values (not detected); the medians are probably a better indication of the true concentration, and these have been shown in Table IV, with the mean deviation to indicate the spread of values. The lead concentrations determined with the graphite-rod atomizer generally compare well with the medians from the S.O.A.P. laboratories, again considering the wide range of reported values.

Tin. With 0.8-μl samples, tin was determined at levels down to 0.1 μg ml^{-1} using the 286.3 nm line. Although the sensitivity is better at 224.6 nm, the low-wavelength source noise was such that better signal:noise ratios were obtained at 286.3 nm. The tin concentrations were less than 1 μg ml^{-1} in all the jet-engine oils studied, and 1–3 μg ml^{-1} in the reciprocating-engine oils. The S.O.A.P. tables generally provided fewer than five flame atomic absorption results, with little agreement among them. Table IV shows the results obtained with the graphite rod, and the range of values from the S.O.A.P. tables. It seems likely that many of the values given by the flame work are the result of scattering, fluctuations in source noise, or enhancement of flame absorption by the oil combustion.

Other Elements. Preliminary work has been carried out on the determination of magnesium and aluminum using the graphite-rod atomizer. With magnesium, the main problem is likely to be in reducing the sensitivity sufficiently while preserving reproducibility. The major difficulties with aluminum are that temperatures of the order of 2300 °C are required for atomization, and the atoms are then lost (*e.g.*, by recombination, condensation) within a short distance of the surface of the rod. Anderson *et al.* (*25*), using limited field viewing conditions, found that in an argon atmosphere, the aluminum atom population became undetectable 1 mm above the rod. Only a small improvement is noted with an argon–hydrogen gas flow (*19*). Without using a limited-field technique, but with the argon/hydrogen/entrained-air flame, we have obtained a value of 20 ± 2 μg ml^{-1} for the aluminum concentration in sample 71-9B (*cf.*, 21.9 ± 8.2 μg ml^{-1} from the S.O.A.P. compilation). At the present time, however, the nitrous oxide–acetylene flame remains the most satisfactory medium for aluminum determination at levels below 5 μg ml^{-1}.

(25) R. G. Anderson, H. N. Johnson, and T. S. West, *Anal. Chim. Acta*, **57**, 281 (1971).

CONCLUSIONS

The graphite-rod atomizer in atomic absorption spectrometry is an effective, simple, rapid, precise, and accurate means of atomizing and measuring Ag, Cr, Cu, Fe, Ni, Pb, and Sn and should also with more study be useful for atomizing and measuring Mg and possibly Al in jet-engine and reciprocating-engine oils. Because of the need to use different dilution ratios and because of the need to measure the absorption signal at different heights for some elements, the time per analysis is slightly larger than with flame atomic absorption spectrometry. However, because of the greater linear range of atomic fluorescence spectrometry, it is possible the same dilution ratio and same height could be used with the fluorescence method and therefore a considerable savings in time would result compared to the absorption method. Atomic fluorescence spectrometry with the graphite filament atomizer should benefit more from the low background than atomic absorption spectrometry; such studies are currently in progress.

ACKNOWLEDGMENT

The authors wish to thank the Perkin-Elmer Corporation, Norwalk, Connecticut, for the loan of a Model 303 Atomic Absorption Spectrophotometer.

RECEIVED for review May 17, 1972. Accepted July 10, 1972. This work was supported by AF-AFOSR-70-1880 H.

High Speed Liquid Chromatography of Derivatized Urinary 17-Keto Steroids

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The 2,4-dinitrophenylhydrazine derivatives of 4 epimeric forms of androsterone and dehydroepiandrosterone have been separated from each other by high speed liquid chromatography. Total analysis time at a flow rate of 0.5 ml/min of isooctane is less than 36 minutes on a 1-meter × 1.8-mm i.d. column. Samples of urine and blood hydrolysates have been derivatized with DNPH and analyzed by the same technique. The 11-hydroxy-17-keto steroids were analyzed by reversed phase liquid chromatography using a "permanently" bonded octadecylsilane support material. The clinical potential of the method is discussed.

