absorption at the maximum of the steroid derivatives investigated (230–280 nm).

The applied voltage across the designated capillary was 20–30 kV (310–465 V cm^{-1}) and the capillary temperature was kept at 10–40°C.

New capillaries were conditioned by rinsing for 5 min with 1 M sodium hydroxide and for 30 min each with 0.1 M sodium hydroxide and background electrolyte.

The CE instrument was programmed to rinse with 0.1 M sodium hydroxide for 2 min and with the background electrolyte for 5 min and to introduce the sample under 5 kPa pressure for 1–5 s prior to each run.

The background electrolyte was automatically replenished after each sample injection to prevent ion depletion and to maintain satisfactory reproducibility of response and migration time.



nortestosterone norethisterone dehydroepiandrosterone ethisterone

2.2. Materials and reagents

The ketosteroids investigated were products of the Chemical Works of Gedeon Richter (Budapest, Hungary), Girard P and T reagents (zur Synthese), ethanol (99.5%, LiChrosolv HPLC grade), glacial acetic acid (100%, pro analysi) and sodium dihydrogenphosphate dihydrate (extra pure) were purchased form Merck (Darmstadt, Germany). Purified water was obtained with a Milli-Q 185 Plus purification system (Millipore, Bedford, MA, USA).

The background electrolyte was 20 mM sodium dihydrogenphosphate set to various pH values (3.0, 4.8 and 6.0).

The reagents were 75 mM solutions of Girard P or T reagent prepared by dissolving sufficient quantities in freshly prepared etahanol–glacial acetic acid (9:1, v/v) using an ultrasonic bath. These solutions are stable without significant decomposition at ambient temperature (20–30°C) for at least one day.

2.3. Methods

The test sample containing 0.015–0.05 mM ketosteroid was dissolved in 10 ml of Girard P or T reagent solution. In the analysis of 4-ene-3-ketosteroids the solution was allowed to stand at ambient temperature for 30 min whereas for 17-ketosteroids the reaction time was 120 min (or alternatively 40 min at 80°C).

The test solutions for electrophoresis were prepared by diluting the above reaction mixture with the background electrolyte in a 1:10 (v/v) ratio. The injection into the CE instrument was carried out immediately after the preparation of the test solution.

Futher details are given in the sections Apparatus and Results and discussion and in the figure captions.

3. Results and discussion

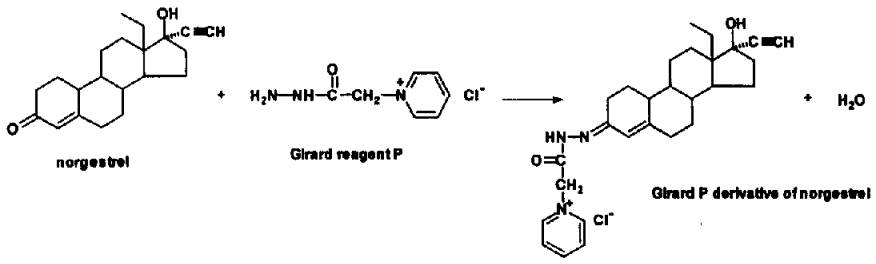
3.1. The derivatization reaction

The reaction taking place between *d,l*-norgestrel (representing the 4-ene-3-ketosteroids) and Girard P reagent is depicted in Scheme 1. Analogus reactions take place with other ketosteroids and Girard T reagent.

In accordance with published data [9], the reactivity of 4-ene-3-ketosteroids is much higher, thus enabling shorter reaction times to be adopted than with 17-tetosteroids. Careful control of the reaction mixture in reaction of norgestrel with Girard T reagent by CE and UV spectrophotometry revealed that the molar absorptivity (ϵ) of the derivative is 25 600 at 280 nm at the time when the CE signal vs. reaction time curve reaches its maximum value. This value is in accordance with those of related hydrazone derivatives [12], indicating that not only is the reaction rapid but also its yield is quantitative if a sufficiently high excess of the reagent (at least 15-fold) is used. No change was observed in the CE profile of the reaction mixtures for up to one day.

