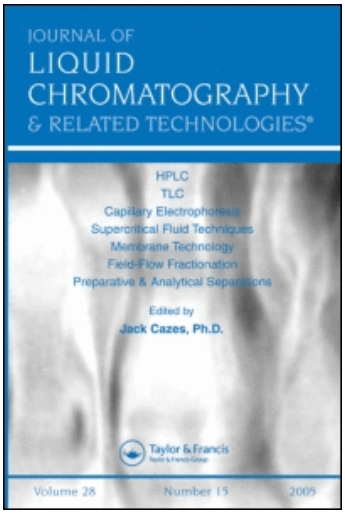

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MONITORING OF FLUORESCENT-LABELLED MAIN PROGESTERONE METABOLITE IN MARMOSET MONKEYS

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ABSTRACT

5α-pregnane-3α,7α-diol-20-one, the main progesterone derivative in marmoset monkeys, shows a very low absorption in UV-light. Labelling of this metabolite prior to HPLC and subsequent monitoring by an UV-detector is essential. We used dansyl-hydrazine for the formation of a fluorescent derivative. The optimum conditions for the reaction of dansyl-hydrazone with hydroxypregnanolone were controlled by HPTLC, chromatographic separation is carried out by HPLC and HPTLC and is described in this paper. When the quantitation of the dansylhydrazones is carried out by HPTLC, the fluorescence intensity of the derivative can be increased by a factor up to 5 by dipping the HPTLC plate into a mixture of paraffin/n-hexane or after treatment with triethanolamin/isopropanol (1:4; by vol). Hydroxypregnanolone from marmoset urine is detected for the first time by liquid chromatography. The derivative is of practical use to determine ovulation and pregnancy in the marmoset monkey. Quantitation by HPLC and thin-layer chromatography is possible in the range of 10 to 1000 ng. The concentrations in marmoset urine during the luteal phase is in the range of 50 to 400 ng/mg creatinine.

INTRODUCTION

5α-pregnane-3α,7α-diol-20-one (hydroxypregnanolone) is excreted in marmoset (Callithrix jacchus) urine as the main metabolite of progesterone. This metabolite appears in considerable amounts and has been proven very useful for the monitoring of ovulation in this primate species [8,10] This steroid shows, however, a very low absorption in UV-light. For this reason, the attempts to determine this excretion product by HPLC have failed up till now. The method described earlier for the determination of hydroxypregnanolone uses postchromatographic derivatization by toluene-sulfonic acid. This procedure, however, can only be applied after thin-layer chromatographic separation, and quantitative results require sufficient instrumentalization.

The 1-dimethylaminonaphthalene-5-sulfonyl (dansyl) group has proven very useful for the quantitation of various substances, e.g. proteins, polyamines, amino acids, phenolic hydoxyl groups [2,15,16,17]. For molecules containing a carbonyl group such as hydroxypregnanolone, dansyl hydrazine is a convenient dye. Initial experiments to form the dansylhydrazones of commercially available 5ß-pregnane-3α,6α-diol-20-one and 5α-pregnane-3α,17α-diol-20-one revealed considerable differences to marmoset hydroxypregnanolone under the conditions for optimum chemical reaction. The primary objective of this work was to establish a derivatization method for unconjugated hydroxypregnanolone isolated from marmoset urine in order to extend the possibilities for the monitoring of this derivative.

MATERIAL AND METHOD

Sample collection

Urine samples were collected from adult pregnant female marmosets (Callithrix jacchus) as described earlier [9] and stored frozen until hydrolysis and purification was performed.

Hydrolysis and extraction of steroids

Samples of 0.1 ml urine were thawed and incubated for 18 h at 37° C with 10 µl 1 M sodium acetate buffer pH 4.5, and 15 µl glucuronidase/arylsulfatase from digestive juice of Helix pomatia (ca. 5.2

U/ml glucuronidase activity, ca. 2.6 U/ml arylsulfatase activity). The extraction with 2.5 ml diethylether was performed by vigorous shaking. The organic and aqueous phases were separated by centrifugation for 12 min at 4500 x g. After removal of the aqueous phase, the residual organic solvent was evaporated.

Derivatization procedure

To the evaporated extracts from 100 µl urine containing the free steroids are redissolved in 100 µl ethanol. To this solution 0.2 ml ethanol containing 2 mg trichloracetic acid (TCA) (stock solution 100 mg TCA/10 ml ethanol) and 0.1 ml dansylhydrazine solution (50 mg dansylhydrazine/10 ml ethanol) are added. The total volume was 400 µl. The test tubes are allowed to stand for 60 min at room temperature. The solvents are then evaporated to dryness. The residue is dissolved in 0.1 ml ethylacetate for chromatographic separation.

