THE PRESENCE OF A POSSIBLE CAFFEINE–STEROID COMPLEX IN HUMAN URINE*

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INTRODUCTION

Observations have been reported in the past[1–5] of an ultraviolet absorbing compound from various sources which has solubility characteristics similar to that of steroids. We have observed that such material can be extracted from human urine and that on occasion it can contain radioactivity when a subject is infused with cortisol-4-^{14}C. Despite the fact that radioactivity can be incorporated into the ultraviolet absorbing region, there were indications that the main part of this material was not a steroid. It therefore was desirable to determine the nature of this compound since it would be of interest to those concerned with corticosteroid work.

METHODS AND MATERIALS

Chemicals, unless otherwise specified, were reagent grade and all solvents were freshly distilled. The caffeine used for comparison purposes was resublimed.

The extraction of small volumes of urine (1 l) was carried out according to the method of EIK-NES[6] for the extraction of Porter-Silber chromogens from plasma. For the extraction of a larger batch of urine (21 l), a liquid–liquid continuous extractor was used. Each batch of 3 l of urine was extracted with methylene chloride for 24 h. The pooled methylene chloride was washed with 0.1 *N* sodium hydroxide, followed by water, dried and evaporated. The residue was partitioned between benzene and water (1:5). The water layer was re-extracted with methylene chloride, dried and the solvent evaporated. This residue from the small volumes of urine of individuals who had received cortisol-4-^{14}C was chromatographed directly on paper, whereas that from 21 l of urine was recrystallized from methanol, ether and finally from ether–hexane (1:2). The yield was 18.6 mg. The material from either source showed the same characteristics and is hereafter referred to as U-272.

Paper chromatography of U-272 was carried out in BUSH B-5[7], ZAFFARONI[8] and in a SCHNEIDER–LEWBART system (No. 6)[9].

Acetylation was done in a mixture of acetic anhydride and pyridine (1:5)[8] allowing the mixture to stand for 18 h. The reaction was then stopped by the addition of 1 ml of methanol and after one hour, the solvents were evaporated at 40° under nitrogen.

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Hydrolysis in alkali was performed by heating for 10 min in a boiling water bath with 0.5 *N* sodium hydroxide and acid hydrolysis was done by treatment with 0.5 *N* sulfuric acid or with Amberlite IR-120 and heating at 45° for 16 h[10]. The aqueous medium was then extracted with ethyl acetate.

The paper chromatograms were scanned for radioactivity in a thin window Geiger strip counter and ultraviolet absorbing spots on the paper were localized by a Haines type scanner[11]. Ultraviolet spectra were determined by a Beckmann DK_2 automatic recording spectrophotometer and the infrared spectra read in a Perkin-Elmer Model 21 recording infrared spectrophotometer.

RESULTS

The running rates of U-272 in various paper chromatographic systems are listed in Table I. In the urine of four out of eleven normal subjects infused* with cortisol-4-^{14}C, radioactivity was found moving with an ultraviolet absorbing compound and this radioactivity could not be separated from the ultraviolet absorbing material in any of the chromatographic systems listed in Table I. The material had an ultraviolet absorption maximum at 272 mμ in methanol. It did not give a positive test with any of the following reagents: Sulfuric acid–ethanol[12], sulfuric acid[13], 2,3,5-triphenyltetrazolium chloride[8], dinitrophenylhydrazine[14] and BARTON's reagent[15].

When acetylation of U-272 was attempted the mobility on paper did not change and the radioactivity remained still with the ultraviolet absorbing area (Table I).

TABLE I

R_F OF U-272 AND ACETYLATED U-272 IN VARIOUS CHROMATOGRAPHIC SYSTEMS

Paper chromatographic systems used	*U-272*	*U-272 after acetylation*	*Caffeine*	*Cortisone*	*Corticosterone*
Bush B-5[7]	0.58	0.60	0.58	0.46	0.78
$CHCl_3$–formamide[8]	0.65	0.66	0.64	0.38	0.72
Benzene–formamide[8]	0.22				
Schneider-Lewbart[9]	0.84				

In a further effort to separate the radioactive material from the ultraviolet absorbing area, U-272 was hydrolyzed with alkali and acid. In alkali, the treatment was obviously too vigorous and the radioactive area now resided at the origin of the chromatogram in the Bush B-5 system and no ultraviolet spot was observed between the R_F's of cortisone and corticosterone. Upon hydrolysis of U-272 with sulfuric acid or following treatment[10] with IR-120, a radioactive area (U-272a) was detected with an R_F of cortisone and an ultraviolet spot (U-272b) was found which moved with an R_F similar to that of unhydrolyzed U-272 in Bush B-5 system of paper chromatography (Fig. 1).