3.2. Spectral characterization of the derivatives

Fig. 1 shows the diode-array UV spectra of Girard P and T reagents and the hydrazone derivatives of *d,l*-norgestrel and dehydroepiandrosterone (3β-hydroxy-5-androstene-17-one) after separation by CE. The spectrum of Girard P reagent is a typical pyridine spectrum



Scheme 1.

($\epsilon_{261_{nm}} = 5060$) [15] whereas in the spectra of the Girard P derivatives the contribution of the pyridine chromophore is hardly recognizable. This is overlapped by the much more intense acyl–hydrazone conjugation bands (see preceding section). In the case of dehydroepiandrosterone this appears at 233 nm, providing excellent possibilities for its selective and sensitive detection, compared with the poor detectability of this spectrophotometrically inactive material by HPLC. In the case of *d*, *l*-norgestrel and other 4-ene-3-keto-steroids, this band is shifted to 277 nm owing to

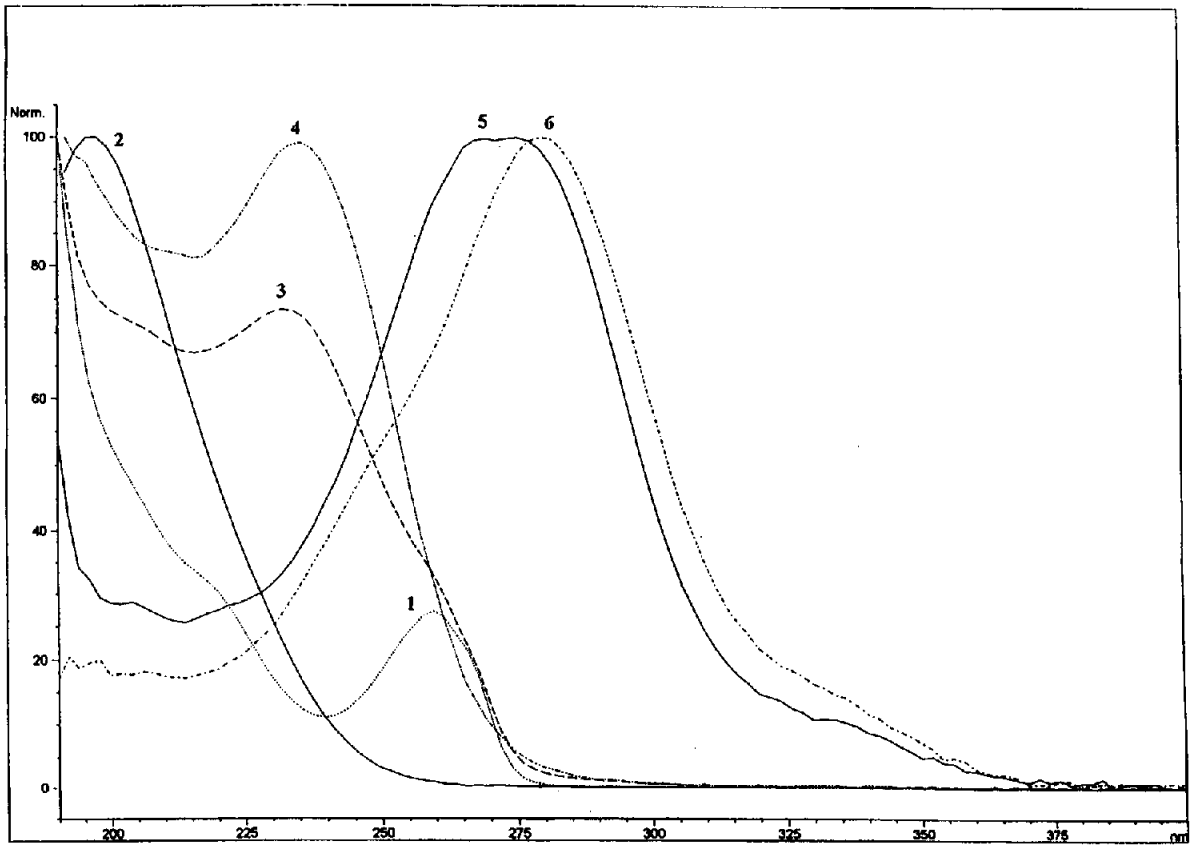


Fig. 1. Diode-array UV spectra of Girard P and T reagents and various ketosteroid Girard bydrazones taken from electropherograms. 1, Girard reagent P; 2, Girard reagent T; 3, dehyroepiandrosterone Girard P derivative; 4, dehydroepiandrosterone Girard T derivative; 5, *d,l*-norgestrel Girard P derivative; 6, *d,l*-norgestrel Girard T derivative.

