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Research Article

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Recurrent Fever of Unknown Etiology: Failure to Demonstrate Association between Fever and Plasma Unconjugated Etiocholanolone

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ABSTRACT A sensitive method for determination of plasma unconjugated etiocholanolone by double-isotope–derivative dilution has been described. The mean values for normal subjects was 0.038 ±0.003 (SEM) μg/100 ml.

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INTRODUCTION

It is well-known that the intramuscular injection of certain 5β-H, A : B *cis* steroids, of which etiocholanolone has been most studied, regularly causes local inflammation and fever in man (1, 2). In 1958 Bondy, Cohn, Herrmann, and Crispell (3) reported elevated plasma concentrations of unconjugated etiocholanolone during recurrent febrile episodes in two patients, and suggested a new clinical syndrome, "etiocholanolone fever." These investigators developed a method for the measurement of unconjugated etiocholanolone in human plasma and established an upper limit of normal of 1.2 μg/100 ml plasma (4) ; the lower limit, less than 0.5 μg/100 ml, was the lowest value detectable. With this method, plasma concentrations of unconjugated etiocholanolone ranging from 2.4 to 14.2 μg/100 ml were reported during febrile episodes in 11 patients (5). Huhnstock, Kuhn, and Oertel (6), using this same method, found values of 6.1–17.2 μg/100 ml during febrile episodes in three patients.

We have developed a double-isotope–derivative dilution method for unconjugated etiocholanolone in plasma which measures as little as 0.01 μg and have used this method to study 20 normal subjects and 40 patients with recurrent fever.

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METHODS

Reagents. The following reagents were obtained in reagent grade: dichloromethane, diethylether, benzene, ethyl acetate, chloroform, toluene, decalin, ethyl alcohol, potassium hydroxide, sodium hydroxide, sodium borohydride. Rhodamine 6G was obtained from the Allied Chemical Corporation, New York. Nitromethane was purified by passage through silica gel, No. 28–No. 200 mesh. Pyridine was purified by addition of calcium hydride (2 g/250 ml) and allowed to stand overnight. The fraction boiling at 115°C was then collected in a fractionating column. Acetic anhydride was refluxed over calcium carbide for 2 hr and then distilled in a fractionating column; the fraction boiling at 139°C was collected. The phosphor solution used for liquid scintillation counting contained 5 mg of 2,5-diphenyloxazole and 500 mg of *p*-bis[2-(5-phenyloxazolyl)-benzene]/liter of reagent-grade toluene.

Thin-layer chromatoplates were prepared by mixing aluminum oxide G for thin-layer chromatography with water (1 : 2, w/w) into a slurry and spreading a layer 250 μ-thick on 20 cm × 20 cm glass plates. The plates were dried overnight at room temperature and stored without further drying or heating.

Carbon 14-labeled acetic anhydride (New England Nuclear Corp., 5 mc/mmole) was obtained in benzene solution (acetic anhydride : : benzene, 20 : : 80, v/v). Its specific activity was determined according to a modification of the method of Kliman and Peterson (7) by acetylation of cortisol, and measurement of pure cortisol acetate-^{14}C by absorption in ethanol at 242 mμ.

2 mg of etiocholanolone were labeled with tritium by the Wilzbach procedure (8) (exposure to 3 c of tritium gas