HPTLC conditions

Aliquots of 10 µl ethylacetate containing the excreted and labelled steroids were sprayed bandwise onto precoated HPTLC silica gel plates with the Camag Linomat III using nitrogen as carrier gas (band-length: 190 mm, distance from the lower edge: 7 mm). Subsequently, the HPTLC plates were developed in a linear developing chamber in horizontal sandwich configuration. The chromatographic conditions were chosen to give sufficient separation of hydroxypregnanolone to the adjacent fluorescent substances which are extracted together with hydroxypregnanolone from hydrolyzed urine samples. The solvent system consisted of cyclo-hexane/ethylacetate/methanol (32:62:6; by vol). The plates were purified before use with the same solvent system. The separation of the sample extract was complete after 12 min.

HPLC conditions

The liquid chromatography was performed with a HPLC device from Knauer, Berlin, FRG. We used OSD2 columns 100x4.5 mm for the

separation of the fluorescent-labelled steroids. The mobile phase consisted of 50% acetonitrile in water with a flow rate of 2 ml/min. The steroid derivatives were detected by UV absorption at 340 nm and by fluorescence at 470 nm.

Reagents

5-[Dimethylamino]naphthalene-1-sulfonylhydrazine and the steroids for reference were purchased form Sigma Chemie, Deisenhofen, FRG. HPTLC silica gel 60 plates, trichloroacetic acid (TCA), trifluoroacetic acid (TFA), all organic solvents, and other analytical grade or chromatographic grade reagents were obtained from Merck, Darmstadt, FRG. Glucuronidase/arylsulfatase was purchased from Boehringer, Mannheim, FRG.

RESULTS

5α-pregnane-3α,7α-diol-20-one was purified from marmoset urine by preparative thin-layer chromatography as described earlier [10]. The isolated steroid was used for the development of the basic conditions for the formation of dansylhydrazones. Various factors were examined to obtain high recoveries of the labelled gestagen metabolite.

The solvents methanol and ethanol were compared in relation to their influence of high recoveries of dansylhydrazones in a first experimental series. Reaction mixtures containing hydroxypregnanolone in methanol, respectively ethanol, were incubated with dansylhydrazine in the presence of different concentrations of TCA. The reaction mixture contained in a total volume of 400 μl 0, 0.5, 1, 2, 3, and 6 mg TCA. Aliquots from each mixture were taken after different incubation times and analyzed for dansyl hydroxypregnanolone. Figure 1 shows the effect of the reaction time and the concentration of trichloracetic acid on the formation of the fluorescent derivative (given as peak area). Ethanol served as solvent. A constant peak area was reached after 60 min in the presence of TCA. The presence of TCA was a prerequisit for the formation of the dansyl-derivative. In a second experiment the solvent ethanol was replaced by methanol and the formation

