ANALYSIS OF URINARY EXTRACTS BY GAS CHROMATOGRAPHY

3α,17-DIHYDROXYPREGNAN-20-ONE, PREGNANE-3α,17,20α-TRIOL AND Δ⁵-PREGNENE-3β,17,20α-TRIOL*

R. S. ROSENFELD, M. C. LEBEAU,
R. D. JANDOREK AND T. SALUMAA
Sloan-Kettering Institute for Cancer Research, New York, N.Y. (U.S.A.)

(Received November 23rd, 1961)

Considerable data have already been accumulated on gas chromatography of steroids and the method appears to hold great potential for steroid analysis of various biological extracts. It is the purpose of this communication to present methods for the estimation of 3α,17-dihydroxypregnan-20-one (17OH-P), pregnane-3α,17,20α-triol (PT) and Δ⁵-pregnene-3β,17,20α-triol (Δ⁵PT) in partially purified urinary extracts. These compounds, frequently present in conditions involving hyperactivity of the adrenal cortex, are difficult to estimate by the more conventional methods.

EXPERIMENTAL

Gas chromatography

Analyses were carried out with a 1.8 m × 5 mm glass column packed with 100–140 mesh Gas Chrom P** coated with SE-30*** (3 % by weight). The column was maintained at 235° with an argon pressure of 30 p.s.i.; an ionization detection system with a radium source was used. Analytical samples of 17OH-P, PT, and Δ⁵PT were used as reference standards. Quantity *versus* the area under each peak was plotted for each compound as described earlier[1] and the curves are shown in Fig. 1. Relative retention times as compared with cholestane (7.8 min) were 0.93 for 17OH-P, 1.08 for PT, and 1.10 for Δ⁵PT. In addition, approximately 1 mg of each standard was chromatographed and the eluate was collected in Teflon tubing attached to the end of the detector cell. The tube was rinsed with acetone and the washings concentrated, and examined by infrared spectrometry. The spectrum of the chromatographic product from 17OH-P was identical with that of 3α,17-dihydroxy-17β-methyl-D-homoetiocholan-17a-one (chloroform, 1150–800 cm^{-1}) while the spectra of the material collected from the chromatography of PT and Δ⁵PT were identical after acetylation with pregnanetriol 3,20-diacetate (chloroform, 1150–800 cm^{-1}) and Δ⁵-pregnenetriol 3,20-diacetate (carbon disulfide, 1200–900 cm^{-1}) respectively.

* This investigation was supported in part by a grant from the American Cancer Society and a research grant (CY-3207) from the National Cancer Institute of the National Institutes of Health, United States Public Health Service.
** General Electric Company, Waterford, New York.
*** Applied Science Laboratories, State College, Pennsylvania.

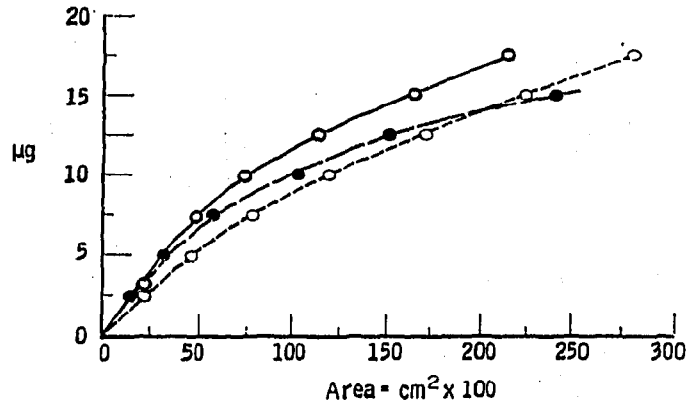


Fig. 1. Relations between quantity and peak area for 17OH-P (○ – – – ○), Δ^5PT (○—○) and PT (●– –●).

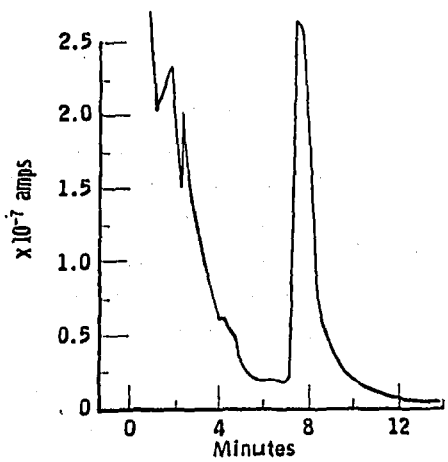


Fig. 2. Gas chromatographic analysis for 17OH-P.