for 14 days) at New England Nuclear Corp., after which the radioactivity in the sample was found to be 142 mc. Readily exchangeable tritium was removed by dissolving the steroid repeatedly in 500 ml of ethyl alcohol, followed by evaporation at 50°C. Further purification was achieved by paper chromatography in two different Bush-type systems (cyclohexane : : methanol : water, 10 : : 10 : 1 and decalin : nitromethane : : methanol, 2 : 1 : : 1). The dried eluate containing radioactive steroid was acetylated with a mixture of equal parts of acetic anhydride and pyridine for 16 hr at 37°C. The acetylation process was stopped by adding 1 ml of 20% alcohol. The sample was extracted with methylene chloride and chromatographed as the acetate in the decalin system. The eluted steroid acetate was taken up in 0.4 ml ethyl alcohol to which 1.6 ml of 0.1 N sodium hydroxide in 70% methyl alcohol was added. After hydrolysis for 18 hr at room temperature the free steroid was extracted into dichloromethane, the dichloromethane steroid solution was washed with 5 ml water; the extract was dried and chromatographed in the same two systems as above. The radioactive steroid was always chromatographed between two samples of etiocholanolane or etiocholanolone acetate, which were identified by spraying with a solution of meta-dinitrobenzene (22 g in 100 ml of ethyl alcohol) and 15% potassium hydroxide (Zimmerman reaction). The purified, tritium-labeled etiocholanolone was stored frozen in benzene. Its specific activity was determined by acetylating an aliquot with carbon 14-labeled acetic anhydride and carrying it through the assay procedure to be described in Methods. Its specific activity was found to be 650 mc/mmole.

Addition of tracer and extraction. Tritium-labeled etiocholanolone, 50,000 cpm, was added to 100 ml of plasma. After thorough mixing, 5 ml of 1 N sodium hydroxide was pipetted into the plasma; after further mixing, the plasma was extracted with 9 volumes of dichloromethane. The extract was washed with $\frac{1}{10}$ its volume of 0.1 N sodium hydroxide, and this procedure was repeated until the aqueous phase was clear. It was then washed twice with $\frac{1}{10}$ its volume of water, dried under an air stream at room temperature, and transferred to a tube and dried for spotting on the chromatoplate.

First chromatography. The unknown sample was taken up in dichloromethane : ethyl alcohol (1 : 1) and applied to a chromatoplate in a streak 4-cm-wide between two spots of known etiocholanolone. The plate was lowered into a tank containing diethyl ether. Ascending, absorption chromatography began immediately and was complete, in this and in all subsequent chromatographic steps, in about 45 min. The plate was removed from the tank, dried in air, and sprayed with rhodamine solution (2 g in 100 ml of ethanol) and then examined under ultraviolet light. Etiocholanolone has an R_f of approximately 0.23 in this solvent. It was identified as an intense orange-yellow spot on a dark yellow background. The area between the two reference standards was marked for elution. Care was taken not to elute beyond the front of the etiocholanolone area, as the "tail" of androsterone (5α-androstane-3α-ol-17-one) may be as little as 1 cm nearer the solvent front. The alumina was scraped from the plate into a tube and eluted by shaking with 5 ml of dichloromethane. After centrifugation at 1000 *g* for 10 min, the supernatant was transferred to a glass-stoppered tube and thoroughly dried under an air stream at 37°C. The sides of the tube were washed down with dichloromethane to concentrate the sample at the tip of the tube before acetylation.

Acetylation. 10 μliters of carbon 14-labeled acetic anhydride and 20 μliters of pyridine were added to each sample and the glass-stoppered tube was sealed with pyridine. After inspection to ascertain that the sample was dissolved, the tube was incubated at 37°C for 16–18 hr. The acetylation was stopped by addition of 1.0 ml of 20% ethanol in water, and the sample was extracted into 5 ml dichloromethane, The extract was washed twice with 1.0-ml portions of water and evaporated to dryness. 50 μg of etiocholanolone acetate was added as visible reference standard.

Second chromatography. The sample was spotted in one corner of an alumina plate and developed in a tank containing toluene. The plate was allowed to dry and then turned on its side and developed in the system, benzene : ethyl acetate, 95 : 5, in a direction perpendicular to that of the toluene run. The plate was sprayed with rhodamine, and the spot was located and eluted into a 50 ml glass-stoppered centrifuge tube. Etiocholanolone acetate has R_f values of 0.43 in toluene and 0.79 in the benzene : ethyl acetate system.