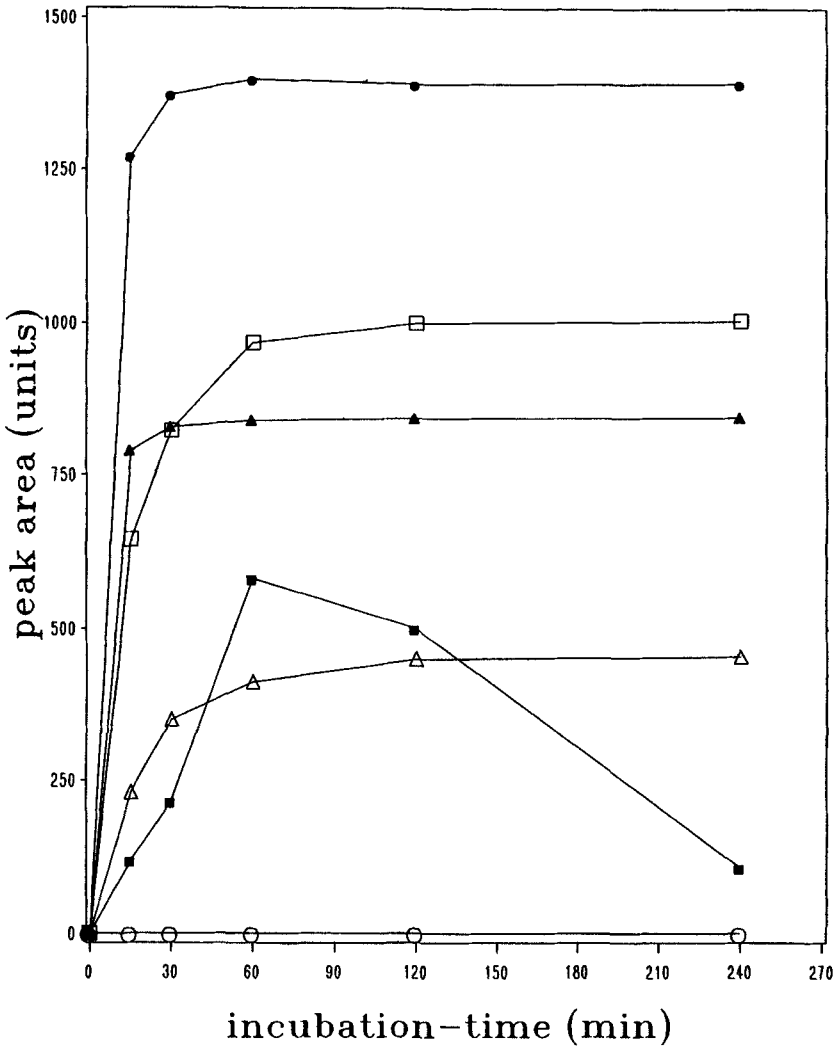

FIGURE 1. Influence of the reaction time and the concentration of trichloroacetic acid on the recovery of fluorescent labelled hydroxypregnanolone in ethanol as solvent. The reaction mixture contained in a volume of 0.4 ml ethanol: 5 mg dansylhydrazine; △ 0.5 mg, □ 1 mg, ● 2mg, ▲ 3 mg, ■ 6 mg of trichloracetic acid; purified hydroxypregnanolone from marmoset urine. The conditions for the chromatographic separation are described in "Material and Methods".

of the fluorescent hydrazones was monitored. Methanolic reaction mixtures yielded samples with a brigth background when chromatographed by HPTLC. We therefore prefered ethanol as solvent in all further experiments.

The influence of the reaction time in the presence of 2 mg TCA in a total reaction volume of 400 μl is given in Figure 2. The reaction time is strongly influenced by the concentration of TCA. Increasing TCA concentrations decrease the reaction time. Replacing TCA by HCl decreased the amount of dansylhydrazones. The chromatographic separation of these samples revealed dansyl-hydroxypregnanolone in a weak and blurred peak. TCA can be replaced by TFA without losses in yield and sensitivity (data not shown).

The effect of the derivatization temperature is demonstrated in Figure 3. The dansylhydrazone of 5α-pregnane-3α,7α-diol-20-one are prepared in ethanolic solution. For reaction conditions we have chosen the best conditions derived from the data in Figure 1. The reaction-mixture contained 2.0 mg TCA and 10 mg dansylhydrazine in a total volume of 0.4 ml. The mixtures were allowed to react for 60 min in a water bath at different temperatures. The results are shown in Figure 3. Optimum reaction temperature was found to be 20° C.

To check the best conditions for the dansylation of hydroxypregnanolone from urine extracts from pregnant marmoset females, we used the hydroxypregnanolone containing fraction which was not further purified prior to use. Equal amounts of extracts were allowed to react with different hydrazine concentrations. The formation of the labelled hydroxypregnanolone revealed a typical saturation curve when the peak area was plotted versus the amount of dansylhydrazine. The highest yield of the fluorescent derivative of hydroxypregnanolone is achieved with a 10-fold surplus of the amount of dansylhydrazine in relation to the amount of hydroxypregnanolone in the urine extract. The detailed conditions are described in Figure 4.

Calibration curve of peak area to hydroxypregnanolone standard is given in Figure 5. The calibration graphs for hydroxypregnanolone are linear in the range of 20 to 1000 ng. The detection limit was determined to be 10 ng. After dipping the plates into paraffin oil - n-hexane (2:1) or triethanolamin-isopropanol (1:4), the fluorescence can be increased by a factor of 5. The