The radioactive U-272a chromatographed like cortisone in both chloroform–formamide and in the Bush B-5 system. One third of U-272a was acetylated and chromatographed in benzene–formamide. The R_F was 0.27 which was identical with that of cortisone acetate run on a parallel strip. The remainder of the radioactive

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material was crystallized to constant specific activity after adding carrier cortisone. The results are given in Table II. The ultraviolet absorbing compound U-272b had, however, the same running rates on paper and the same ultraviolet maximum as before the acid hydrolysis.

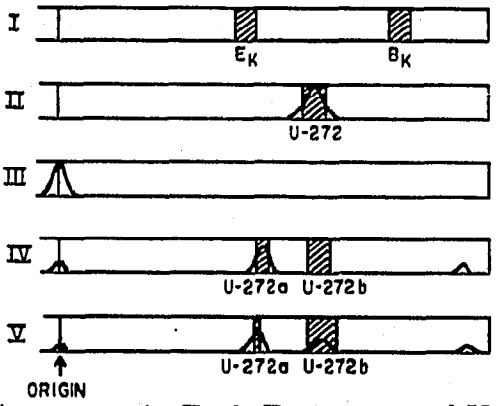

Fig. 1. Paper chromatographic pattern in Bush B-5 system of U-272 before and after hydrolysis. Shaded area represents ultraviolet absorption and the curve indicates radioactivity. (I) The relative mobilities of cortisone (E_K) and corticosterone (B_K); (II) mobility of U-272; (III) after hydrolysis with 0.5 *N* sodium hydroxide; (IV) after acid hydrolysis with 0.5 *N* sulfuric acid; (V) after treatment with Amberlite IR-120.

An elemental analysis of U-272b obtained after acid hydrolysis gave an empirical formula of $C_8H_{10}N_4$* which agrees with caffeine. The R_F's of caffeine in several systems of paper chromatography were similar (Table I). The melting point of U-272b and caffeine were identical (234.5–235.5°) and a mixed melting point determination showed

TABLE II

CRYSTALLIZATION TO CONSTANT SPECIFIC ACTIVITY OF THE RADIOACTIVITY FROM ACID-HYDROLYZED U-272

No. of crystallization	*c.p.m./mg*	*Solvent*
0	112	
	117	
1	108	Methanol
	111	
2	124	Ether–hexane (1:2)
	122	
3	126	Ethanol
	124	
4	123	Methanol
	117	

the two compounds to be identical. The melting point of the picrate of caffeine and of U-272b were also the same (144–145°) and admixture of the two did not lower the melting point. The ultraviolet and infrared spectra of U-272b were identical in all respects with those of caffeine.

Finally, urine from an individual maintained on a caffeine-free diet yielded no U-272 when extracted and processed as outlined.

* Done by Elek Micro Analytical Laboratories, Los Angeles, Calif.

DISCUSSION

The finding of caffeine in urine or in plasma is not new[16, 17], but it appeared interesting since in some cases steroid was observed bound to caffeine. The complexing of steroids with other substances which absorb ultraviolet light and/or which migrate on paper chromatograms like the corticosteroids have been observed by other workers. BERLINER *et al.*[2] have found that placental extracts contain a substance similar to caffeine. STAPLE *et al.*[3] reported a complexing of progesterone, sometimes as much as 75%, with a substance in beef adrenal. This complex migrated on paper between cortisone and corticosterone. The progesterone could be readily liberated by treatment with dilute acid.

The complexing of a number of steroids with purines has been studied by MUNCK, SCOTT AND ENGEL[18] and the postulate of ENGEL[19] on the physiological significance of such a complex is interesting. The physiological importance of the steroid–caffeine complex found in our investigation is questionable since complexing was not found to be general. In fact only about 36% of the normal individuals studied showed this phenomenon. However, that such a complex can be found in human urine and also that caffeine migrates similar to corticosteroids on paper chromatograms suggests that care must be exercised in the interpretation of quantitative and qualitative estimation of these steroids extracted from biological fluids and subjected to ultraviolet measurements only.

SUMMARY

A compound which behaves like a steroid in terms of solubilities and chromatographic behaviour in paper systems commonly used for the separation of C-21 steroids has been isolated and characterized. This compound was shown to be caffeine. Further, in four out of eleven cases where cortisol-4-^{14}C had been infused, cortisone-^{14}C was found bound to caffeine.

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