Sodium borohydride reduction. The dried sample was taken up in 0.2 ml of ethyl alcohol, and sodium borohydride (0.3 ml of a freshly prepared solution containing 2 g in 100 ml of 60% ethyl alcohol) was added. The tube was stoppered and allowed to stand for 30 min at room temperature, after which the reaction was stopped by adding 5 ml of 5% (w/v) acetic acid. The sample was extracted into 25 ml of dichloromethane, washed twice with 5 ml of water, and the extract evaported to dryness under air.

Third chromatography. The sample was spotted in one corner of an alumina plate and developed in a tank containing benzene : ethyl acetate, 95 : 5. After development, the plate was dried, turned on its side, and developed in chloroform. Etiocholane, 17-ol,3 acetate has R_f values of 0.23 in the benzene : ethyl acetate system and 0.77 in chloroform. The eluate was transferred to a counting vial, evaporated to dryness, and taken up in 10 ml of phosphor solution for counting in a liquid scintillation spectrometer.

Counting and calculations. Counting, by the "screening method" of Okita (9), was done first at a voltage low enough (840 v) so that an insignificant percentage of counts from ^3H are counted. The amount of ^3H was determined by subtracting from the total counts above background at the higher voltage the amount of ^{14}C appearing at the higher voltage. This in turn was determined from the ratio of the count from a ^{14}C standard at the higher voltage to that at the lower voltage. For statistical reliability, the ^3H : ^{14}C count ratio at the higher voltage should be greater than 1. By this method we have found a ^{14}C efficiency of 55% and an ^3H efficiency of 35%. Calculations were as follows:

μg of etiocholanolone in plasma sample

$$= \frac{c \cdot a}{(m - cr) \cdot s} \times \text{mol wt of etiocholanolone}$$

where c = cpm minus background at lower voltage (i.e., ^{14}C); a = cpm of etiocholanolone tracer added to plasma sample, at higher voltage (i.e., ^3H); m = cpm minus background at higher voltage (i.e., ^{14}C and ^3H); r = cpm of ^{14}C standard at higher voltage/cpm of ^{14}C standard at lower voltage; and s = specific activity of etiocholanolone tracer (cpm/μmole, ^3H).

Evaluation of method

The precision of the method was evaluated with 10 100-ml samples of pooled human plasma. Etiocholanolone was added to five of these. The mean value for the five replicates with added etiocholanolone was 0.70 μg ±0.055 (SEM). The mean of the five replicates without added etiocholanolone was 0.061μg ±0.006.

The accuracy of the method was evaluated with duplicate 100-ml samples of pooled human plasma. Approximately 0.8 μg of etiocholanolone was added to a control tube without plasma and to one of the plasma samples. The etiocholanolone content of the three samples was 0.11 μg (plasma alone), 0.86 μg (etiocholanolone alone), and 0.94 μg (plasma with etiocholanolone added).

Over-all recovery in the method, as indicated by the per cent of added ^{3}H which was found in the final sample, has ranged between 2–25%, with a mean recovery of 7%. In the initial extraction step, as much as 50% of the added ^{3}H counts were lost. There was a consistent blank value of 6–8 cpm ^{14}C above background. Whether this is significant in a particular determination depends upon the amount of etiocholanolone in the plasma sample and the recovery obtained. When that amount was 0.1 μg or less and the recovery was 5% or less, the blank value was significant. When either the amount of etiocholanolone or the recovery was increased, the contribution of the blank became less significant. The amount of "etiocholanolone" was determined in a 38-ml plasma sample from a woman with Addison's disease and was found to be 0.000–0.003 μg. This apparent "biological blank" compares favorably with a reagent blank of 0.005 μg.

The specificity of the method was evaluated by addition of ^{19}C steroids resembling etiocholanolone. 5 μg of either testosterone, androsterone, or dehydroepiandrosterone was added to one of three 100-ml aliquots of pooled human plasma which had been previously found to contain 0.061 μg of etiocholanolone/100 ml. The values for etiocholanolone were 0.051, 0.073, and 0.057 μg, respectively, for the three aliquots. Within the precision of the method, these values do not differ from the value of 0.061 μg for the plasma alone.