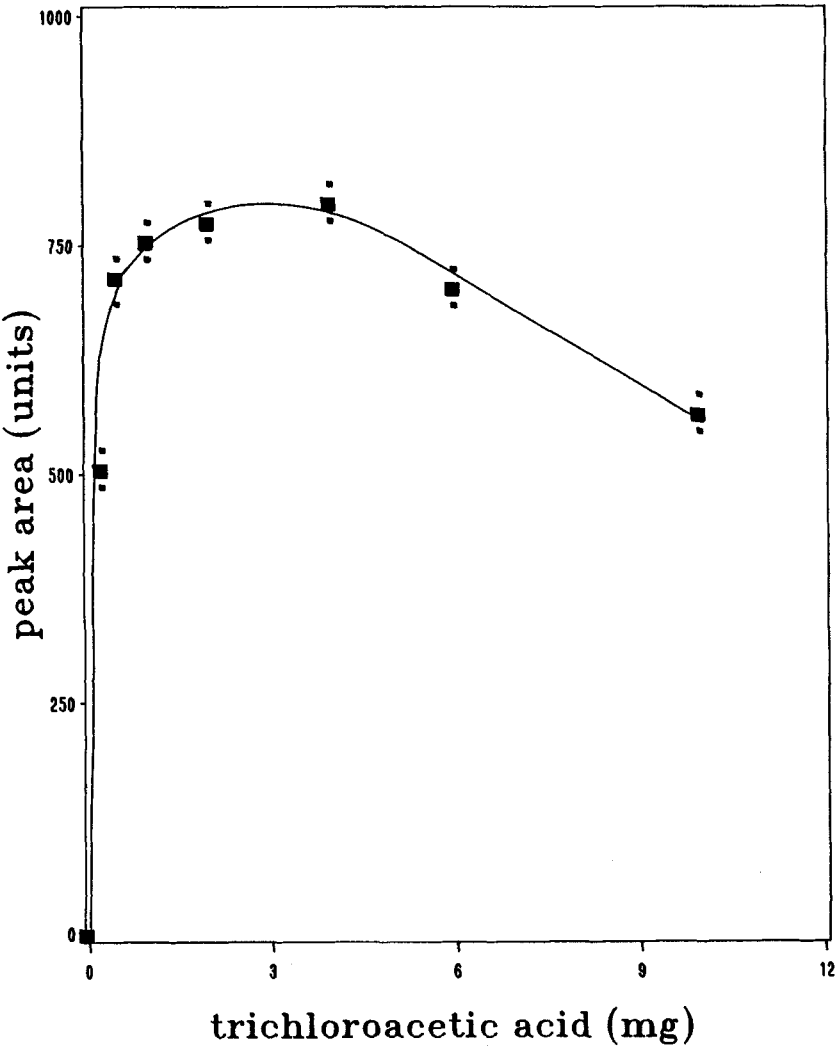

FIGURE 2. Effect of the variation of trichloroacetic acid on the formation of the dansyl derivative of 5α-pregnane-3α,7α-diol-20-one. The reaction time was 60 min, the concentration of purified hydroxypregnanolone was kept constant, and the dansylhydrazine concentration was 5 mg in a a total volume of 0.4 ml ethanol.