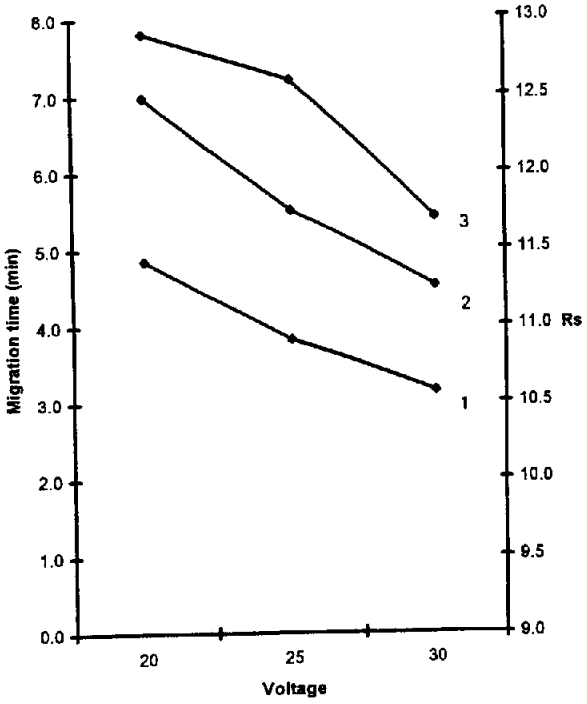


Fig. 2. Dependence of the migration times of Girard P reagent (1) and *d,l*-norgestrel–Girard P hydrazone (2) and their resolution (3) on the applied voltage. pH = 6; temperature, 30°C.

the presence of the 4,5-double bond, which lengthens the conjugation chain.

As is also seen in Fig. 1, the situation is essentially the same with the spectrophotometrically practically inactive Girard T reagent; maxima of the derivatives with dehydroepiandrosterone and *d, l*-norgestrel are at 237 and 282 nm, respectively. The absence of the pyridine chromophore results in narrower bands, but this does not affect the excellent detectability of both steroids. It is worth mentioning that another effect of the absence of the pyridine chromophore is that in the case of monitoring near 280 nm the peak of the large excess of the reagent is not detectable.

In the case of 4-ene-3,17-dione derivatives (e.g. androstenedione) which form bis-Girard hydrazones with two maxima around 245 and 280 nm, monitoring of the electropherogram at 250 nm is advisable.

3.3. Electrophoretic properties of the derivatives

Fig. 2 shows the voltage–electrophoretic mobility plot of the Girard P derivative of *d, l*-norgestrel and the excess of the reagent. It is seen that the migration time difference between the peaks of the low molecular weight reagent and high molecular weight steroid derivative, both possessing one positive charge, is large enough ($R_S > 11$) at any of the investigated voltages to provide space for peaks of related steroid derivatives. Similar observations were made also with the Girard T derivative. A voltage of 25 kV (388

