# GAS–LIQUID CHROMATOGRAPHY OF THE TETRAHYDRO DERIVATIVES OF CORTISOL ISOLATED FROM URINE*

JOHN A. LUETSCHER AND R. GORDON GOULD

*Department of Medicine, Stanford University School of Medicine, Palo Alto, Calif. (U.S.A.)*

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VANDENHEUVEL, SWEELEY AND HORNING[1] reported that various androstane, pregnane and cholestane derivatives, containing oxygen atoms in the 3, 11, or 20 positions or as 17-ketosteroids, could be separated by gas chromatography on Chromosorb W coated with 2 to 3 % silicone gum (SE-30) at 222°. Cortisol and other pregn-4-en-17, 21-diol-3, 20-dione compounds appeared as the corresponding 17-keto-steroids[2]. LIPSKY AND LANDOWNE[3] found no evidence of epimerization of hydroxyl groups or of hydrogen on carbon position 5 of various steroids, but noted that compounds of the pregnan-21-ol-20-one series were unstable. In earlier work in this laboratory, DALLMAN AND GOULD showed that the chief cortisol metabolites, reduced at carbons 3, 4 and 5, could be separated by gas chromatography, each giving a single peak with the retention time of the corresponding 17-ketosteroid.

In fractions obtained from liquid-liquid chromatography of extracts of urine, the principal metabolites of cortisol, pregnane-3α,17α,21-triol-11,20-dione (tetrahydrocortisone), allopregnane-3α,11β,17α,21-tetrol-20-one (allotetrahydrocortisol), and pregnane-3α,11β,17α,21-tetrol-20-one (tetrahydrocortisol) are eluted sequentially, but the first two may not be regularly or completely separated. The present studies were undertaken to determine whether these metabolites could be separated and measured by gas liquid chromatography as individual standards, in mixtures, and in fractions eluted during liquid-liquid chromatography of urine extracts.

## METHODS

A model 15 Barber-Coleman gas chromatograph was used. The glass U-tube (8 ft. long × 5 mm internal diam.) was packed with Chromosorb-W (acid washed), which had been silanized and coated with SE-30 (0.75 %). Temperature around the column was 217°, and at the vaporizer 250°. Argon flow rate was 50 ml/min. The ionization detector utilizing $^{90}Sr$ was operated at 750 volts.

Authentic reference steroids were obtained from Dr. W. KLYNE (through the courtesy of Mr. M. GRAFF and the Endocrinology study Section, N.I.H.) and from U.S.P. Reference Standards. The neutral steroid fraction, extracted from urine incubated with β-glucuronidase, was transferred to a Celite column (60 cm long × 1 cm internal diam.) with 50% methanol as the stationary phase and a mobile phase

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of one part of ethyl acetate in 15 parts of toluene. An aliquot of each fraction, which contained material reducing blue tetrazolium[4] and giving the SILBER-PORTER reaction[5], was dried in a stream of air and rinsed down into the point of a conical, glass stoppered tube. Measured amounts of cholestane and ethanol were added to each standard or sample to bring the concentration of cholestane to 1.0 $\mu$g/$\mu$l and the concentration of steroid to between 2 and 4 $\mu$g/$\mu$l. Aliquots of 2 to 8 $\mu$l were injected from a Hamilton 10 $\mu$l syringe into the vaporizer chamber.

Areas under recorded peaks of detector response were recorded by a disc integrator and calculated from peak height. Agreement was satisfactory with standard steroids or when urine fractions consisted of well defined and separated components.

## RESULTS

### *Retention time of standards*

As noted in Table I, tetrahydrocortisone, tetrahydrocortisol, and allotetrahydrocortisol yielded peaks with retention times identical with the corresponding 17-ketosteroid analogs. As the pregnane compounds showed no greater tendency to