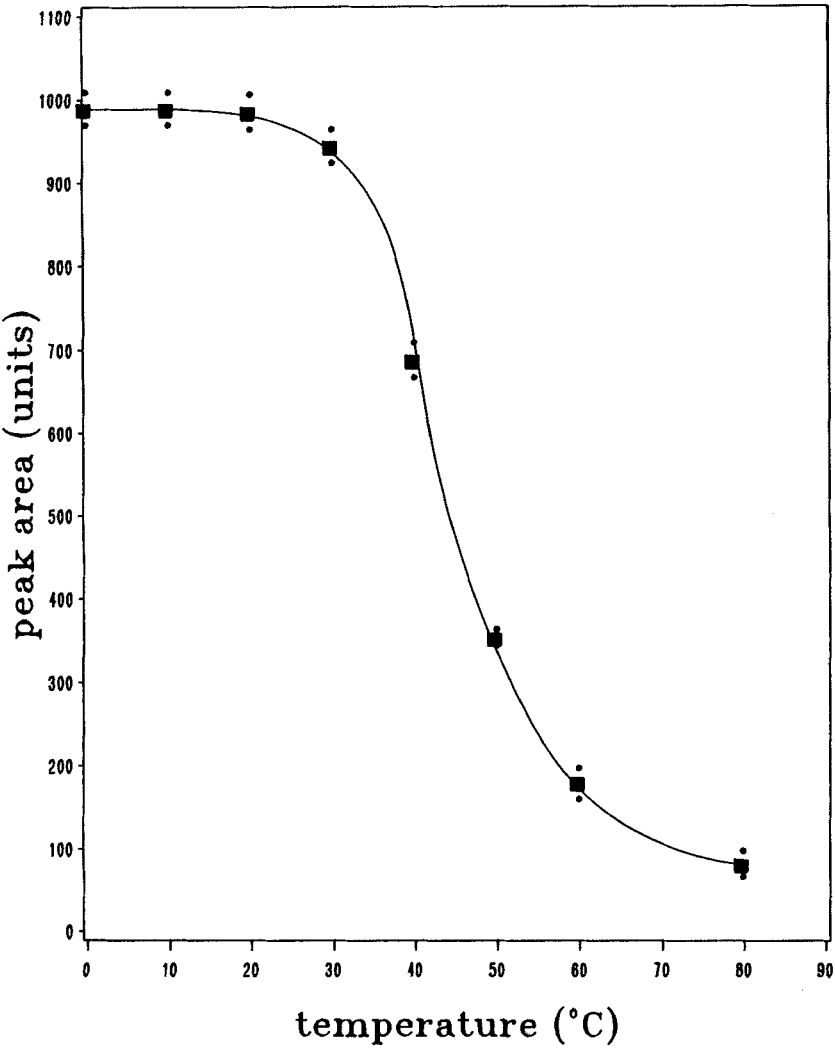

FIGURE 3. Influence of the reaction temperature on the formation of the fluorescent derivative of hydroxypregnanolone. The reaction mixture contained: 2.0 mg trichloroacetic acid ; 5 mg dansylhydrazine, and a constant amount of 10 μg hydroxypregnanolone from marmoset urine extract in a total volumume of 0.4 ml ethanol.

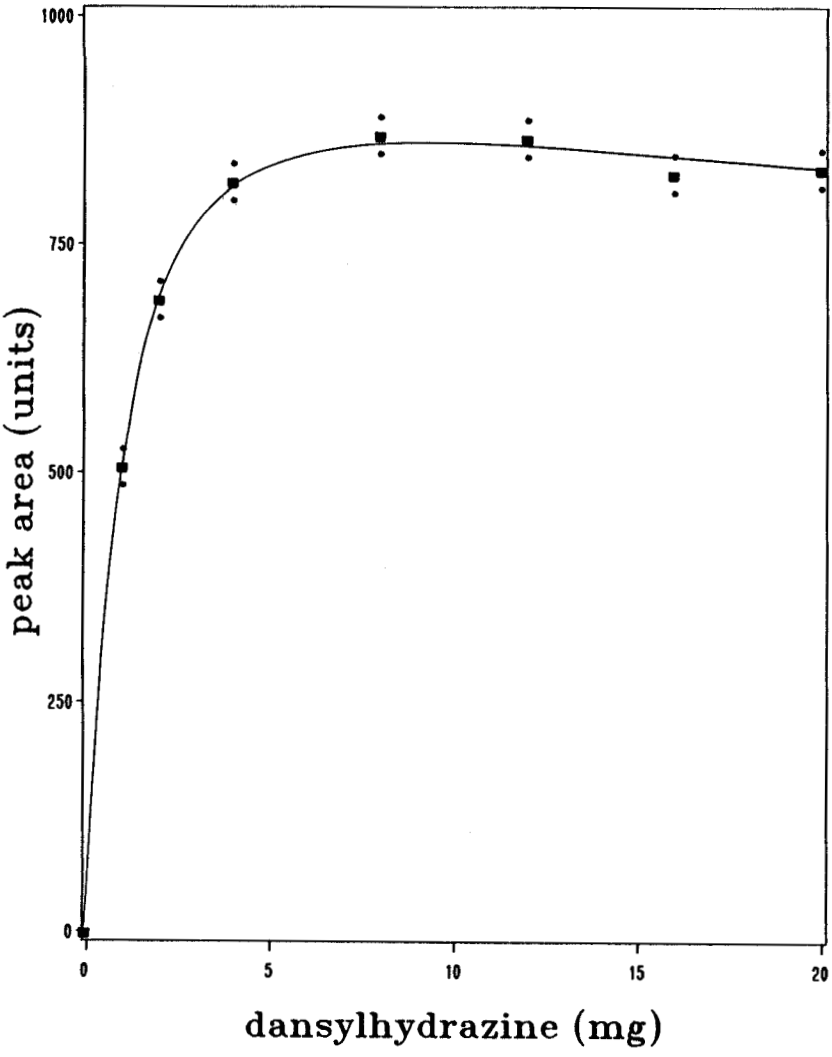

FIGURE 4. Optimization of the dansylhydrazine amount per 0.4 ml reaction volume for the formation of labelled hydroxypregnanolone. The reaction mixture contained a urine extract from a pregnant marmoset female with 10 μg hydroxypregnanolone. All other conditions were kept as described in Figure 3.