Evidence for radiochemical purity of the ^{3}H etiocholanolone tracer was sought by derivative-formation and chromatography. A sample of pure etiocholanolone was acetylated with ^{14}C-labeled acetic anhydride and the radioactive steroid acetate purified by paper chromatography in the systems, cyclohexane : : methanol : water, 10 : : 10 : 1, and decalin : nitromethane : : methanol, 2 : 1 : : 1. After this purification, a paper chromatogram was run on a radiochromatogram strip counter, and this showed a single radioactive peak without "shoulders." A sample of the ^{3}H etiocholanolone tracer was acetylated with acetic anhydride and then combined with a sample of purified etiocholanolone acetate ^{14}C. If the ^{3}H etiocholanolone were radiochemically pure, the two steroid acetates should represent a single species labeled with ^{3}H and ^{14}C, and the $^{3}H : {}^{14}C$ ratio should remain constant through subsequent procedures. The combined samples were carried through one two-dimensional thin-layer chromatography, acetylated, and then carried through two more two dimensional thin-layer chromatographic steps. $^{3}H : {}^{14}C$ ratios did not change significantly with purification (Table I). Aliquots of four samples at the end of the assay procedure were counted and the remainders of the samples were acetylated to form the etiocholane-3,-17 diacetate and chromatographed on two, two dimensional alumina thin-layer chromatography plates in toluene (Table I). After elution and counting, the mean $^{3}H : {}^{14}C$ ratio was 0.090, as compared to 0.076 obtained at the end of the assay procedure.

TABLE I

^{3}H:^{14}C Ratios of Etiocholanolone Acetate Showing Radiochemical Purity of Etiocholanolone Tracer

Sample	Two-dimensional system No.		
	2	3	4
A	0.246	0.086	0.094
B	0.200	0.061	0.097
C	0.231	0.076	0.084
D	0.199	0.079	0.085

Subjects

21 samples of 250-ml of heparinized plasma were obtained by plasmaphoresis from 10 normal women and 10 normal men (age range, 21–55 yr). Nine of these plasma samples were obtained in the morning and the others in the afternoon. One plasma sample from a woman with Addison's disease was also studied.

40 patients with histories of illnesses characterized by recurrent fever as a major symptom comprised the patient group (10). All but two of these patients were hospitalized for study of recurrent fever at the NIH; one was seen in consultation; one patient was hospitalized elsewhere, and the plasma was sent to us. 20 of the patients (16 men, 4 women, ages 17–55 yr) had familial Mediterranean fever (FMF), characterized by recurrent episodes of fever, peritonitis, and/or pleuritis. The other 20 patients (14 men, 6 women, aged 15–60 yr) were referred as cases of "periodic fever" or recurrent fever of unknown etiology. Whereas all of these 20 patients had had episodes of fever for at least a year before admission, definitive diagnoses were later established in fourteen of these patients. The diagnoses included recurrent urinary tract infection, hypothalamic disorders, granulomatous disease of the liver, regional enteritis, tuberculosis, carcinoma of the colon, and juvenile rheumatoid arthritis. Complete clinical descriptions of these patients will be published elsewhere.

Heparinized blood was obtained by plasmaphoresis during febrile episodes. The blood was drawn into plastic bags in ice, centrifuged at 4°C, and the plasma was immediately frozen at −20°C; the red blood cells were reinfused into the patient. In almost all cases, a second plasma sample was obtained when the patient was afebrile at the same time of day as that sample obtained when the patient was febrile. The plasma was kept frozen until analyzed. All determinations were done on 100 ml of plasma, except two determinations which were done on 72 and 52 ml of plasma.

TABLE II

Plasma Concentrations of Unconjugated Etiocholanolone in 20 Normal Subjects

No. of samples	Etiocholanolone concentration			Significance
	Mean	SEM	Range	
	μg/100 ml			
Males (10)	0.035	±0.004	(0.016–0.064)	
Females (11)*	0.041	±0.005	(0.017–0.074)	$P \geq 0.3$‡
a.m. (9)	0.041	±0.005	(0.021–0.074)	
p.m. (12)*	0.036	±0.004	(0.017–0.064)	$P = 0.5$

Over-all grand mean ±SEM = 0.038 ±0.003.
* 2 samples drawn at different times from the same donor.
‡ *t* test, unpaired.