TABLE I

RETENTION TIME AND MOLAR AREA OF STANDARD STEROIDS RELATIVE TO CHOLESTANE

| *Standard* | *Relative retention time*[a] | *Relative molar area*[b] |
|---|---|---|
| Pregnan-3$\alpha$,17$\alpha$,21-triol-11,20-dione (tetrahydrocortisone) | 0.47 | 0.33 |
| Etiocholan-3$\alpha$-ol-11,17-dione | 0.47 | 0.50 |
| Pregnan-3$\alpha$,11$\beta$,17$\alpha$,21-tetrol-20-one (tetrahydrocortisol) | 0.62 | 0.30 |
| Etiocholan-3$\alpha$,11$\beta$-diol-17-one | 0.61 | 0.50 |
| Allopregnan-3$\alpha$,11$\beta$,17$\alpha$,21-tetrol-20-one (allotetrahydrocortisol) | 0.69 | 0.33 |
| Androstan-3$\alpha$,11$\beta$-diol-17-one | 0.70 | 0.50 |
| Cholestane | 1.00[a] | 1.00[b] |

[a] Observed retention time 18.6 $\pm$ 0.7 min for cholestane under operating conditions described.
[b] Area under recorded peak per micromole steroid divided by area per micromole cholestane. In each case, area represents integrated detector response under operating conditions described.

trailing, it seems likely that loss of side chain occurs almost immediately after injection into the vaporizer. The order of appearance (retention time shorter for 11-ketone than for the corresponding 11$\beta$-OH compound, and shorter for 5$\beta$-H than for 5$\alpha$-H) conforms with findings on other steroids[1,3,6].

### *Detector response to standards*

The greatest response per microgram was given by cholestane, followed by the 17-ketosteroids (Fig. 1). The lowest response was observed in the pregnane series, especially with tetrahydrocortisol. Results, presented in Table I as integrated detector response per micromole of steroid, supported the thesis of SWEELEY AND CHANG[6] that increasing oxygen content was associated with decreasing relative detector response,

and that the lowest relative response was obtained from pregnane derivatives which lost carbons 20 and 21 during gas chromatography, even when conditions which minimized variations in molar response were selected.

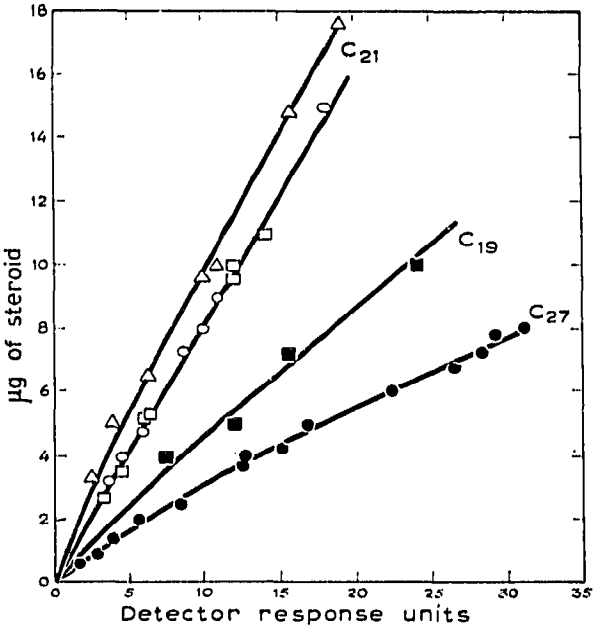


Fig. 1. Area under recorded peak of detector response after injection of various steroids. Cholestane ($C_{27}$) is indicated by solid circles; 17-ketosteroids ($C_{19}$) by solid squares; and in the pregnane series ($C_{21}$), tetrahydrocortisone by open circles, allotetrahydrocortisol by open squares, and tetrahydrocortisol by open triangles.

*Fractions from partition chromatograms of urine extracts*

Fig. 2 presents records of detector response during four gas-liquid chromatograms. In each case, the double spike on the left represents air and solvent and the last peak on the right is due to cholestane. In the pair of records on the left side, standard tetrahydrocortisone (above) is compared with a fraction obtained from chromatography of urine extract (below). The responses are quite similar and indicate that tetrahydrocortisone is the chief component of each sample. The pair of records on the right side of Fig. 2 represent gas-liquid chromatograms of standard allotetrahydrocortisol (above), and a urine fraction (below), containing tetrahydrocortisone (large peak), allotetrahydrocortisol (smaller peak corresponding to peak in standard run above), and several unidentified components.