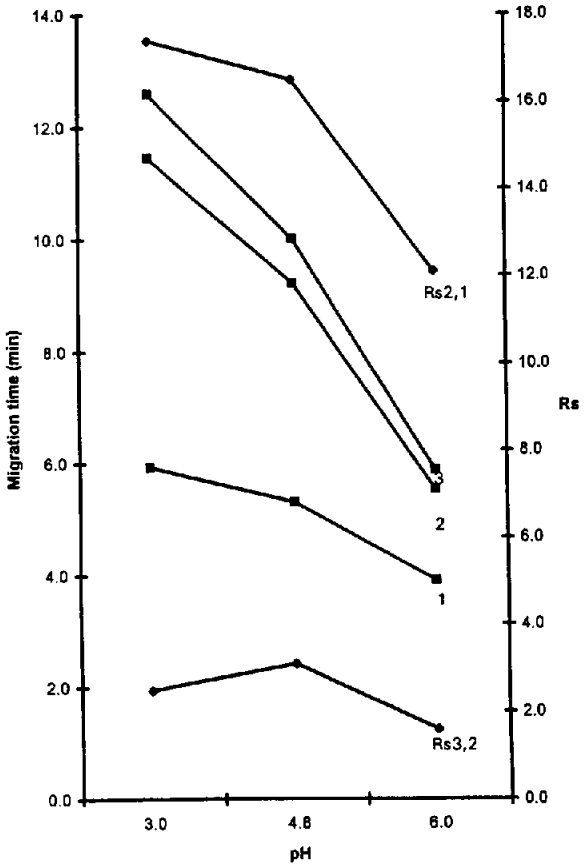


Fig. 3. Dependence of the migration times of Girard P reagent (1), norethisterone–Girard P hydrazone (2) and norethisterone oenanthate–Girard P hydrazone (3) and their resolution on the pH of the background electrolyte Voltage = 25 kV; temperature, 30°C.