TABLE III

Plasma Concentrations of Unconjugated Etiocholanolone in 40 Patients with Recurrent Fever

Subjects	State	No. of samples	Etiocholanolone concentrations			Significance
			Mean	SEM	Range	
				μg/100 ml		
FMF*	Afebrile	19	0.115	±0.027	0.018–0.530	
FMF	Febrile	18	0.105	±0.024	0.030–0.462	$P = 0.5$
Recurrent fever	Afebrile	19	0.077	±0.012	0.023–0.218	
Recurrent fever	Febrile	20	0.105	±0.034	0.039–0.718	$P = 0.5$

* FMF = familial Mediterranean fever.

RESULTS

1. Normal Subjects. The results of the analysis of samples obtained from the normal volunteers are presented in Table II. The mean plasma concentration of unconjugated etiocholanolone in the 10 women (11 samples) was 0.041 μg/100 ml ±0.005 (SEM); the mean for the 10 men was 0.035 ±0.004. The mean values do not differ significantly ($P = 0.3$, Student's t test). The mean plasma concentrations for morning and afternoon specimens were 0.041 ±0.005 and 0.036 ±0.004, respectively. These values do not differ significantly ($P = 0.5$). Since there was no significant difference between men and women or between morning and afternoon specimens, the mean for the entire normal population may be given as 0.038 ±0.003 μg/100 ml.

The plasma from a young women with documented Addison's disease was found to contain from 0.000 to 0.008 μg/100 ml of unconjugated etiocholanolone.

2. Patients. The results of the samples obtained from the 40 patients are analyzed in Table III. The mean plasma-free etiocholanolone concentration of the FMF patients when afebrile was 0.115 ±0.027 and when febrile, 0.105 ±0.024 μg/100 ml. The mean concentration for the recurrent fever patients when afebrile was 0.077 ±0.012 and when febrile, 0.105 ±0.034 μg/100 ml. Neither of these differences was statistically significant. The mean of all patients when afebrile was 0.096 ±0.015 and when febrile, 0.106 ±0.021 μg/100 ml. The over-all mean of all the samples from the patients was 0.101 ±0.040 μg/100 ml. This mean value is significantly higher than normal ($P < 0.001$).

Of 29 pairs of samples from patients, the higher value of plasma unconjugated etiocholanolone occurred during the febrile episode in fifteen instances, during the afebrile period in fourteen instances. Of eighteen samples with values above 0.100 μg/100 ml, only ten were drawn during fever. The highest value recorded, 0.718 μg/100 ml, was obtained during fever in a 60 yr old man with carcinoma of the colon; after removal of the primary carcinoma, the patient remained afebrile until 8 months later when he died.

DISCUSSION

Despite the great interest aroused by the original report of "etiocholanolone fever" in 1958 (3), very little has been done to clarify the relationship between etiocholanolone and spontaneously occurring fever. This may be attributable largely to the difficulty inherent in the measurement of plasma-free steroids appearing in minute concentrations. The colorimetric method of Cohn, Bondy, and Castiglione, for example, could not detect values below 0.5 μg/100 ml (4) (Table IV). Kirschner, Lipsett, and Collins, using gas chromatography were able to detect lower concentrations than those previously detected (11) (Table IV). With the development of more sensitive and reproducible techniques for the measurement of such steroids, the possible role of etiocholanolone in spontaneous fevers has been reevaluated. Double-isotope dilution methods offered a clear advantage over previously available colorimetric ones, and we report such a method here; a similar method was developed by Gandy and Peterson (12). It is clear (Table IV) that such methods allow a further definition of the lower limit of normal.

