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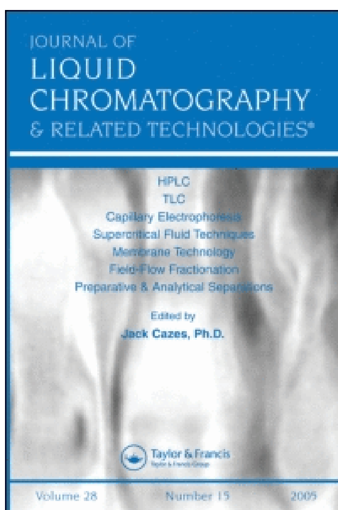

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Analysis of Norethindrone In Plasma by High-Performance Liquid Chromatography

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ANALYSIS OF NORETHINDRONE IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A sensitive specific high-performance liquid chromatographic procedure for the determination of norethindrone in plasma is described. The organic solvent extract from plasma is chromatographed on a reversed phase column using a high-performance liquid chromatograph fitted with an ultraviolet detector (254 nm); quantitation from plasma samples containing 2 ng/ml norethindrone is reported. Metabolites and endogenous substances do not interfere with the assay. The determination of norethindrone concentrations in plasma following administration of single oral dose to a mini-pig is described.

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INTRODUCTION

At present the analytical procedures for the determination of norethindrone in plasma samples are based on radio-immunological principles(1-4). Recently, it was reported that some radio-immunological assays lacked specificity for the oral contraceptive drug(4).

This report describes a high performance liquid chromatographic (HPLC) method that is sufficiently sensitive and specific for the determination of plasma samples containing 2 ng/ml norethindrone.

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MATERIALS

Norethindrone (Searle Chemical Inc., Chicago, Ill.) was used for the preparation of standard solutions. The solvents used for the mobile phase were methanol, HPLC grade (Fisher Scientific, Montreal, Canada) acetonitrile, HPLC grade (Fisher Scientific, Montreal, Canada) and double glass-distilled water.

Hexane, UV grade (Burdick & Jackson Labs, Muskegon, Michigan, U.S.A.) and methylene dichloride, HPLC grade (Fisher Scientific, Montreal, Canada) were used in the extraction procedure in borosilicate glass tubes (16x150 mm) equipped with screw caps (Corning Glass Works, Corning, N.Y). Norethindrone [6,7 - ^{3}H] with a specific activity of 40 Ci/mmole (New England Nuclear, Montreal, Canada) was used in the extraction study.

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METHODS

Chromatographic Procedure

A high-performance liquid chromatograph (Waters Assoc., Milford, Mass., U.S.A.) containing a model 6000A pump, U6K injector and model 440 detector at 254 nm was used at a nominal attenuation of .005 absorbance units full scale (AUFS) and fed into a 5 mV recorder thus giving an overall response of 0.0025 AUFS. The column (250 x 4.6 mm I.D. 316 stainless steel) was packed with 5 μm Li Chrosorb RP-8 (Brownlee Labs, Santa Clara, California, U.S.A.), which is octyl silane covalently bonded to totally porous, irregularly shaped microparticulate silica gel. The mobile phase consisting of 20% methanol and 30%

acetronitrile in water (V/V) was prepared fresh daily. A flow-rate of 120 ml/h (3400 P.S.I.) was used.

Preparation of Standard Solutions

Norethindrone, accurately weighed, was dissolved in redistilled ethanol to a volume of 5 ml. An aliquot of this solution was diluted with ethanol to produce a final solution of the desired concentration. Spiked plasma solutions (2 to 20 ng/ml) were prepared by addition of various volumes of standard norethindrone solution using a 10-µL syringe (Hamilton, Reno, Nevada, U.S.A.).

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General Procedure

A 2.0 ml aliquot of plasma sample was extracted with 5 ml of a mixture of hexane-methylene chloride (50:50). A teflon-lined screw cap was used to seal the tube which was then shaken for 10 min. in an Evapo-Mix constant speed shaker (Buchner, Fort Lee, N.J., U.S.A.) and centrifuged (10 min.) at 2000 R.P.M. (International Model HN-S, Needham Heights, Mass.). A 4.0 ml aliquot of the organic phase was transferred to a 5 ml conical tube and evaporated under nitrogen in a constant-temperature (55°) bath. The residue was immediately reconstituted with 300 µl of mobile phase and stored at 4°C prior to analysis. A 150 µl aliquot of the sample was chromatographed.

Radioactive Recovery Experiment

(^{3}H)-Norethindrone (113000 D.P.M.) was added to 2.0 ml of plasma containing from 2 to 20 ng/ml of norethindrone and was extracted using the procedure described as above. The organic extract was transferred into a scintillation vial and evaporated to dryness. Ten milliliters of

cocktail (BBS-3 Beckman, Fullerton, California, U.S.A.) were added and the radioactivity was determined on a Beckman LS 150 scintillation counter equipped with an automatic quench correction device.

RESULTS AND DISCUSSION

The reproducibility and efficiency of the extraction procedure were determined using ^{3}H-norethindrone. Results summarized in Table I, show that the extraction efficiency and reproducibility are comparable at plasma concentrations ranging between 2 and 20 ng/ml.