USE OF HIGH SPEED LIQUID CHROMATOGRAPHY as a technique for the separation and analysis of steroid hormones has been recently demonstrated (*1*, *2*). Using liquid chromatography, Huber (*3*) has shown an analysis of urinary estriol present in high titer during the third trimester of pregnancy. Detection of estradiol and estrone was also achieved. Vestergaard and Sayeh (*4*) were able to separate a synthetic mixture of 50 μg each of seven different 17-keto steroids using capillary alumina columns and gradient elution in 2.5 hours. Vestergaard (*5*) has also been successful in separating Zimmerman chromophores of the 17-keto steroids in urine by column chromatography. No successful attempts at the isolation of 17-keto

(1) S. Siggia and R. Dishman, ANAL. CHEM., **42**, 1223 (1970).

(2) R. Henry, J. Schmit, and J. Dieckman, *J. Chromatogr. Sci.*, **9**, 513 (1971).

(3) J. F. K. Huber, J. Hulsman, and C. Meijers, *J. Chromatogr.*, **62**, 79 (1971).

(4) P. Vestergaard and J. Sayeh, *ibid.*, **24**, 442 (1966).

(5) P. Vestergaard, *Clin. Chem.*, **16**, 651 (1970).

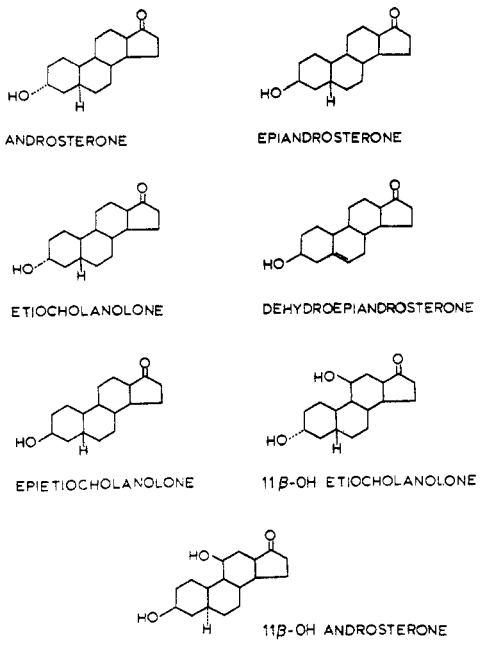

Figure 1. Steroid structures

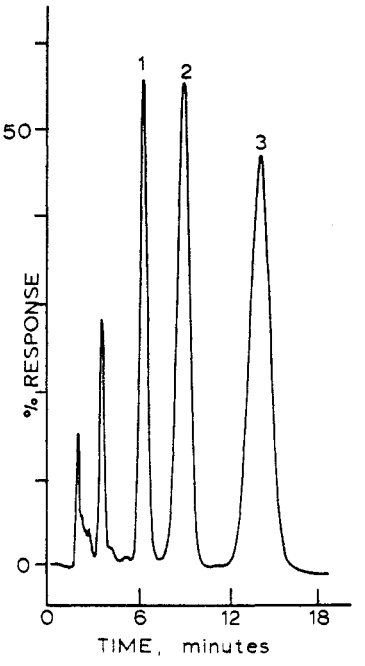

Figure 2. 1 meter, 1.5% ODPN on Zipax, 0.70 ml/min isooctane, UV = 0.16 AUFS ambient temperature. 1 = A, 2 = EPA, 3 = DHA

steroids from physiological fluids by modern high speed liquid chromatography have been reported.

The problems which are associated with the analysis of urinary steroids and which have prevented a more widespread application of this technique are due to a number of facts. The ultraviolet active Δ^4-3-ketone system present in many steroids is reduced by metabolic processes to a saturated A ring with a 3-hydroxy group, thus minimizing or eliminating the response of most commercial ultraviolet detectors at their operating wavelength of 254 nm. The inherent sensitivity of the refractive index detector and other commercially available detectors is still too poor to be of substantial value for detection of the small amounts of steriods isolated from complex biological fluids.

Since the steroids are excreted for the most part in the form of salts of glucopyranosiduronic acid or sulfuric acid conjugated at the C-3 position, the analysis is further complicated by the necessity of an enzymatic or acid hydrolysis or a solvolysis step to convert them to a form preferred for analysis. The extent of artifact production and steroid degradation due to the hydrolysis have been extensively documented (*6–8*), and it is this step which appears to be the limiting one in terms of time and accuracy.