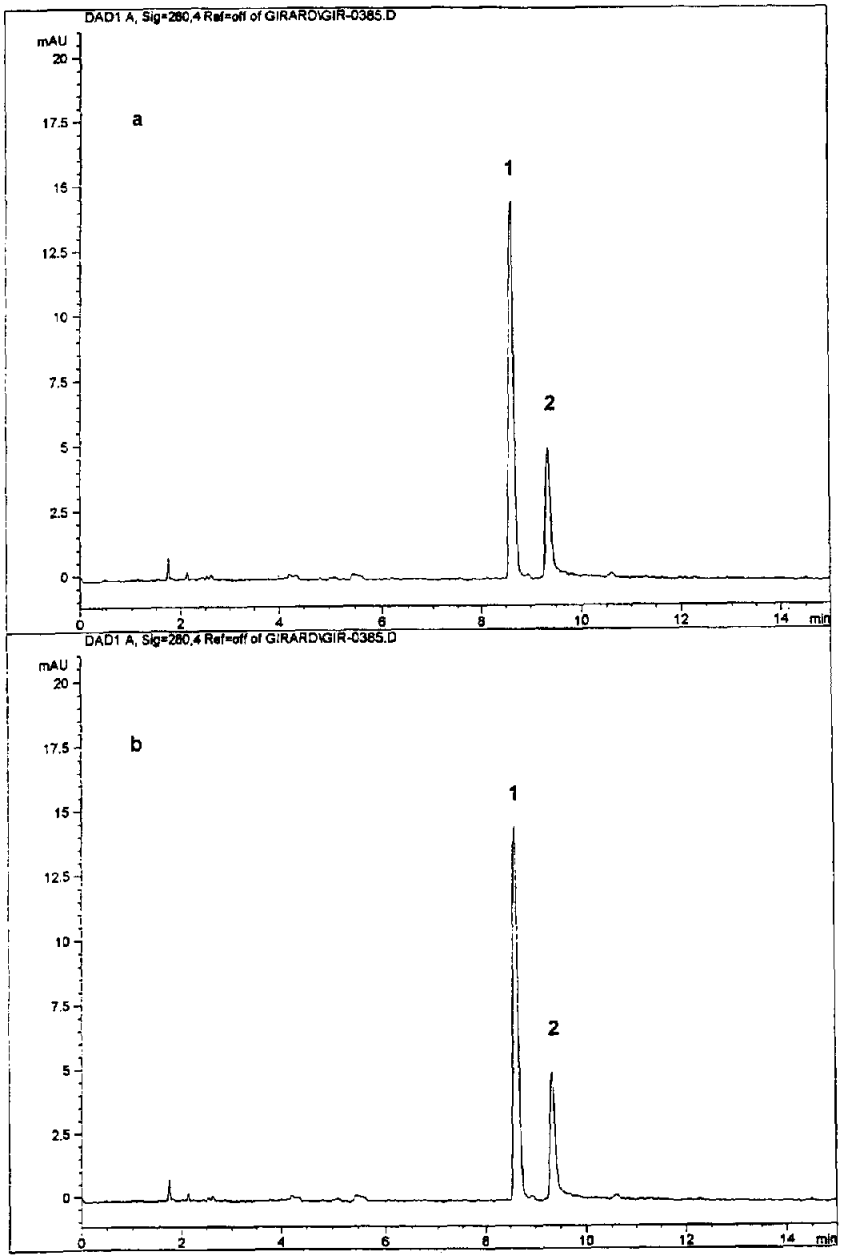


Fig. 4. CE separation of 17-hydroxy-4-ene-3-ketosteroids and their ester derivatives after derivatization with Girard T reagent. (a) Norethisterone (peak 1) and norethisterone oenanthate (peak 2). (b) Nortestosterone (nandrolone) (peak 1) and nandrolone phenylpropionate (peak 2). pH = 4.8; voltage, 25 kV; temperature, 30°C; injection time, 5 s; UV detection at 280 nm.

V cm^{-1}) was selected for further studies.

The effect of pH on the electrophoretic mobilities, the efficiency and the selectivity is demonstrated in Fig. 3 with the example of the Girard P derivative of norethisterone and norethisterone oenanthate. Although changes in the pH of the background electrolyte do not effect the ionization of the quaternary ammonium cation-type reagent and derivative, a decrease in the electroosmotic flow by decreasing the pH results in an

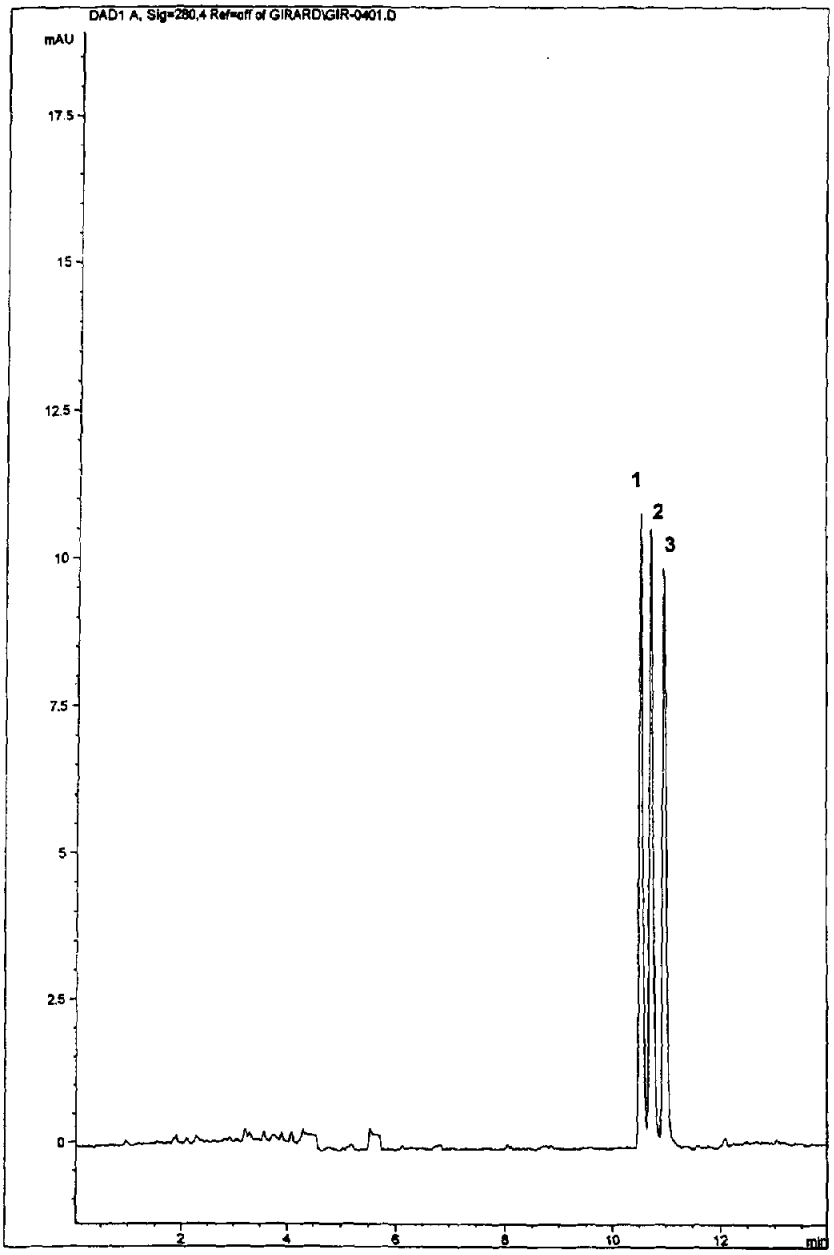


Fig. 5. CE separation of a model mixture of 19-nor-4-ene-3-ketosteroids after derivatization with Girard T reagent. 1, Nortestosterone; 2, norethisterone; 3, *d*, *l*-norgestrel. pH = 4.8; voltage, 25 kV; temperature, 15°C; injection time, 3 s; UV detection at 280 nm.

