The Isolation, Characterization, and Measurement of Steroid Glucuronides in Human Plasma*

GEORGE L. COHN† AND PHILIP K. BONDY

From the Department of Internal Medicine, Yale University School of Medicine, New Haven, Connecticut

(Received for publication, April 21, 1958)

Since one of the important pathways in the metabolism of adrenal cortical steroids involves reduction and conjugation, the study of steroid conjugates in plasma seems an important step in understanding the fate of these substances in the body. Most investigators have measured conjugated steroids, after hydrolysis with acid or by specific enzymes, in normal individuals and in patients with liver disease (1-3), before and after the administration of cortisone (1), cortisol, tetrahydrocortisone and its mono-
glucuronide, tetrahydrocortisol, and dihydrocortisol (4, 5), and after the administration of adrenocorticotropicin (5, 6). In these previous studies the conjugated steroids have not been isolated or characterized. The methods have depended for their specificity upon the nature of the hydrolytic process and the characteristics of the steroids. Since the effectiveness of a given enzyme in hydrolyzing a variety of substrates may not be uniform, since the purity of the enzyme preparation cannot be guaranteed, and since there are difficulties in determining the completeness of hydrolysis, the results thus obtained require confirmation by more specific methods.

The technique to be described involves the use of paper electrophoresis and paper chromatography for the direct isolation of steroid conjugates from plasma. The glucuronides obtained can then be measured by the carboxazone reaction. An isotope dilution method with tetrahydrocortisone-4-C14-glucuronide used to correct the results to 100 per cent recovery is also reported. The method is sufficiently sensitive to be useful in measuring the concentration of H4FG1 and H3EG in normal human plasma.

EXPERIMENTAL

Isolation—50 ml. of heparinized blood were centrifuged within 15 minutes after collection as suggested by Bush (7). 0.1 ml. of a standard solution of tetrahydrocortisone-4-C14-glucuronide (8-μg.) in methanol was pipetted into a 60-ml. centrifuge tube and evaporated to dryness. From 20 to 25 ml. of plasma were added, and the tube was shaken for about 1 minute to insure complete mixing of the radioactive steroid glucuronide and the plasma, after which 1.5 to 2.0 volumes of absolute ethanol at room temperature were added. The precipitated protein was removed by centrifugation and further washed three times with 60 ml. of redistilled n-butanol saturated with water. The ethanol and butanol extracts were pooled and dried in a rotating vacuum evaporator. The residue was redissolved in 25 ml. of 70 per cent methanol and washed three times with equal volumes of redistilled hex-
ane. The hexane layers were discarded and the washed methanol fraction was taken to dryness in a vacuum. The residue was then dissolved in 1.0 ml. of 70 per cent redistilled methanol and applied as a streak 12.7 cm. from the cathodal end of a strip of Whatman No. 3MM paper, 47 cm. in length and 10.0 cm. in width. Reference spots of approximately 20 μg. of H3EG and androster-
one sulfate2 were applied on opposite edges of the paper and ad-
jaent to the streak. Electrophoresis was carried out by the method of Levin and Davies (8) in a Plexiglass (Rohm and Haas Company) chamber connected to a regulated power supply for 8 to 10 hours at 4° in 0.1 mM Veronal (Winthrop Laboratories) buffer, pH 8.0, with 20 per cent of absolute ethanol added. The potential was 250 volts and the current, 8 to 10 ma. At the termination of the run the paper was air-dried.

The glucuronide band was located by dipping the pilot strip which contained the glucuronide reference standard into a fresh mixture of 1 volume of 2 per cent triphenyltetrazolium chloride and 4 volumes of 10 per cent NaOH in 60 per cent methanol (9). The glucuronides migrated 8 to 10 cm. as a band toward the anode, whereas the neutral (nonconjugated) steroids remained at the origin. Sulfate conjugates migrated 11 to 14 cm. toward the anode (Fig. 1).

The sulfate conjugate standard was located by staining the appropriate pilot strip with Zimmermann reagent (10). The corresponding band was cut from the paper and eluted with 70 per cent methanol. However, no further characterization was performed on the sulfate conjugates, since no triphenyltetra-
zolium staining material was present in this region of the paper.