In a recent review by Bondy, Cohn, and Gregory, thirteen previously reported cases of "etiocholanolone fever" were discussed (5). The determinations of etiocholanolone had been done by the authors themselves in eleven of these; in the twelfth the datum was derived from a personal communication; in the thirteenth, from the publications of another laboratory that used colorimetric methods. These thirteen patients were divided into three groups, viz., two with virilizing congenital adrenal hyperplasia, six with familial Mediterranean fever, and five with a miscellany of disorders including Hodgkin's disease and adrenal carcinoma. It is noteworthy that the groups of patients reported in the present study are strikingly similar to those reported by others (5).

TABLE IV

Normal Concentrations of Plasma Unconjugated Etiocholanolone

Authors	No. of normals	Plasma concentration
		μg/100 ml
Cohn, Bondy, and Castiglione (4)	6 (4 men, 2 women)	0.5–1.2
Kirschner, Lipsett, and Collins (11)	7 men	0.62 ±0.17
	4 women	0.31 ±0.02
Gandy and Peterson (12)	22 men	0.070 ±0.014
	6 women	0.056 ±0.009
George, Wolff, Diller, and Bartter	10 men	0.035 ±0.004
	10 women	0.041 ±0.004

In 1966 Huhnstock, Kuhn, and Oertel reported three patients with recurrent fever of unknown etiology in whom plasma etiocholanolone concentrations were increased during episodes of fever, as compared to interim values (6). The methods employed were colorimetric ones and the normal range was not given. The patients in the present study fall into groups similar to those in previous reports (5, 6), except that none had adrenal hyperplasia. Of the thirteen patients reported by Bondy et al., eight had blood drawn for etiocholanolone when they were afebrile, and of these, three had the abnormally high plasma values of 1.9, 2.0, and 28.7 μg/100 ml (5). The last value was obtained from one of the patients with virilizing adrenal hyperplasia (13–15) in whom a measurement was never made while he was febrile. It is noteworthy that this value of 28.7 μg/100 ml, which followed infusion of ACTH, is the highest value ever reported. One would expect this patient to have been febrile indeed, if the concentration of plasma unconjugated etiocholanolone were directly related to fever.

It is well established that etiocholanolone is a potent pyrogen when administered intramuscularly to man. That plasma concentrations need not be directly related to fever is evident from two principal observations. First, it is well-known that the half-time for clearance of etiocholanolone from plasma is very short–about 20 min (5, 16, 17), although there is a latent period of from 6 to 8-hr between administration of the steroid and the onset of fever (1, 2). Thus it was reported by Kappas and Palmer (18) that, in patients with fever induced experimentally with etiocholanolone, there was no temporal relationship between plasma concentration of steroid and maximum temperature elevation; indeed, most of the steroid had been excreted in the urine by the time the temperature had reached its peak. Secondly, a local tissue response is probably required for development of fever from exogenous etiocholanolone, since hydrocortisone suppresses the fever of administered etiocholanolone only when it is injected at the same site (19). Thus the pathogenesis of experimental etiocholanolone fever appears to require an inflammatory response, and/or the release of a secondary (possibly leukocytic) pyrogen (10, 20).

An interesting finding in the present studies was the over-all mean value of plasma etiocholanolone in the patients as opposed to the normal subjects: the mean for the patients, whether febrile or afebrile, was significantly higher ($P < 0.001$) than that in the normals. This finding is unexplained; it may represent a nonspecific response to illness. The underlying disease, for example, might activate tissue β-glucuronidase or inhibit UDP glucuronic acid transferase activity; both actions might increase circulating free etiocholanolone (21). Alternatively, this observation may be relevant to the basic underlying pathogenesis of these diseases. Clearly the observation deserves further study, and the methodology described in this paper may serve to stimulate future investigations in this area.

Although we report some values for plasma etiocholanolone above the normal range, we have not demonstrated any relationship whatsoever between the plasma concentration and the presence of fever. Thus, it appears appropriate to reconsider the use of the term "etiocholanolone fever" and to question whether such a syndrome really exists.

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