17-Keto Steroids. A group of steroids which are of clinical interest in certain diseased states are the neutral 17-keto steroids (17-KS). In the normal male, approximately two-thirds to three-fourths of the total urinary 17-KS are derived from the adrenal cortical hormones with the remainder resulting from the metabolism of testosterone by the testes. In the normal female, essentially all of the 17-KS are of adrenocortical origin with a trace amount contributed by the ovaries. The urinary 17-KS level in males is therefore a composite measure of adrenal and testicular function while the level in the normal female is indicative of adrenal activity alone. The major 17-keto steroid produced by the adrenal gland is dehydroepiandrosterone (DHA) sulfate and the principal metabolites appearing as conjugates in the urine are androsterone (A), epiandrosterone (EPA) and etiocholanolone (E). Some DHA is also excreted unchanged. Their structures are shown in Figure 1. The 11β-hydroxy and 11-keto derivatives of androsterone and etiocholanolone are also found in urine. These 11-oxygenated keto steroids are mainly due to the effect of hepatic enzymes on cortisol. Trace amounts of epietiocholanolone (EPE) may be present also.

The most widely employed method for 17-KS analysis at present is the group estimation technique (*9*) based on the chemical reaction of a methylene group adjacent to a carbonyl with *m*-dinitrobenzene in alkaline solution, the absorptivity of the resulting solution being measured at 520 nm. The Zimmerman reaction, being nonspecific, is subject to interferences not only from nonketonic artifacts but also from steroids with carbonyl groups at positions other than C-17 on the steroid nucleus. The use of correction factors based on absorbances at several wavelengths only partially compensates for these difficulties. Modifications of the Zimmerman reaction have been applied directly to urine and plasma extracts; however, the method is most useful only when a prior fractionation step has been included to eliminate interferences. The obvious disadvantage of the group estimation method is that it does not reflect the relative concentrations of the various steroids. A popular method for assessment of individual 17-KS has been thin layer or paper chromatography followed by spectrophotometric measurement of the eluted spot material or else direct scanning of the developed plate. The disadvantages associated with this approach such as long analysis times and loss of material through mechanical trans-

(6) L. Cawley, B. Musser, W. Faucette, *et al.*, *Clin. Chem.*, **11**, 1009 (1965).
(7) L. Cawley and B. Musser, *Amer. J. Clin. Pathol.*, **39**, 301 (1963).
(8) A. Schindler, V. Ratanosopa, and W. Hermann, *Clin. Chem.*, **13**, 186 (1967).
(9) W. Zimmerman, *Hoppe-Seyler's Z. Physiol. Chem.*, **233**, 257 (1935).

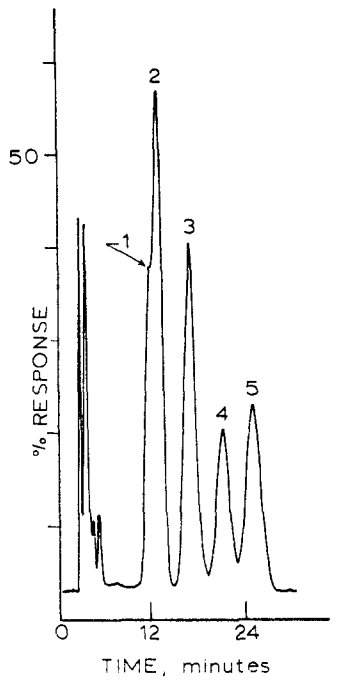

Figure 3. Same as 2 except 0.50 ml/min. 1 = EPE, 2 = A, 3 = EPA, 4 = E, 5 = DHA

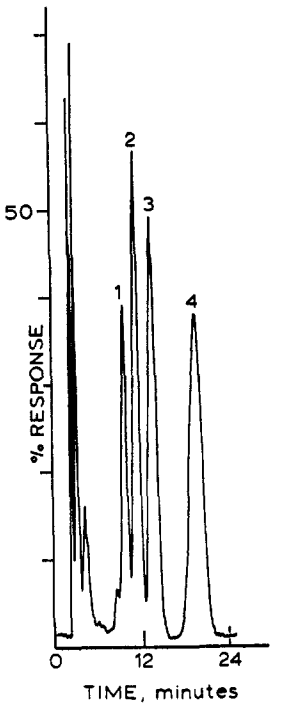

Figure 4. 1 meter 0.75% ODPN on Zipax; other conditions same as Figure 3. 1 = EPE, 2 = A, 3 = EPA, 4 = DHA and E