The glucuronide band was eluted by shaking with 15 ml. of 70 per cent methanol for 2 hours at 36°. The methanol solution was decanted after centrifugation, and the paper was washed three times with 70 per cent methanol. The combined extracts were reduced to approximately 1.0 ml. in a rotating vacuum evaporator and applied as a streak at 10 cm. from one end of a 55-cm. length of Whatman No. 1 filter paper which had been washed in methanol-ethyl acetate (3:1) for 72 hours in a Nolan

* This investigation was supported in part by a grant from the James Hudson Brown Fund, Yale University, and in part by Grant No. 254 (C5), from the Division of Grants, National Insti-
tutes of Health, United States Public Health Service.
† Trainee, National Institute of Arthritis and Metabolic Dis-
eease, United States Public Health Service.

1 The abbreviations used are: H4FG, tetrahydrocortisol glu-
curonide, which is 3α-β-α-glucuronide), 11β,17α,21-trihydroxy-
pregnane,20-one; and H3EG, tetrahydrocortisone glucuronide, which is 3α-β-α-glucuronide), 17α,21-dihydroxyprog

2 Dr. John J. Schneider, Jefferson Medical College, Philadel-
phia, Pennsylvania, generously supplied the pure steroid glu-
curonides and sulfates.
The separation of steroid glucuronides and sulphates

**FIG. 1.** The separation of H₄FG and H₄EG by paper electrophoresis and paper chromatography. Rp refers to the ratio of the mobility of the compounds from the origin to the solvent front which traveled to the bottom of the paper.

Reference compounds of H₄EG and H₄FG were also applied adjacent to the streak. The paper was then equilibrated at room temperature for 16 hours in a sealed glass tank which contained some of the mobile phase in the bottom. After this the mobile phase of n-butyl acetate-n-butanol-10 per cent acetic acid (80:20:100) was added to the trough (9). Descending chromatography was carried out for 6 to 8 hours at room temperature for 2 hours before they were read. A duplicate standard curve was constructed on each occasion with crystalline H₄EG, Beer's law was followed from 0.3 to 1.2 μg. The optical density reading of the samples was 2.5 to 5 times that of the paper blanks.

The remaining 0.5-ml aliquot intended for radioactive measurement was pipetted into a 30-ml, optically clear vial and evaporated to dryness. The sample was dissolved in 15 ml. of 0.4 per cent 2,5-diphenyloxazole and 0.005 per cent 1,4-di(25-phenyloxazolo)benzene in toluene and counted for three 30-minute periods in a liquid scintillation counter (Technical Measurements Company, model LP-2, New Haven, Connecticut) which had a counting efficiency of 65 per cent.

**Calculation**—The values obtained by the carbazole reaction were corrected for the blank readings and dilution. The percentage of recovery was obtained with the use of an isotope dilution technique described by Bondy et al. (12). In the case of the H₄EG a standard amount of this labeled compound was added at the beginning of the isolation. A correction was applied for this addition by subtracting this value from the final yield. The final results in μg per 100 ml. thus accounted for any losses incurred during the isolation procedure. Although no comparable experiments were carried out with radioactive H₄FG, a correction for recovery was applied on the assumption that the percentage of recovery of the tetrahydrocortisonc-4-C⁴-glucuronide was identical with that of H₄FG since identical isolation and measurement procedures were applied to the two compounds. Further work is now in progress to substantiate the validity of this assumption. The aliquots of the eluates from the paper blanks had only background radioactivity so that no further correction for the paper blanks was necessary.

**Recovery**—The over-all radioactive recovery was 72.4 ± 7.2 per cent (mean ± standard deviation (Table I)). Attempts were made to locate the missing radioactivity by elution, with 70 per cent methanol, of individual 2-cm. strips from the entire electropherogram after removal of the glucuronide and sulfate bands. The eluates were pooled, taken to dryness in a vacuum, and quantitatively transferred to 2.0-square centimeter areas on aluminum planchettes. The radioactivity was counted in a windowless gas flow counter which had a background of 1 c.p.m. Approximately 5 to 8 per cent of the total radioactivity was found at the origin of the paper. Performance of the same experiments after chromatography revealed that 8 to 10 per cent of the radioactivity migrated with the solvent front.