Estimates of the quantity of reduced cortisol metabolites in fractions of urine extracts by gas-liquid chromatography agree well with results of colorimetric analysis when the quantity of the cortisol metabolite is sufficient to give an easily measurable peak, well separated from other components of the fraction. When these conditions are not satisfied, one or more of the following problems may interfere with the analysis or with recovery of known steroid added to urine extracts: elevated or irregular baseline of detector response may make quantitation difficult; or retention time may vary when a large amount of contaminant is present; or a small amount of a known pregnane compound added to a heavy residue (obtained by pooling a number of urine fractions) may fail to give the expected peak, suggesting delay or interference

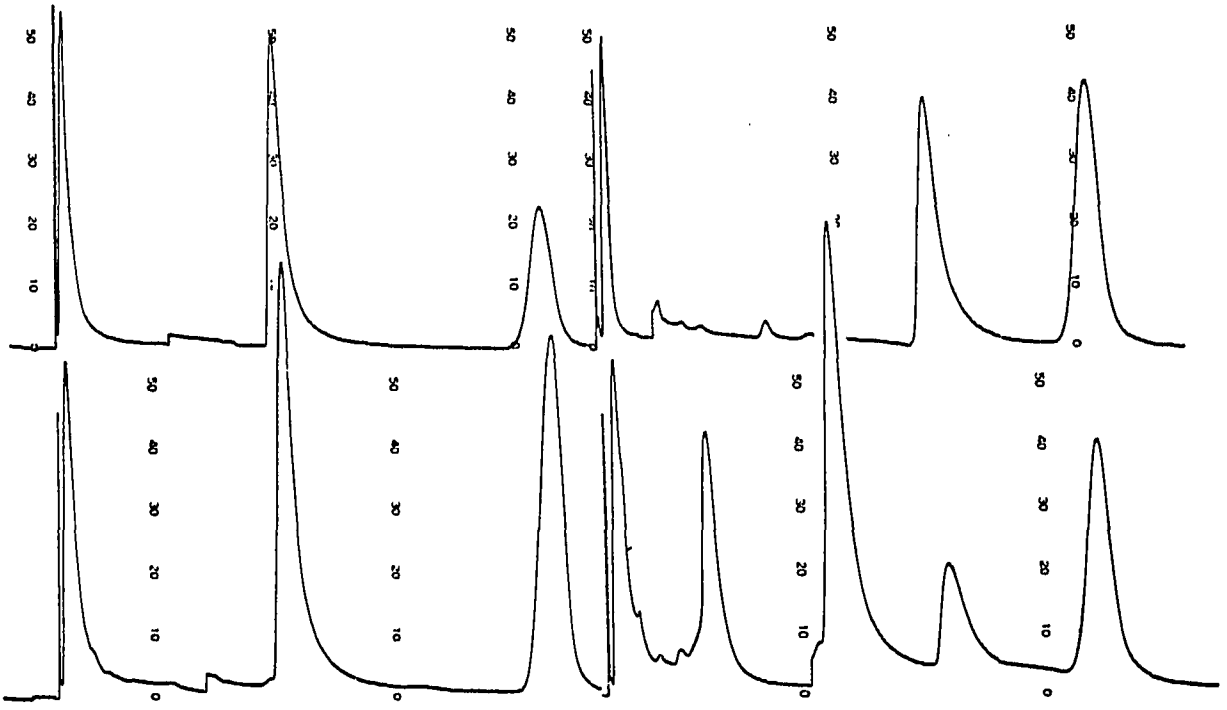

Fig. 2. Detector responses recorded during four gas-liquid chromatograms. See text for explanation.

with the rapid and reproducible conversion to 17-ketosteroid observed when a standard compound is injected.

The technique described has proved quite useful in monitoring fractions from liquid-liquid partition columns in order to differentiate between tetrahydrocortisone and allotetrahydrocortisol, which are not easily separated on the liquid-liquid column, but which separate readily on gas chromatography.

## SUMMARY

Tetrahydrocortisone, tetrahydrocortisol, and allotetrahydrocortisol can be separated by gas-liquid chromatography and quantitated with an argon ionization detector. The retention times of these cortisol metabolites are identical with those of their 17-ketosteroid analogs. This technique has been used to monitor the composition of fractions from liquid-liquid chromatograms of urine extracts.

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