increase in the migration times of both the reagent and the analytes with a simultaneous improvement of the efficiency and selectivity. The situation is similar with the Girard T derivatives. pH 4.8 was selected for further investigations, where the resolution of the two steroids is optimal. At pH values below 4 the stability of the test solution is not sufficient for practical work since the Girard hydrazones tend to hydrolyse to the reagent and the parent ketosteroid under these

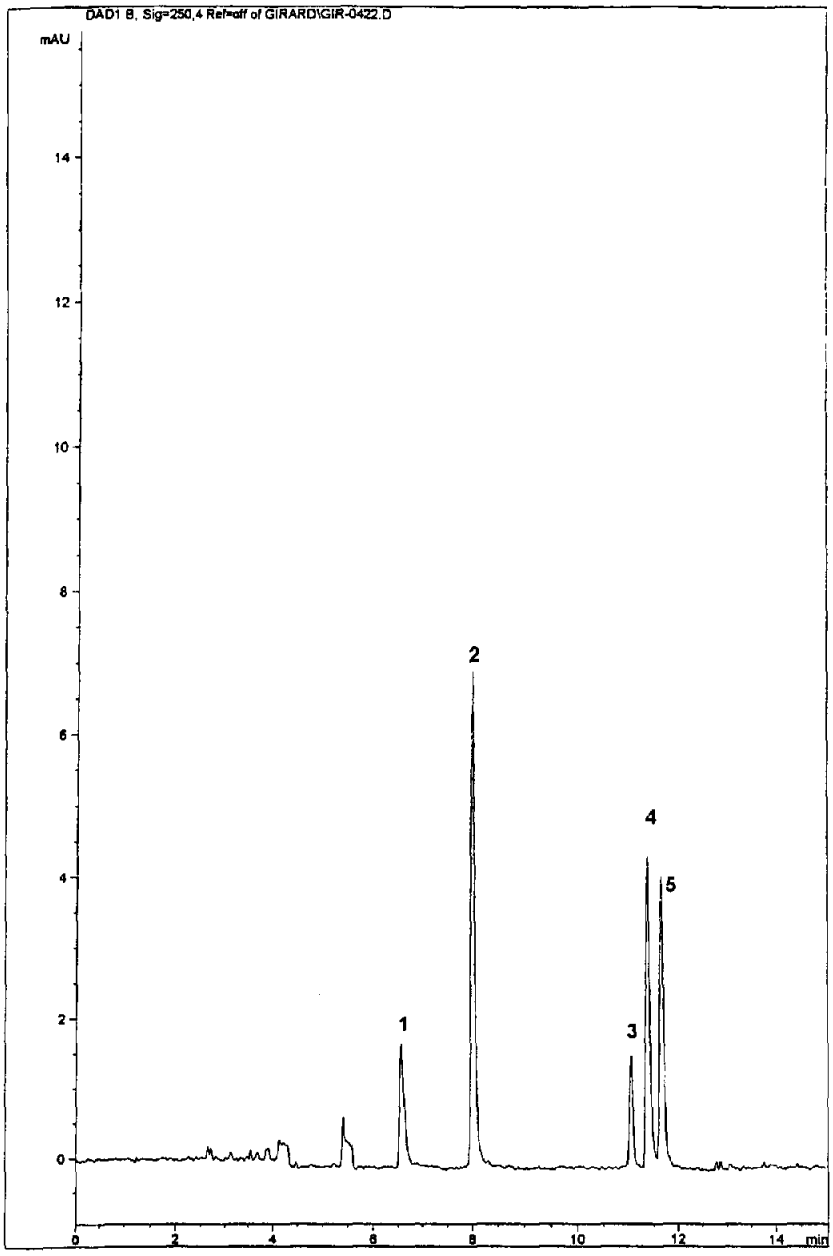
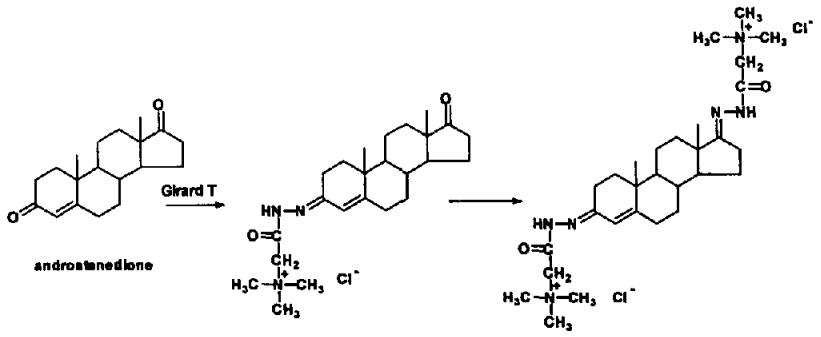