**TABLE I**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Sex</th>
<th>Age</th>
<th>H₄EG</th>
<th>H₄FG</th>
<th>Isotope recovery</th>
<th>H₄FG</th>
<th>H₄EG</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. F.</td>
<td>F</td>
<td>23</td>
<td>3.1</td>
<td>5.7</td>
<td>72</td>
<td>4.3</td>
<td>7.9</td>
<td>12.2</td>
</tr>
<tr>
<td>C. P.</td>
<td>F</td>
<td>23</td>
<td>2.8</td>
<td>7.2</td>
<td>63</td>
<td>4.4</td>
<td>11.4</td>
<td>15.8</td>
</tr>
<tr>
<td>E. T.</td>
<td>F</td>
<td>26</td>
<td>5.6</td>
<td>10.1</td>
<td>81</td>
<td>6.8</td>
<td>12.4</td>
<td>19.2</td>
</tr>
<tr>
<td>C. C.</td>
<td>F</td>
<td>29</td>
<td>2.4</td>
<td>3.4</td>
<td>81</td>
<td>2.9</td>
<td>4.2</td>
<td>7.1</td>
</tr>
<tr>
<td>D. K.</td>
<td>M</td>
<td>25</td>
<td>2.1</td>
<td>3.0</td>
<td>75</td>
<td>2.8</td>
<td>4.0</td>
<td>6.8</td>
</tr>
<tr>
<td>D. M.</td>
<td>M</td>
<td>27</td>
<td>3.5</td>
<td>6.1</td>
<td>88</td>
<td>5.1</td>
<td>8.9</td>
<td>14.0</td>
</tr>
<tr>
<td>J. M.</td>
<td>M</td>
<td>27</td>
<td>3.2</td>
<td>4.0</td>
<td>75</td>
<td>4.3</td>
<td>5.3</td>
<td>9.6</td>
</tr>
<tr>
<td>R. P.</td>
<td>M</td>
<td>28</td>
<td>4.2</td>
<td>7.0</td>
<td>66</td>
<td>6.4</td>
<td>10.6</td>
<td>17.0</td>
</tr>
<tr>
<td>H. K.</td>
<td>M</td>
<td>29</td>
<td>2.3</td>
<td>3.5</td>
<td>81</td>
<td>2.8</td>
<td>4.3</td>
<td>7.1</td>
</tr>
<tr>
<td>P. M.</td>
<td>M</td>
<td>31</td>
<td>3.5</td>
<td>4.7</td>
<td>63</td>
<td>5.6</td>
<td>7.5</td>
<td>13.1</td>
</tr>
<tr>
<td>G. C.</td>
<td>M</td>
<td>32</td>
<td>3.6</td>
<td>8.5</td>
<td>86</td>
<td>5.5</td>
<td>12.9</td>
<td>18.4</td>
</tr>
<tr>
<td>M. C.</td>
<td>M</td>
<td>33</td>
<td>2.8</td>
<td>9.0</td>
<td>89</td>
<td>4.1</td>
<td>13.0</td>
<td>17.1</td>
</tr>
<tr>
<td>G. C.</td>
<td>M</td>
<td>42</td>
<td>3.5</td>
<td>4.9</td>
<td>84</td>
<td>4.2</td>
<td>5.8</td>
<td>10.0</td>
</tr>
<tr>
<td>G. M.</td>
<td>M</td>
<td>51</td>
<td>2.2</td>
<td>2.2</td>
<td>69</td>
<td>3.2</td>
<td>3.2</td>
<td>6.4</td>
</tr>
</tbody>
</table>

Mean .......... | 72.4 | 4.45 | 7.96 | 12.41
Standard deviation .. | ±7.2 | ±1.29 | ±2.70 | ±4.92

Subject = Sex: M = male, F = female; Age in years; μg./100 ml. uncorrected; μg./100 ml. corrected.
labeled H,EG was prepared biosynthetically. 100 mg. of
with the mobility characteristics of etiocholane-3a, 1 l/3, diol ,17-
one (derived from tetrahydrocortisol), and etiocholane 3a, 1\beta, -
absence of an absorption peak at 238 to 242 nm in ethanol and
4-ene, 3-one, in the tetrahydro derivatives was indicated by the
had chromatographic mobilities identical to standard tetra-
hydrocortisol and tetrahydrocortisone. No other compounds,
duction reactions confirmed the presence of an oc-ketol. Bis-
such as allotetrahydrocortisol, were detectable. The lack of a
by the failure of the compounds to fluoresce in alkali. The
yielded, on hydrolysis with \( \text{glucuronidase} \), compounds which
were studied for alkaline
reaction. 250,000 Fishman units of enzyme were added to ten
samples of plasma. 8 pg. of pure H,EG were added
to alternate plasma samples and processed simultaneously by
the methods described. The mean percentage of chemical
recovery of H,EG was 68.7 ± 10.2, which is not significantly
different from the recovery of 72.4 ± 7.2 as measured by isotope
dilution.