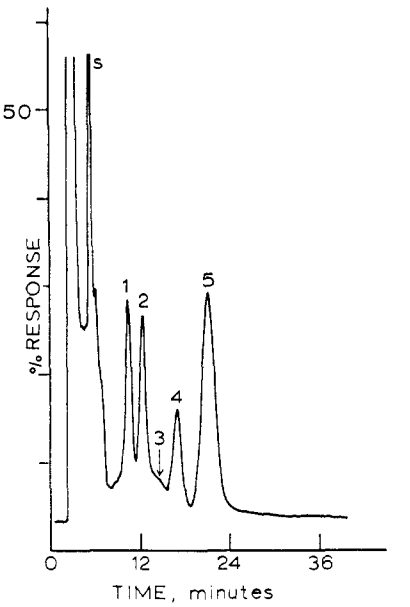

Figure 5. Same as Figure 3 except UV 0.08 AUFS. Male urine, acid hydrolysate. S = ethanol, 1 = A, 2 = Unknown, 3 = EPA, 4 = E, 5 = DHA

fer are well known. Gas chromatographic methods (*10*, *11*) have been reported but have not been utilized extensively for routine clinical determinations. The primary reason for this appears to be due to the fact that extensive work up procedures often employing TLC or column chromatography are necessary to obtain a urinary extract "clean" enough for injection into a gas chromatograph. Unlike gas chromatographic methods which are employed only as a final sensitive measuring technique, the liquid chromatographic method described contributes both resolving power and sensitivity to the problem of steroid analysis.

Recently, Henry, Schmit, and Dieckman (*2*) have shown the advantage of using a dinitrophenylhydrazine derivative to enhance the sensitivity of carbonyl compounds to ultraviolet light. The reaction between DNPH and the carbonyl group is fast, selective, and quantitative, thus making it an excellent choice for the formation of a steroid-DNPH derivative. Using this approach, Henry, Schmit, and Dieckman were able to separate the DNPH derivatives of a synthetic mixture of A and DHA. This approach has been extended in our laboratory to include the separation of four epimeric forms of A in addition to DHA and also the detection of these compounds as DNPH derivatives in human urine and plasma.

EXPERIMENTAL

Apparatus. A commercially available high pressure liquid chromatograph, Varian L. C. Series 4100, was used throughout this investigation. The instrument has a flow capability of 0–200 ml/hr delivered by a positive displacement syringe pump at pressures of up to 5000 psi. The chromatograph is equipped with both ultraviolet and refractive index detectors. The ultraviolet detector operates at a single wavelength, 254 nm, produced by a low pressure mercury lamp and the appropriate filters. Column materials were $^1/_8$-in. stainless steel tubing with an internal diameter of 1.8 ± 0.2 mm. End fittings of porous stainless steel frits imbedded in reducing unions were used as retainers for the column packing.

Reagents. Zipax support material (DuPont) was used for normal phase partition chromatography, and Corasil C_{18} (Waters Associates) a packing material consisting of a Corasil base with a C_{18} silane permanently bonded to the surface was used for the reversed phase chromatographic system. Solvents were reagent grade and were used as supplied except where otherwise noted. Injections were made with a Hamilton HP 305 5-μl syringe. Oxydipropionitrile stationary phase is available from a number of commercial sources. Reagent grade 2,4-dinitrophenylhydrazine was obtained from Eastman Kodak and recrystallized from *n*-butanol as a precautionary measure before using.

Procedure. Analytical columns were prepared by dissolving an appropriate amount of β,β′-oxydipropionitrile (ODPN) in methylene chloride and adding this to a weighed amount of Zipax to give a 1.5% loading by weight. After evaporation of the methylene chloride, the dry material was packed in a straight 1-meter × $^1/_8$-in. o.d. stainless steel tube. Complete procedural details of this operation can be found in "Modern Practice of Liquid Chromatography" (*12*). Our experience has been that column configurations other than straight exhibit decreased efficiency. A 30-cm × $^1/_8$-in. o.d. s.s. pre-column of 15% ODPN on Anakrom AB (100–110 mesh) or Porasil C (200–400 mesh) was found to be optimal to create a proper degree of pre-saturation of the mobile phase

(10) M. Sparagna, E. Kruetman, and W. Mason, ANAL. CHEM., **35**, 1231 (1963).
(11) W. Van den Heuval, E. Horning, and B. Greech, *Anal. Biochem.*, **4**, 191 (1962).
(12) J. Kirkland, "Modern Practice of Liquid Chromatography," J. Wiley and Sons, New York, N. Y., 1971.