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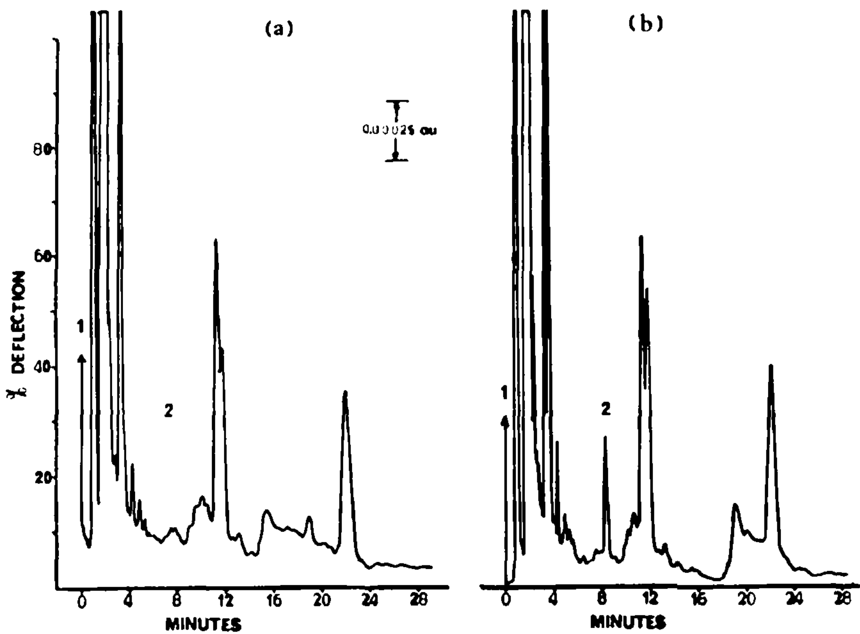

FIGURE 1. High Performance Liquid Chromatogram of (a) Human Blank Plasma Extract and (b) Extracted Human Plasma Spiked with Norethindrone (5 ng/ml).

1 - Injection, 2 = Norethindrone

TABLE I

Extraction Recoveries of (6,7 - ^{3}H) norethindrone from Plasma

CONCENTRATIONS ng/ml	RECOVERY (%)	COEFFICIENT OF VARIATION (%) (n=4)
2	95.25	2.35
5	94.15	1.10
20	100.35	2.36

The chromatogram obtained from the analysis of a spiked sample containing 5 ng/ml of norethindrone using the system described above is illustrated in Fig. 1b and Fig 1a shows the chromatogram obtained from pooled blank plasma from 8 human volunteers. No interfering compounds were extracted using the described procedure.

A calibration curve was obtained by plotting the baseline corrected peak height versus the concentration of norethindrone in spiked plasma. The plot is linear and is forced through the origin ($y = mx$) over the concentration range of 2 to 20 ng/ml, since linear regression analysis of the data indicated that the intercept was not significantly different from zero ($P > .05$). The slope value is 0.5111 and its upper and lower 95% confidence limits are 0.5274 and 0.4958 respectively. The regression coefficient of determination (r^2) is 0.998.

Table 2 lists the results obtained from the analysis of a number of spiked plasma samples ranging from 2 ng/ml to 20 ng/ml. These data indicate the accuracy and precision of the method.

The plasma norethindrone concentrations of a mini-pig following oral administration of 40 mg norethindrone dissolved in ethanolic saline

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TABLE 2

Estimation of Norethindrone (2,5 and 20 ng/ml) in Plasma

Theoretical(T) (ng/ml)	Estimated(E) (ng/ml)	E/T%	Coefficient of Variation (%) (n=4)
2.179	2.285	104.8%	± 10.90
5.179	5.37	103.7%	± 6.96
20.179	19.60	97.2%	± 3.88

are summarized in Table 3. Blood was collected in heparinized 10 ml Vacutainers (Becton-Dickinson, Toronto, Canada) and the plasma, separated by centrifugation, was transferred to a 10 ml glass tube and stored at -20°C prior to use.

The plasma drug concentration can be readily detected. Nygren et al. (1) have reported that following oral ingestion of 3 mg of norethindrone to a female volunteer, the plasma drug concentration, assessed by

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TABLE 3

Plasma Norethindrone Concentrations in a Mini Pig
Following Oral Administration of 40 mg Dose in Ethanol Saline Solution

TIME (h)	NORETHINDRONE (ng/ml)
0.08	5.2
0.25	36.0
0.50	47.0
0.75	63.0
1.00	124.0
2.00	70.0
3.00	70.0
4.00	41.2

RIA, was in the range 2-30 ng/ml. Thus, the HPLC method described is sensitive enough for the determination of norethindrone in plasma following single dose (3 mg) administered to humans.

The major metabolites, 3,5-tetrahydro norethindrone and 5-dihydro norethindrone isomers, do not possess a strong chromophore with absorption at 254 nm and thus do not interfere with the assay. Bedolla-Tovar et al. (4) reported that both metabolites interfered significantly with radio-immunological assays and thus the new method is more specific than the reported RIA assays.

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