Determination of 17-Hydroxycorticosteroids in Human Urine

W. FEINSTEIN and J. J. SCIARRA ˣ

Abstract □ A study was undertaken to develop a method that could be used as an indication of the absorption of steroids. It was demonstrated that 17-hydroxycorticosteroids in urine could be quantitatively determined *via* absorptivity values obtained through the use of standard hydrocortisone-alcohol solutions or standard solutions of hydrocortisone in urine utilizing the blue tetrazolium assay method. This method is dependent upon the hydrolysis of conjugated urinary steroids by beef liver glucuronidase. The resultant concentration of 17-hydroxycorticosteroids is determined colorimetrically using blue tetrazolium.

Keyphrases □ 17-Hydroxycorticosteroids—colorimetric analysis in human urine □ Steroids—colorimetric analysis of 17-hydroxycorticosteroids in human urine □ Colorimetry—analysis, 17-hydroxycorticosteroids in human urine

It has been shown by others that significant percutaneous absorption of steroids can take place from topical dosage forms. The percutaneous absorption that occurred when pharmaceuticals were applied over large body areas was investigated (1). The results from two patients treated with fluocinolone acetonide led to the conclusion that the depression of urinary steroid excretion was caused by suppression of endogenous adrenocorticosteroid production and that this suppression was a result of the absorption of the drug. One phenomenon associated with the administration of steroids is the decrease in concentration of 17-hydroxycorticosteroids in the urine.

Burch and Migeon (2) stated that this phenomenon presumably reflects a partial shutdown of adrenal gland activity because of the absorbed steroid. The extent of percutaneous absorption of this drug was noted to be dependent upon the lipid solubility of the drug. The biotransformation of the topically applied steroid to a compound of greater water solubility was studied by way of the urine concentration profiles of measured 17-hydroxycorticosteroids.

DISCUSSION

The 17-hydroxycorticosteroids that occur primarily in urine as conjugates of glucuronic acid can be hydrolyzed. Drekter *et al.* (3) developed a simplified method for the determination of urinary 17-ketosteroids by means of acid hydrolysis. They replaced the routine aqueous alkali wash with a solid alkali pellet wash to remove urine pigments, such as the acidic, phenolic, and pigmented constituents of hydrolyzed urine, which normally interfere with the results. Therefore, this technique reduces the blank readings significantly. In addition, they found that no interaction occurred between the solid sodium hydroxide pellets and the steroids present.

Several methods for hydrolyzing the steroid conjugates and extracting the free compounds were presented (4). It was noted that urine contains various reducing agents and the specificity of the assay method depends upon the efficiency of the extraction and the purification of the extract. The free 17-hydroxycorticosteroids resulting from the hydrolysis reaction can then be oxidized with sodium iodate or sodium bismuthate on the C_{20},C_{21}-dihydroxy position. The evolved formaldehyde then can be determined (5).

The oxidation reaction also produces 17-ketosteroids, which can be analyzed by the modified Zimmermann reaction (the ketosteroids are removed by borohydride reduction to the corresponding alcohols). The 17-ketogenic steroids are then oxidized by bismuthate and the resulting concentrations are determined either directly (6) or by the Pettenkoffer reaction (reaction between acetic acid solutions and the steroid when treated with furfural and sulfuric acid) (7). The normal range of 17-ketosteroids also gives this reaction.

The Kober reaction (involving acid hydrolysis, ether extraction, separation of ethers by chromatography, colorimetric measurement, and spectrophotometric correction for interfering chromogenic material) (8) is occasionally utilized for the analysis of free 17-hydroxycorticosteroids. However, all 17-hydroxyestrogens interfere with this reaction.

The only two specific reagents are those used in the Porter–Silber reaction (9), in which all 17,21-dihydroxy-20-one type steroids give a positive reaction, and the blue tetrazolium reagent (10), which is specific for primary ketol side chains. The Porter–Silber reaction requires 18 hr for color development and employs concentrated sulfuric acid to give increased values for the urine blank determination. The blue tetrazolium method employs no sulfuric acid and takes 90 min for color development (11).

The quantitative determination of 17-hydroxycorticosteroids in urine was studied using beef liver glucuronidase to hydrolyze conjugated urinary steroids and subsequently treating the sample with blue tetrazolium to measure the concentration of 17-hydroxycorticosteroids colorimetrically.

Table I—Absorbance as a Function of Hydrocortisone Concentration in Alcohol and Human Urine Using Blue Tetrazolium Assay

Alcohol		Human Urine	
Hydrocortisone, μg/ml	Net Absorbance (525 nm)	Hydrocortisone Added, μg/ml	Net Absorbance (525 nm)
1.667	0.210	0.62	0.075
3.333	0.408	1.24	0.150
5.000	0.620	1.86	0.250
8.333	1.020	3.10	0.350

EXPERIMENTAL

Test Solutions—For the blue tetrazolium solution, exactly 125 mg of blue tetrazolium was dissolved in 25 ml of absolute methanol. For the tetramethylammonium hydroxide test solution, 10 ml of a 10% tetramethylammonium hydroxide solution was diluted to 100 ml with absolute methanol.