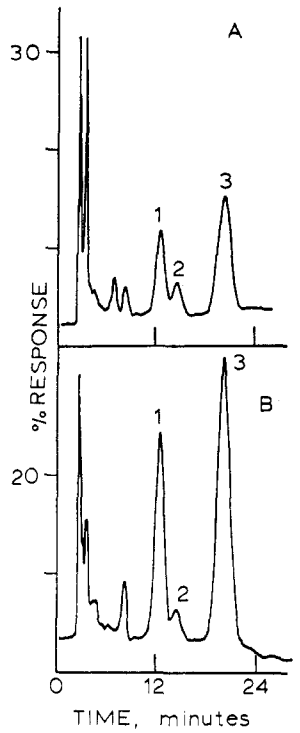

Figure 6. Same as Figure 5. A = Urine, No. 163; B = Urine No. 195, 1 = A, 2 = EPA, 3 = E

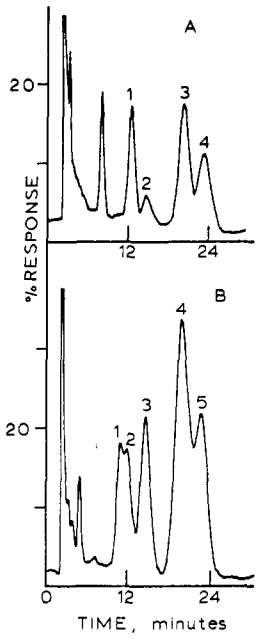

Figure 7. Same as Figure 5. *A* = Acid hydrolysis urine M2. 1 = A, 2 = EPA, 3 = E, 4 = DHA; *B* = standard mixture, 1 = EPE, 2 = A, 3 = EPA, 4 = E, 5 = DHA

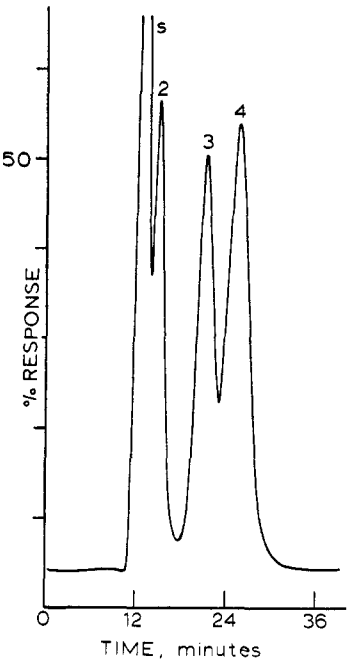

Figure 8. 1.2-meter Corasil C_{18} × 1.8-mm i.d., 0.16 ml/min 50 : 50 v/v ethanol:water, UV = 0.16 AUFS, S = solvent, 2 = DNPH, 3 = 11β-OH etiocholanolone, 4 = 11β-OH androsterone

with the stationary phase. The pre-column is necessary to prevent arbitrary and uncontrolled removal of the stationary phase from the analytical column with resultant irreproducible retention data. Isooctane was used as the mobile phase in all cases with ODPN as the stationary phase. The solvent was degassed prior to use to prevent spurious signals due to bubble formation in the detector.

Pure steroids were reacted with 2,4-dinitrophenylhydrazine (DNPH) according to the method of Siggia (*13*). After complete reaction the steroid derivatives were dissolved in a known amount of ethanol or chloroform. Carbonyl-free methanol used in the reaction was prepared by refluxing over DNPH for 1 hour followed by distillation.