The analytical procedure takes 4 days to complete but can be
interrupted at any stage when the compounds are completely
dried. The dried materials may then be stored in the Deep Freeze
without any quantitative loss of the compounds.

Identification of Plasma Glucuronides—H,EG and H,FG were
identified after hydrolysis with \( \beta \)-glucuronidase (Glusulase,
Endo Products, Richmond, Virginia) in order to be sure that only
these compounds were being measured by the carbazole
reaction. 250,000 Fishman units of enzyme were added to ten
pooled alcohol and butanol extracts which represented a total
of 300 ml. of original plasma. The hydrolysis was performed at
pH 4.6 in 0.2 m acetic buffer for 15 hours. The temperature was
47°. The steroids thus released were extracted three times with
2 volumes of redistilled chloroform. The chloroform
extract was evaporated in a vacuum at 35°.

The free compounds were chromatographed on washed What-
man No. 1 filter paper in parallel with reference standards of
tetrahydrocortisone and tetrahydrocortisol for 3 days in toluene-
propylene glycol (13) and for 6 hours in 50 per cent methanol-
benzene (14). The substances isolated were studied for alkaline
fluorescence (15), ultraviolet absorption, Porter-Silber reaction
(16), blue tetrazolium (17), and triphenyltetrazolium reactivity
(9). Portions of the tetrahydrocortisone and tetrahydrocortisol
were oxidized by the method of Norymberski (18) with sodium
bismuthate. The resulting 17-ketosteroids were characterized by
paper chromatography in heptane-propylene glycol (19),
with the Zimmermann reagent (10) used to locate the steroids.

The substances identified according to their mobility in the
butyl acetate-n-butanol-acetic acid system as H,FG and H,EG
yielded, on hydrolysis with \( \beta \)-glucuronidase, compounds which had
chromatographic mobilities identical to standard tetra-
hydrocortisol and tetrahydrocortisone. No other compounds,
such as allotetrahydrocortisol, were detectable. The lack of a
4-ene,3-one, in the tetrahydro derivatives was indicated by the
absence of an absorption peak at 238 to 242 m\( \mu \) in ethanol and
by the failure of the compounds to fluoresce in alkali. The
presence of a dihydroxyacetone side chain was indicated by a
positive Porter-Silber reaction. The positive tetrazolium re-
duction reactions confirmed the presence of an \( \alpha \)-ketol.
Bismuthate oxidation resulted in Zimmermann reacting materials
with the mobility characteristics of ctocholanol-3\( \alpha \),11\( \beta \),diol,17-
one (derived from tetrahydrocortisol), and ctocholanol 3\( \alpha \), 11,17-dione (from tetrahydrocortisone).

Preparation of Radioactive Standard for Isotope Dilation—The
labeled H,EG was prepared biosynthetically. 100 mg. of
cortisol\(^{4} \), which contained a total of 8.8 \( \times \) 10\(^{6} \) c.p.m., were
administered intravenously over a 15-minute period to a normal
volunteer. The radioactive H,EG was isolated according to
the method of Schneider and Lewbart\(^{4} \) from a 24-hour collection of
the subject's urine.

The urine was first passed through a 150 to 200-gm. column (60 \( \times \) 2.0 cm.) of Amberlite 1R-120 (Rohm and Haas Company)
in the hydrogen form. The effluent was adjusted to pH 3 to
3.5 with a sodium carbonate solution, saturated with \( n \)-butanol,
and then further extracted with water-saturated \( n \)-butanol.
The combined extracts were backwashed with very small
amounts of wet butanol and the aqueous washings were dis-
carded. The pH was adjusted to 6.5 to 7.5 and the washed,
neutral butanol extract was then concentrated to approxi-
mately 50 ml. in a vacuum. The crude material was recovered
by centrifugation, washed with dry \( n \)-butanol, and then dried
in a vacuum over calcium chloride.

After this preliminary preparation was completed the dried
sodium salt was purified by sublimation in a vacuum by gradual
elevation of the temperature from 200 to 218°, for 4 days at an
ultimate vacuum of 0.05 mm. On the 4th day a small quantity
of crystalline material was obtained which was further purified
by paper electrophoresis and paper chromatography as described
previously. The material was identified as H,EG and contained
5000 c.p.m. per mg. The sublimation procedure did not alter
the chemical structure of the H,EG as determined by the electro-
phoretic and chromatographic characteristics.