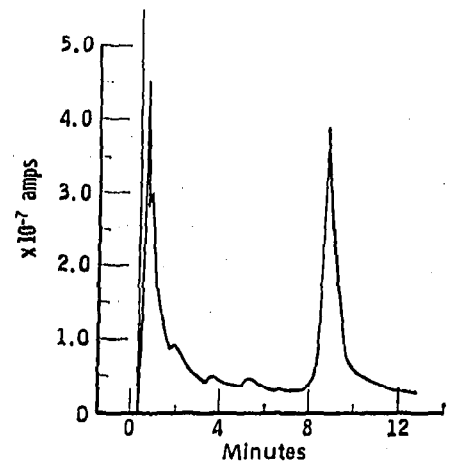
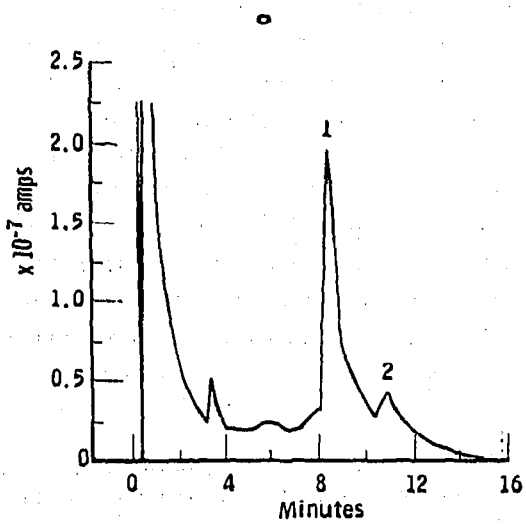


Fig. 3. Gas chromatographic analysis for Δ^5PT.



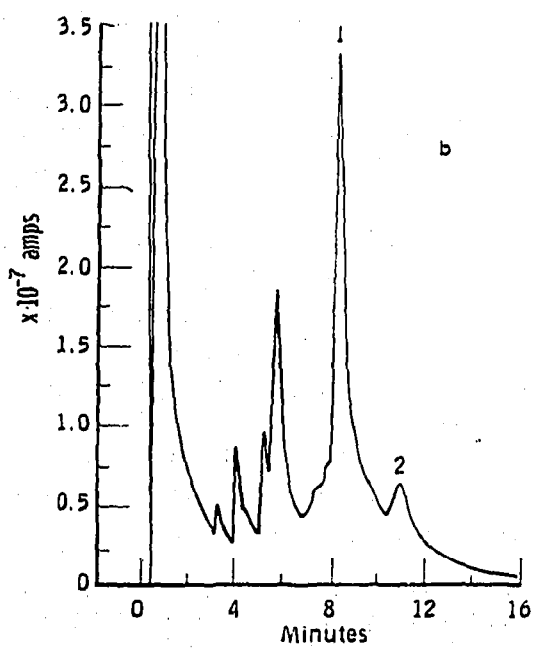


Fig. 4. Gas chromatographic analysis for PT. (a) Extract from paper chromatogram. (b) Non-ketonic extract from urine. 1 = PT; 2 = probably 11-ketopregnane-3α,17,20α-triol.

Isolation and measurement of compounds

Fractions containing 17OH-P, PT, or Δ^5PT were obtained from urine by methods described by FUKUSHIMA and his associates[2,3]. Chromatography of the fractions containing 17OH-P was carried out on Whatman No. 1 paper (18 × 118 cm) at 24° for 48 hours. The stationary phase was methanol–water (3:1) and the mobile phase was a mixture of 2,2,4-trimethylpentane–toluene (3:1). The 17OH-P area (46–50 cm from origin) was located by a pilot strip. PT and Δ^5PT were isolated by chromatography of the appropriate extract in the system 2,2,4-trimethylpentane–toluene–methanol–water (3:5:4:1) in which Δ^5PT had migrated 45–49 cm while PT was located 60–64 cm from the origin in 32 hours[4]. The areas containing the triols were cut out and extracted with methanol. A portion of each was analyzed for triols by the method of Cox[5]; another portion, dissolved in 1:1 chloroform–methanol, was injected into the column. Typical gas chromatographic patterns of extracts containing 17OH-P, Δ^5PT, and PT are shown in Figs. 2, 3, and 4a respectively. For quantitation, the area under each peak was measured and compared with the reference standards[6].

RESULTS AND DISCUSSION

Paper chromatography of the extracts containing 17OH-P in the system employed separated this steroid from the others present in the urine fraction and gas chromatography of the eluate afforded a single reproducible peak for the compound. The small ionization peaks superimposed on the high background between 1 and 6 minutes (Fig. 2) were due to impurities washed from the paper. Values for 24 hour urinary excretion of 17OH-P by this method ranged from 0–0.26 mg in 6 subjects with apparently normal adrenals and were 2.1, 7.1 and 8.9 mg in 3 patients with adrenocortical hyperactivity.