Physiological Fluids. Urine samples were prepared for analysis by several methods. Urine solvolyzed by the method of Burstein and Lieberman (*14*) and hydrolyzed enzymatically, was extracted with ethyl acetate as described and the solutions were stored at −4 °C until ready for analysis. Known volumes of ethyl acetate were then evaporated to dryness and the residue was reacted according to the same procedure as the pure steroid standards. Care must be taken in the derivatization reaction that the pH does not fall below 0.8 or else dehydroepiandrosterone may be converted to 6β-OH-3,5-cyclo-androstan-17-one or 3β-chloroandrost-5-en-17-one. A number of urine samples were also hydrolyzed by the method of Vestergaard and Claussen. This technique involves an acid hydrolysis and simultaneous extraction of the liberated free steroid into benzene under reflux conditions. These conditions have been previously evaluated by gas chromatography and found suitable (*6*). The Vestergaard and Claussen extract was treated analogously to the steroid standards. Although the resultant extract contained chromogenic artifacts, no interferences were noted in the separation of steroid DNPH derivatives for urine aliquots of 100 ml or less. Urine aliquots of 200 ml or greater generally contained enough ultraviolet active impurity to produce a "solvent shoulder" which interfered with early eluting steroid peaks.

(13) S. Siggia, "Organic Analysis via Functional Groups," J. Wiley and Sons, New York, N. Y., 1963, pp 85–7.
(14) S. Burstein and S. Lieberman, *J. Biol. Chem.*, **233**, 331 (1958).

RESULTS AND DISCUSSION

Figure 2 shows a separation of a synthetic mixture of androsterone, epiandrosterone, and dehydroepiandrosterone-DNPH with the conditions as noted. Individual peaks were identified by comparison of retention data with known standards. Figure 3 shows the separation of four major urinary 17-keto steroids: androsterone, epiandrosterone, etiocholanolone, and dehydroepiandrosterone. The shoulder appearing on the upward slope of the androsterone peak was later found to be an impurity in the etiocholanolone standard identified as epietiocholanolone (5β-androstan-3β-ol-17-one). Figure 4 illustrates the improved separation of the early eluting peaks by altering the % loading of the stationary phase. All components were identified by comparison of the retention times of individual steroids. With 0.75% loading of ODPN on Zipax, epietiocholanolone, androsterone, and epiandrosterone are base-line resolved. Unfortunately, at this loading, etiocholanolone and DHA elute at the same retention volume. Figure 5 shows the chromatogram resulting from an acid hydrolyzed, derivatized urine sample (100 ml). Androsterone, epiandrosterone, and etiocholanolone were present in addition to high levels of DHA. Figures 6 and 7 show additional urinary hydrolysate chromatograms; a standard mixture is superimposed in Figure 7. The urine volume in both cases was 25 ml. Enzymatic hydrolysis and solvolysis were used in these examples. The working volume before injection was 0.5 ml.

The physiologically important 11β-hydroxylated analogs of androsterone and etiocholanolone exhibited excessively

Table I. Qualitative Analysis of Urinary 17-Keto Steroids

Hydrolytic method	Sample[a]	A	EPA	E	DHA	EPE	11 OH
Enzymatic-solvolysis	No. 195	+	+	+	?	−	+
Enzymatic-solvolysis	No. 163	+	+	+	?	−	+
Acid	Pooled	+	+	+	+	−	+
Acid	M 1	+	+	+	+	−	+
Acid	M 2	+	+	+	+	−	

[a] + = found; − = not found; ? = questionable. M 1, M 2 refer to individual male urine samples. No. 195 and No. 163 refer to patient number of samples obtained from Peter Bent Brigham Hospital.

long retention times on the β,β′-ODPN column with pure isooctane as the mobile phase, evidently due to the influence of the polar 11-hydroxy group. This behavior suggested the use of reverse phase chromatography to circumvent the retention time problem. Figure 8 illustrates the isolation of 11β-hydroxy keto steroids from the more lipophilic 11-deoxy steroids. The relatively nonpolar steroids such as androsterone and its epimers were retained for more than 30 minutes at a flow of 0.5 ml/min on a 1.2-meter × 1.8-mm i.d. column of Corasil C_{18} with 50:50 v/v ethanol/water as the mobile phase. On the same column it was possible to resolve 11-β-hydroxy-androsterone from 11-β-hydroxy-etiocholanolone. Figure 9 represents the detection of 11β-hydroxy keto steroids in urine sample No. 195. Table I lists the qualitative results for a number of different urine samples along with the hydrolytic method employed.