Fig. 6. CE separation of ethisterone and its synthetic precursors after derivatization with Girard T reagent (model mixture). 1, Girard T reagent; 2, androstenedione–bis-Girard T derivative; 3, androstenedione–tris-Girard T derivative; 4, dehydroepiandrosterone; 5, ethisterone. pH = 4.8; voltage, 25 kV temperature, 15°C; injection time, 3 s; UV detection at 250 nm.

circumstances [9]. As an illustration, the separation of the Girard T derivatives of norethisterone and its oenanthate and also nortestosterone and its phenylpropionate at pH 4.8 is shown in Fig. 4. (As already mentioned, the peak of the excess reagent (at 5.0 min) is not visible because it does not absorb at 280 nm).

The effect of temperature on the efficiency and



Scheme 2.

selectivity was also investigated over the range 10–40°C. The migration times increase with decreasing temperature together with a slightly improved separation of structurally related steroids. For this reason, working at subambient temperatures is useful in the solution of delicate separation problems.

Fig. 5 shows the electropherogram of a model mixture of 19-nor-4-ene-3-ketosteroids after derivatization with Girard T reagent obtained by adopting conditions which are considered to be the best at the present stage of the optimization, i.e., relatively low pH and temperature and also a short injection time, which is advantageous from the point of view of the efficiency and hence the separation of related ketosteroids (but at the same time naturally disadvantageous in any kind of quantitative work).

Another example is presented in Fig. 6, where the separation of a model mixture of ethisterone and its synthetic precursors (dehydroepiandrosterone and androstenedione) is presented. It is worth mentioning that the migration time of the bis-Girard T derivative of androstenedione bearing two positive charges is very short (peak 2) and the intermediate 3-mono-Girard T derivative is also separated (peak 3) (see Scheme 2).

3.4. Quantitative aspects

Although this paper does not contain applications of the described method for quantitative analytical purposes, the prospects of such applications seem to be good. The relative standard deviation (RSD) of the peak area of norgestrel–Girard P hydrazone calculated from five independent weighings and reactions was found to be 2.5%. This value is only slightly higher than the usual value for peak-area precision in CE without a preliminary derivatization reaction, RSD 1–2% [16]. The precision of the migration times (RSD $< 1\%$) is also good.

As a consequence of the good UV absorption properties of the derivatives and the good column efficiency, the limit of quantitation (defined as the peak height of the steroid derivative equal to ten times the signal-to-baseline noise ratio) is as low as 6 pg per injection. This offers good possibilities for the determination of ketosteroids in low-dose pharmaceutical preparations and biological samples.

4. Conclusions and future work

The derivatization of ketosteroids with Girard P and T reagents involves quantitative reactions and the properties of the derivatives thus formed are suitable for separation and quantitation by CE.

Although the method seems to be suitable for the solution of various simple analytical problems even in its present form, its selectivity is inferior to that of MEKC [2,3,5,7] and it is not suitable for the separation of structurally closely related steroids. Plans for future work include further optimization of the separation, extension

of the work to other types of ketosteroids and quantitative applications.

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