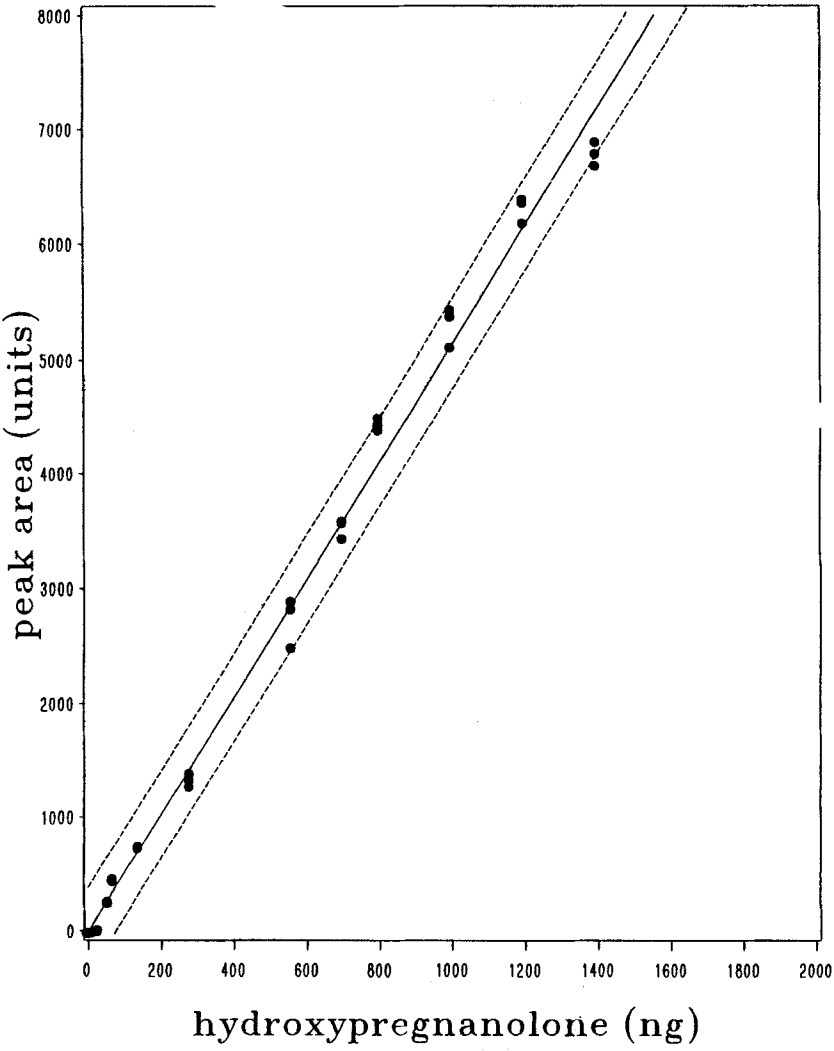

FIGURE 5. Calibration curve of a hydroxypregnanolone standard. The graph shows a linear range from 10 ng to 1000 ng. The detailed reaction mixture for the derivatization procedure is described in Figure 3.

concentrations of hydroxypregnanolone in the urine of marmoset females during follicular and luteal phase varies between 20 and 400 ng/10μl urine. The dipping procedure of the HPTLC plates for the routine monitoring is, therefore, not necessary. If amounts below 20 ng hydroxypregnanolone only have to beb determined, the sensitivity of the method has to be increased by dipping the plates into triethanolamine or paraffine oil. The detection limit was found then to be 2 ng.

Figure 6 demonstrates the chromatographic separation of the dansyl-hydroxypregnanolone of a urine sample derived from a pregnant marmoset female. 5α-pregnane-3α,7α-diol-20-one-dansylhydrazone migrates with an Rf value of 0.37 in the solvent system consisting of cyclo-hexane/ethylacetate/methanol (32:62:6; by vol). Conjugated hydroxypregnanolone did not react with dansylhydrazine under the previously described conditions. For these reasons, the urine must be hydrolyzed prior to prechromatographic derivatization to contain the unconjugated steroid.

Figure 7 presents the typical hydroxypregnanolone profile during one ovulatory cycle from one marmoset monkey. The day of ovulation is defined "day 0".

A typical separation of fluorescent derivatives of a urine sample obtained from a pregnant marmoset female by HPLC is shown in Figure 8. The detection limit with liquid chromatography in the fluorescence mode was found to be 10 ng. In order to control the derivatization procedure and the exact quantitation, an internal standard should be added. We used for this purpose 5ß-pregnane-3α,6α-diol-20-one. This steroid reacts sufficient similar to the marmoset hydroxypregnanolone, but shows a clear difference in the Rf-value [11] respectively Rt-value.

DISCUSSION

The sensitivity of several chromatographic methods is not sufficient for the determination of numerous steroids in biological fluids. Special problems arise if substances with low absorption in UV-light must be quantitatively determined by using an UV-detector. In those cases when direct quantitation is not possible different analytical procedures attach chromogenic or