Urinary Conjugates. The fact that one can alter constituents in complex physiological fluids to better suit one's method of analysis—for example, conversion of the steroid conjugates to the free steroids—does not necessarily mean that the substrate measured is actually the substrate of significance. It is known that steroids are conjugated in the blood and are also associated with proteins (*15*) by some type of weak interaction. The ideal analytical method therefore would measure the compound of interest in its functioning form. Of course, incomplete knowledge of the nature of the mechanism of steroid action at present obviates this problem. We have obtained some results in this direction by isolation of the conjugates from their natural matrix prior to conversion and detection as the free steroids.

Two hundred milliliters of a mixed urine pool were treated by the method of McKenna and Norymberski (*16*) to extract the urinary steroid conjugates. The urine was made 0.3*M* in pyridinium sulfate and extracted one time with 100 ml of chloroform. The extract was evaporated to dryness and solvolyzed (*14*) to cleave the steroid sulfates present. After evaporation, the solvolysate was reacted with 2,4-DNPH and chromatographed. Figure 10 shows the resultant chromatogram. As might be expected, the relative amounts of epiandrosterone and dehydroepiandrosterone are greater in this case since they are predominately conjugated as sulfates. Androsterone and etiocholanolone are predominately conjugated as salts of glucopyranosiduronic acid and therefore have only a small tendency to be extracted into the chloroform phase. Glucuronide conjugates extracted would also require an acid or enzymatic hydrolysis for cleavage to the free steroid. Figure 11 shows the chromatogram of a pooled urine sample from which the conjugates were isolated by the method of Bush and Gale (*17*) prior to acid hydrolysis. Androsterone, epiandrosterone, and etiocholanolone peaks are evident, however the "background" is much higher in this in-

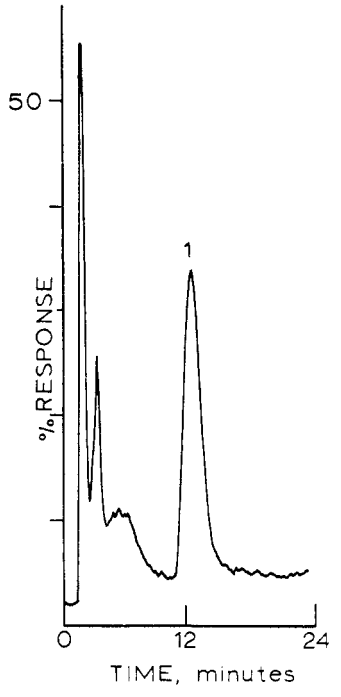

Figure 9. 1.2-m Corasil C_{18} × 1.8-mm i.d., 0.35 ml/min 50 : 50 v/v ethanol : water. UV 0.04 AUFS, Urine = No. 195, 1 = 11β-OH 17 KS

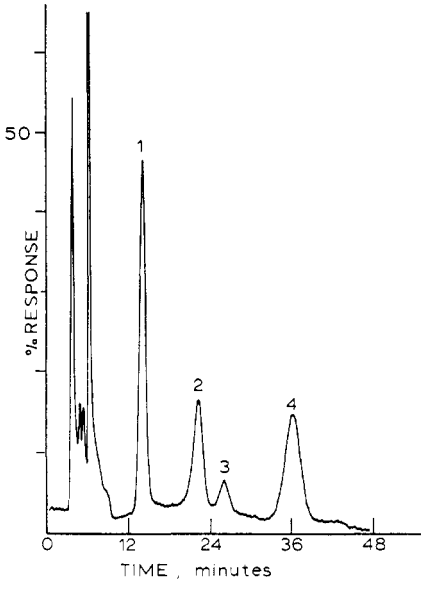

Figure 10. 1.5% ODPN on Zipax, 1-meter, 0.33 ml/min isooctane, UV 0.04 AUFS. Pooled urine, 1 = unknown, 2 = EPA, 3 = E, 4 = DHA

(15) C. Gray and A. Bacharach, "Hormones in Blood," Academic Press, New York, N. Y., 1961.
(16) J. McKenna and S. Norymberski, *Biochem. J.*, **76**, 60P (1960).
(17) J. Bush and M. Gale, *ibid.*, p 10P.