Standard Preparation—Approximately 100 mg of hydrocortisone USP, accurately weighed, was dissolved in alcohol USP to make exactly 100 ml. Then 1 ml of the solution was diluted to exactly 100 ml with alcohol. This standard solution contained approximately 10 μg/ml.

Sample Preparation—Urine specimens were collected from normal, healthy adults, and the pH of the urine was immediately adjusted to 5.0 with acetic acid. The urine was divided into two portions; one portion was retained for use as the untreated specimen. The second portion was subdivided and to each aliquot were added varying amounts of hydrocortisone and urine to achieve final hydrocortisone concentrations of approximately 0.5, 1, 2, and 3 μg/ml.

Procedure—Aliquots of 2, 4, 6, and 10 ml of the standard solu-

tion were pipetted into separate 35-ml centrifuge tubes and the volume was brought to 10 ml with alcohol. To each standard solution and blank, 1 ml of blue tetrazolium solution was added. The tubes were mixed and exactly 1 ml of tetramethylammonium hydroxide test solution was added. The solution was mixed, allowed to stand for 90 min, and then placed into 1-cm silica cells. The absorbance of each standard solution was read against the blank in a suitable spectrophotometer at 525 nm (Table I).

Exactly 10 ml of the untreated urine from each specimen was pipetted into three 35-ml centrifuge tubes. A blank was prepared with 10 ml of purified water adjusted to pH 5.0 with acetic acid. Four milliliters of beef liver glucuronidase solution[1] was added to each sample and blank. These tubes were mixed, stoppered, and incubated overnight at 37°. After cooling to room temperature, 10 ml of carbon tetrachloride was added and the tubes were shaken for 5 min at very low agitation and centrifuged for 5 min. The aqueous phase was transferred to a second 35-ml centrifuge tube, and the extraction was repeated with 10 ml of carbon tetrachloride.

Twelve milliliters of the aqueous phase was transferred to a 125-ml separator, extracted with 50 ml of methylene chloride, and allowed to settle for 30 min. The organic phase was then transferred to a 125-ml flask. Thirty pellets of sodium hydroxide were added, the flask was shaken for 5 min, and the sample was then allowed to settle. The solution was filtered through filter paper[2] into a 50-ml centrifuge tube.

Twenty milliliters of the filtrate was pipetted into a 35-ml centrifuge tube and evaporated to dryness in a water bath at 30° with the aid of a gentle stream of air. The residue was dissolved in 10 ml of alcohol. The sample solutions and their blank were then treated with the blue tetrazolium solution and tetramethylammonium hydroxide solution, as indicated for the standard solution; the absorbance was read in a similar manner. The hydrocortisone-treated urine samples were assayed similarly, starting with the addition of the beef liver glucuronidase solution to 10-ml aliquots (Table I).

RESULTS

The analytical method (12) used for the determination of 17-hydroxycorticosteroids in urine was initially evaluated by adding known amounts of hydrocortisone to urine specimens. These evaluations indicated possible difficulties with the method. Therefore, this method was modified by omitting the sodium hydroxide backwash, substituting a solid sodium hydroxide wash, and utilizing the blue tetrazolium method in place of the Porter–Silber reaction. The results of the two standardizations are shown in Table I. These data demonstrate that there is no difference between the two standardization studies; that is, the absorptivity value for the hydrocortisone–blue tetrazolium reaction is essentially identical whether the solution is prepared in alcohol or urine.

Table II reports the results on 17-hydroxycorticosteroid recovery in untreated urine and is based on the absorptivity value calculated from the data presented in Table I. These studies demonstrate that 17-hydroxycorticosteroids in untreated human urine can be quantitatively determined *via* absorptivity values obtained through the use of standard hydrocortisone–alcohol solutions or standard solutions of hydrocortisone in urine.

Table II—17-Hydroxycorticosteroid Levels of Randomly Taken Urine Samples from Selected Subjects

Subject	Urine Voided, ml	Net Absorbance (525 nm)	17-Hydroxycorticosteroid	
			μg/ml	mg/500 ml
A (am)[a]	105	0.780	6.33	3.17
B (pm)	300	0.280	2.27	1.14
C (am)	270	0.570	4.62	2.31
D (pm)	250	0.192	1.56	0.78
E (am)	75	0.800	6.49	3.25
F (pm)	50	0.800	6.49	3.25

[a] Time of urine collection appears in parentheses.

[1] Ketodase solution (5000 Fishman units/ml), General Diagnostics, Division of Warner-Lambert Co., Morris Plains, N.J.
[2] Whatman No. 4.

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[x] To whom inquiries should be directed. Present address: Brooklyn College of Pharmacy, Long Island University, Brooklyn, NY 11216