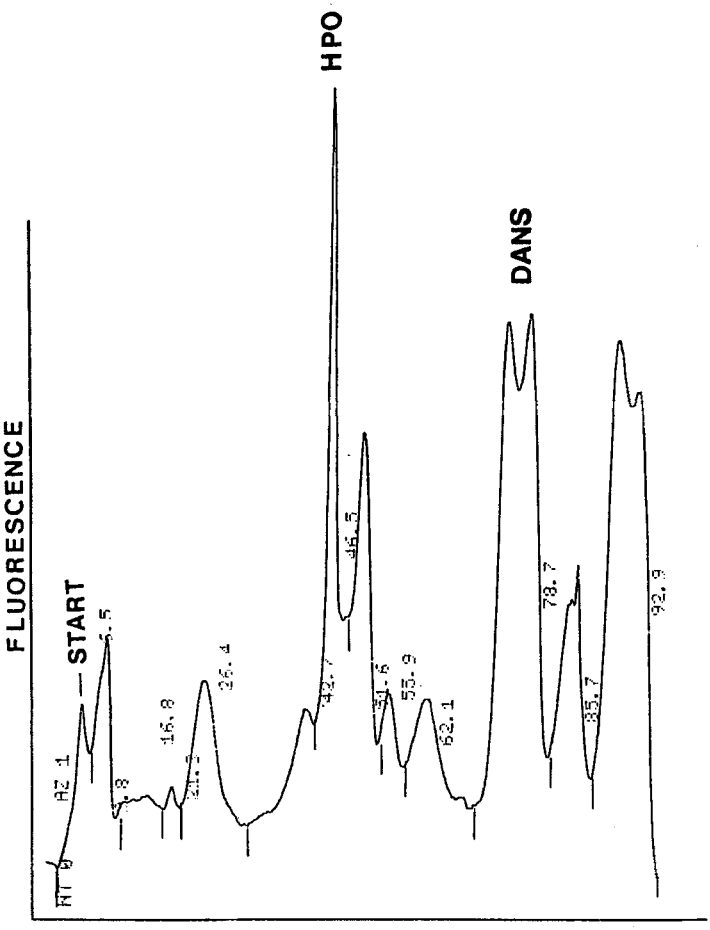

FIGURE 6. HPTLC separation of the dansyl-hydroxypregnanolone of a urine sample derived from a pregnant marmoset. The solvent system consisted of cyclohexane/ethylacetate/methanol (32:62:6; by vol.) HPO - 5α-pregnane-3α,7α-diol-20-one; DANS - dansylhydrazine. The reaction conditions for the derivatization are described in Figure 3.

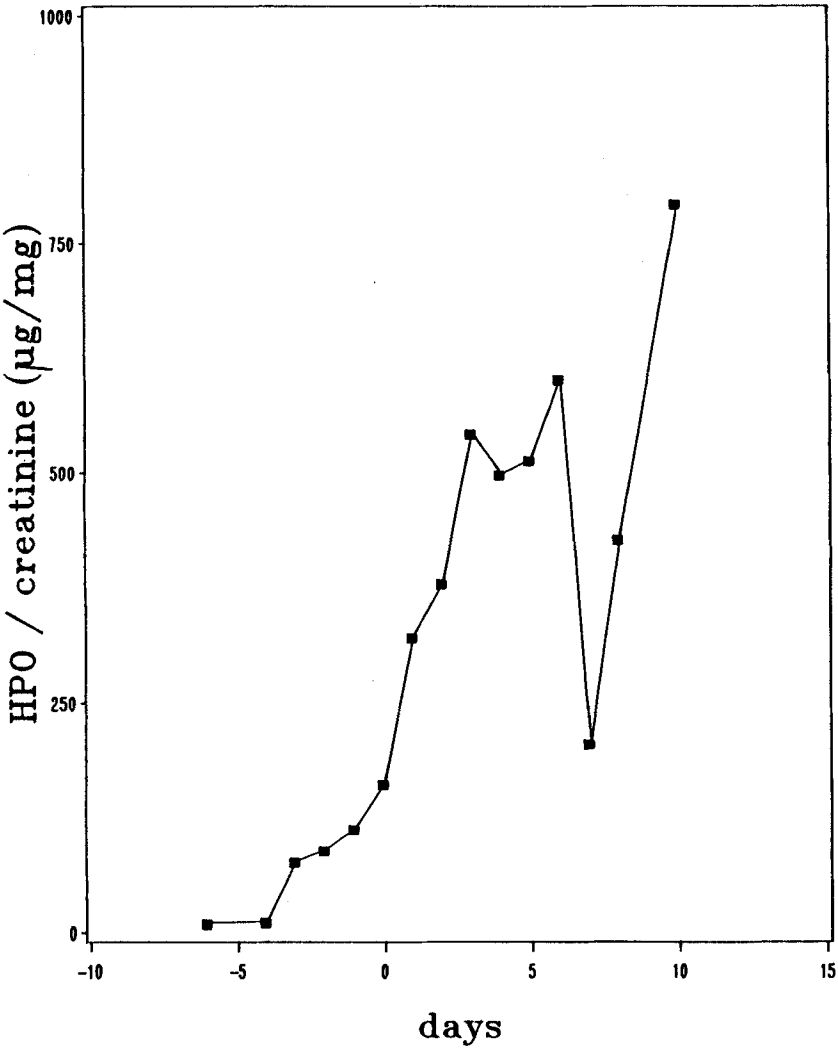

FIGURE 7. Typical example of the excretion profile of hydroxypregnanolone during one ovulatory cycle in a marmoset female. The day of ovulation is defined "day 0". HPO = 5α-pregnane-3α,7α-diol-20-one. The derivat-ization are described in Fige 4.