Identification of Radioactive H,EG—Paper chromatography of
the crystalline radioactive H,EG along with a pure reference
standard in two systems, \( n \)-butanol-buty1 acetate-10 per cent
acetic acid, and butyl acetate-methanol-0.1 barbital in 50 per
cent aqueous methanol, \( \text{pH} 8.2 \) (150:50:50, monophasic (9),
revealed only one radioactive area after the entire paper had
been cut into 2-cm. strips, eluted with 70 per cent methanol,
and counted in the low background windowless counter. Paper
chromatography and enzyme hydrolysis of the compound with
subsequent paper chromatography in the Zaffaroni and Bush
systems (13, 14), and chemical tests as described, followed by
oxidation to the corresponding C 10 steroid, ctocholanol 3\( \alpha \)-
ol,11,17-dione, were considered sufficient criteria to substantiate
the characteristics of the compound.

RESULTS

14 subjects, including 4 women and 10 men who ranged in age
from 23 to 51 years and who were normal with respect to liver,
adrenal, renal, and thyroid function were studied to determine
the normal range of values for plasma H,EG and H,FG. The
samples were taken at approximately 10 a.m. The results
are presented in Table I.

DISCUSSION

Since plasma steroid conjugates represent important inter-
mediary products of cortisol metabolism, a direct, simple method
for the isolation and measurement of these glucuronides seems
useful. Steroid glucuronides and sulfates were separated by
paper electrophoresis but no steroids with the dihydroxyacetone
side chain were detectable in the sulfate region. However, C-19

\(^{4} \text{Cortisol-4-C}^{14} \) was supplied by the Endocrinology Study Sec-
tion, National Institutes of Health.

\(^{a} \) Personal communication.
steroids conjugated as sulfates were found and separated by paper chromatography; these studies will be reported later. Nonconjugated C-21 plasma steroids remained at the origin and were easily eluted with methanol-chloroform (1:1) and separated by paper chromatography. When compounds such as cortisol, corticosterone, and progesterone were added to plasma, they were easily identified by their mobility characteristics with paper chromatography. However, quantitative recoveries and measurement of the nonconjugated steroids were not performed on the subjects studied.

The specificity of the method depends upon the separation of the steroid glucuronide from any other compounds in plasma which might react with carbazole. It would have been equally possible to measure the steroid moiety but attempts to apply Porter-Silber and blue tetrazolium reactions were unsuccessful because of the very high values of the paper blanks. Since no attempt was made to characterize the glucuronide portion of these substances, it is not possible to state with assurance that the materials measured are glucuronides. However, these compounds were cleaved by β-glucuronidase, and it seems unlikely that any other carbazole-reacting substance(s) which could combine with these steroids would produce compounds with electrophoretic and chromatographic mobilities identical with glucuronide standards.

This direct method avoids the difficulties inherent in the hydrolysis of steroid glucuronides and sulfates and results in the isolation of the compounds as they occur in physiological or pathological conditions.

SUMMARY

A method is presented for measurement of the glucuronides of tetrahydrocortisone and tetrahydrocortisol in plasma. The conjugated steroids are separated from nonconjugated steroids by paper electrophoresis and paper chromatography. The measurement of the glucuronides is carried out by the carbazole reaction. An isotope dilution technique is used for the correction of losses. The range of plasma concentrations of tetrahydrocortisone glucuronide is 3.2 to 12.9 mg/liter and tetrahydrocortisol glucuronide, 2.8 ± 6.8 mg/liter.

Acknowledgment—We should like to express our deep appreciation to Dr. John J. Schneider and Dr. Marvin Lewbart for their advice and permission to publish in advance of publication their method for the isolation of steroid glucuronides in urine. We are indebted to Miss Fritze Pederson for technical assistance and to Dr. Samuel Gordon who supplied us with Glusulase. We also wish to thank Dr. Melvin V. Simpson for helpful criticism in the preparation of the manuscript.

ADDENDUM

It has recently been observed that a cleaner extract is obtained if the plasma is washed 3 times with 30 ml of redistilled chloroform before the ethanol, butanol extractions, and hexane partition steps. The chloroform-soluble material can be set aside for determination of unconjugated steroids. This initial procedure does not alter the recovery of H2EG and H2FG.

REFERENCES

The Isolation, Characterization, and Measurement of Steroid Glucuronides in Human Plasma
George L. Cohn and Philip K. Bondy

J. Biol. Chem. 1959, 234:31-34.

Access the most updated version of this article at http://www.jbc.org/content/234/1/31.citation

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/234/1/31.citation.full.html#ref-list-1