The formation of 3α,17-dihydroxy-17β-methyl-D-homoetiocholan-17a-one from 17OH-P during gas chromatography was expected since FUKUSHIMA and co-workers obtained this compound by heating above the melting point[7]. This rearrangement, being quantitative, does not affect the accuracy of the determination; it may be added to those transformations of steroids during gas chromatography already recorded[8].

Several methods for the estimation of PT and Δ^5PT in urine, all of which involve quantitation of suitable column or paper chromatographic fractions[5,9–14], have been reviewed[11] and the most specific appears to be the acetaldehydogenic steroid method of Cox[5]. Quantitation of PT and Δ^5PT by gas chromatography is rapid, convenient and in reasonable agreement with data obtained by the Cox procedure. In analyses carried out on paper extracts from the non-ketonic fractions of 26 urine collections from subjects with a variety of clinical conditions, the PT values ranged from 0.75 to 25.2 mg per day and averaged about 10% lower than acetaldehydogenic analyses carried out on the same fractions. For 21 analyses for Δ^5PT, the range was from 0.27 to 33.5 mg per day and averaged 5% lower than the values determined by the Cox method. From time to time, however, gas chromatographic analyses were as much as 4 times lower than by the acetaldehydogenic procedure. When this gross discrepancy occurred, there was always an additional large ionization peak at 3.3–3.6′ which was either absent or barely present in the samples where the two methods agreed. This was probably due to either an impurity in the urinary extract which was not completely separated by paper chromatography and yielded acetaldehyde or to some un-

controlled, intermittent variable in the column that caused decomposition of the steroid. In Fig. 4a, peak 2 (11 min) has a retention time identical with that of 11-keto-pregnane-3α,17,20α-triol. Since the extract was shown to contain this compound by other methods, it is probable that the secondary peak was due to the triolone. Preliminary work indicates that in urine of patients with clinical conditions associated with the excretion of very large amounts of PT (Fig. 4b), gas chromatographic analysis may be carried out on extracts before paper chromatography and, while less accurate, suggestive information may be obtained.

ACKNOWLEDGEMENTS

The interest and support of Dr. T. F. GALLAGHER are gratefully acknowledged.

SUMMARY

Analysis by gas chromatography on the non-polar phase SE-30 has been used to determine the quantity of 3α,17-dihydroxypregnan-20-one, pregnane-3α,17,20α-triol and Δ^5-pregnene-3β,17,20α-triol in eluates of paper chromatograms of urinary extracts. During the process, the 3α,17-dihydroxypregnan-20-one rearranges to 3α,17-dihydroxy-17β-methyl-D-homoetiocholan-17a-one.

REFERENCES

1 D. B. SILVERSMIT, C. C. SWEELY AND H. A. NEWMAN, *Circulation Research*, 9 (1961) 235.
2 D. K. FUKUSHIMA, T. F. GALLAGHER, W. GREENBERG AND O. H. PEARSON, *J. Clin. Endocrinol. and Metabolism*, 20 (1960) 1234.
3 D. K. FUKUSHIMA, H. L. BRADLOW, L. HELLMAN, B. ZUMOFF AND T. F. GALLAGHER, *J. Clin. Endocrinol. and Metabolism*, 21 (1961) 765.
4 D. K. FUKUSHIMA, H. L. BRADLOW, L. HELLMAN, B. ZUMOFF AND T. F. GALLAGHER, *J. Biol. Chem.*, 235 (1960) 2246.
5 R. I. COX, *J. Biol. Chem.*, 234 (1959) 1693.
6 R. S. ROSENFELD, M. C. LEBEAU, S. SHULMAN AND J. SELTZER, *J. Chromatog.*, 7 (1962) 293.
7 D. K. FUKUSHIMA, S. DOBRINER, M. S. HEFFLER, T. H. KRITCHEVSKY, F. HERLING AND G. ROBERTS, *J. Am. Chem. Soc.*, 77 (1955) 6585.
8 W. J. A. VANDEN HEUVEL AND E. C. HORNING, *Biochem. and Biophys. Research Communs.*, 3 (1960) 356.
9 H. WILSON, M. B. LIPSETT AND D. W. RYAN, *J. Clin. Endocrinol. and Metabolism*, 21 (1961) 1304.
10 M. I. STERN, *J. Endocrinol.*, 16 (1957) 180.
11 A. M. BONGIOVANNI AND W. R. EBERLEIN, *Anal. Chem.*, 30 (1958) 388.
12 W. NOWACZYNSKI, E. KOIW AND J. GENEST, *J. Clin. Endocrinol. and Metabolism*, 20 (1960) 1503.
13 C. DECOURCY, *J. Endocrinol.*, 14 (1956) 164.
14 R. I. COX, *Biochem. J.*, 52 (1952) 332.