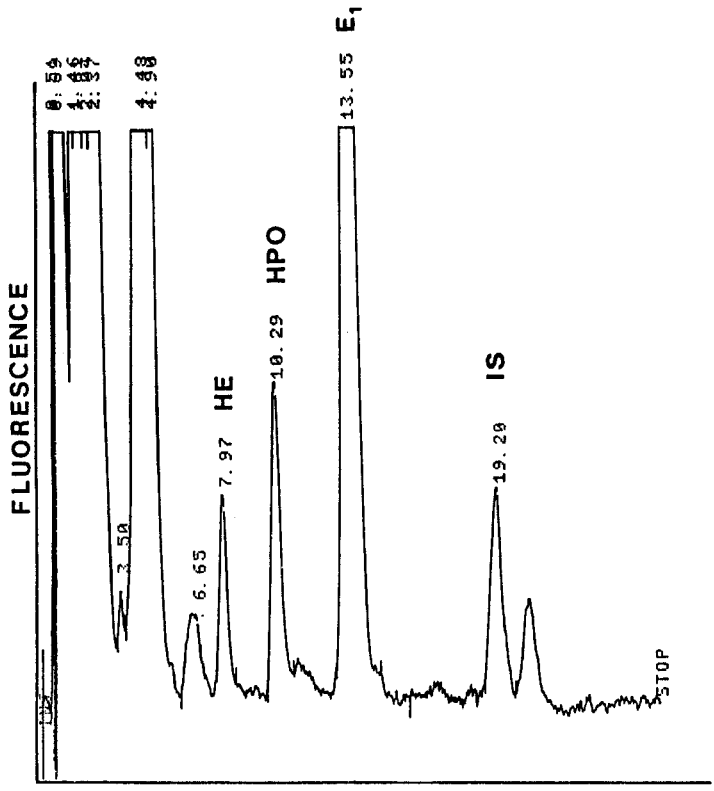

FIGURE 8. Separation of the fluorescent derivatives of a urine sample from a marmoset female during the luteal phase by liquid chromatography. HE - Hydroxyestrone; HPO - 5α-pregnane-3α,7α-diol-20-one; E_1 - Estrone; IS - 5ß-pregnane-3α,6α-diol-20-one. Column ODS2; 100 x 4.5 mm. Mobile phase: 50% acetonitrile in water with a flow rate of 2 ml/min.

fluorescent groups at the molecule. The accuracy and sensitivity for certain substances can be enhanced by these methods. Goto et al. [7] derivatized the C-3 hydroxyl group of bile acids with 1-anthroyl nitrile into a fluorescent compound. Knapp [14] labelled the keto-group of different steroids with 2,4-dinitrophenylhydrazine. Chayen et al. [1], Funk et al. [3] and Kawasaki et al. [12,13] introduced the dansyl group as fluorophores into various ketosteroids, e.g. testosterone and cortisol. The technique of fluorescence-labelling of the steroids proved to be a specific and highly sensitive method [17,5]. The

fluorescent hydrazones may then easily be separated and detected by subsequent monitoring with an UV-detector.

The dansylhydrazone can be formed with 5α-pregnane-3α,7α-diol-20-one as with other steroids containing a carbonyl group. A method of prechromatographic-labelling of this steroid is described in this paper. The derivatization conditions are clearly different from those described for the fluorescence-labelling of cortisol [3]. Since the hydroxypregnanolone has no conjugated-double binding in relation to its carbonyl group, the derivatization requires room temperature and the presence of elevated concentrations of TCA or TFA. Funk et al. [3,5] found cortisol to be much more reactive. The formation of hydrazone is complete after 10 min and the reaction is independent of temperatures between 0° and 60° C. The derivatization of 5α-pregnane-3α,7α-diol-20-one shows a long linear working curve up to 1 μg. This metabolite was found to be excreted as main progesterone derivative by marmoset monkeys [11]. It is an important steroid metabolite, indicating the luteal phase in female marmosets. The dansyl-labelled steroid shows a high fluorescence and can be quantitated for the first time by HPLC. The detection limit is sufficiently low for the determination of the ovulatory cycles of marmoset females.

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