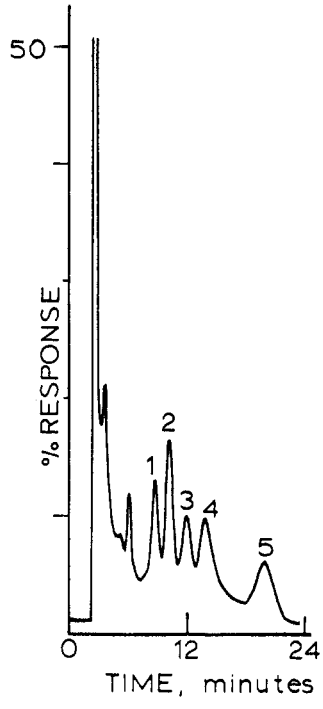

Figure 11. 1-meter, 0.75% ODPN on Zipax, 0.50 ml/min isooctane, UV 0.08 AUFS, Pooled urine, 1, 4 = unknown, 2 = A, 3 = EPA, 5 = E

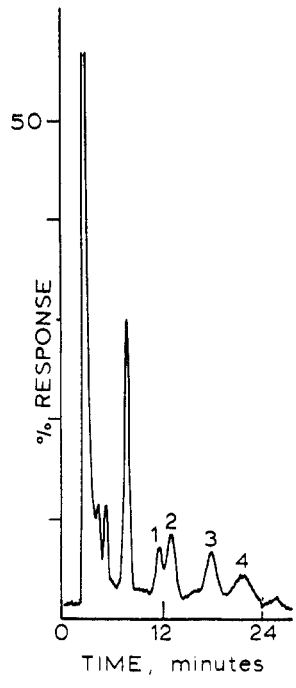

Figure 12. Same as Figure 5, 17-KS from plasma. 1 = A, 2 and 4 = unknown, 3 = DHA

stance lending cause for uncertainty in the positive identification of peaks. Another method of isolating the conjugates with Sephadex was attempted (*18*), but the product isolated was very "dirty". Hopefully a direct analysis on the urinary conjugate derivatives can be developed, thus eliminating the hydrolysis step completely.

17-Keto Steroids in Blood. An effort was made to determine 17-keto steroids in blood plasma. Twenty milliliters of blood plasma were shaken with 100 ml of 1:1 ethanol–acetone and filtered after 30 minutes at 37°C to remove proteins. The filtrate was evaporated to dryness and 20 ml of 70% methanol was added to the residue. The mixture was then stored at −10°C for 12 hours. The cold mixture was centrifuged for 5 minutes and the supernatant decanted, lipids remaining behind. After evaporation to dryness, 10 ml of water, 0.1 ml of concentrated sulfuric acid, and 2 grams of NaCl were added to the residue. This solution was extracted twice with 15 ml of ethyl acetate. Solvolysis of the steroids was achieved by 15-hour incubation at 37°C. The solvolysate was washed with 5 ml of 2*N* NaOH. The alkaline washing and the aqueous acid phase were then combined and heated with 2 ml of 50% sulfuric acid for 20 minutes on a boiling water bath to hydrolyze any glucuronides present. The cooled hydrolysate was extracted twice with 15 ml of ethyl acetate. The solvolysate and the hydrolysate were combined and the total extracts were washed with 5 ml of water. The extracts were then evaporated to dryness and reacted with DNPH according to the same procedure as that of urine. Figure 12 shows a chromatogram of a derivatized blood extract. Androsterone and DHA which occur predominately in blood were identified by comparison of retention times.

(18) D. Gupta and J. Goodwin, *Steroids Suppl.*, 195 (1966).

CONCLUSIONS

The more common 17-keto steroids have been detected as their DNPH derivatives from urine and plasma. Preliminary results on quantitative analysis of these steroids indicates a precision of 10% relative standard deviation at the microgram and lower ranges. Because alterations in the excretion level of the 17-keto steroids are indicative of a number of diseased states, it is believed that this method may have clinical potential. The time factor involved is less than gas or thin layer chromatography. Future investigations should be useful in evaluating its potential as a quantitative technique more completely.

ACKNOWLEDGMENT

The authors express their appreciation to James N. Little of Waters Associates and William Zeronsa of Pfizer for their help. We would also like to thank the laboratory staff of the University of Massachusetts Infirmary for supplying pooled urine samples.

RECEIVED for review March 30, 1972. Accepted July 21, 1972. This work was sponsored in part by an American Chemical Society Analytical Division Summer Fellowship sponsored by the Carle Corporation.