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53. Toshihiro Nishina, Ikuko Hariya, and Michiya Kimura : Fundamental Studies on Clinical Chemistry. X.*1 A New Micro Method for the Colorimetric Determination of Urinary Dehydroepiandrosterone.

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Abnormally high excretion values of 17-ketosteroids are often found in patients with interstitial cell tumours, masculizing ovarian tumors, and adrenal hyperfunction. In adrenocortical tumors the proportion of dehydroepiandrosterone (DHEA) may, often but not always, be extensively increased in relation to the rather normal levels of other 17-ketosteroids such as androsterone and etiocholanolone.

DHEA differs from the other principal constituents of the androgen series in three significant respects, each of which may provide a basis for the determination. In contrast to androsterone and etiocholanolone, the substance is first in the 3β-hydroxysteroid series, secondly unsaturated between C_5 and C_6, and thirdly excreted mainly as a sulfate conjugate. Several procedures for the determination of urinary DHEA are available. Most popular colorimetric methods based upon the Zimmermann reaction are employed usually with the fractionation by chromatography[1,2] or the precipitation of digitonide.[3,4] Polarographic methods were also reported,[5,6] in which the half-wave potential of Girard's hydrazone of androst-4-en-3,17-dione derived from DHEA by Oppenauer oxidation were measured. Recently, micro methods have been developed by using gaschromatography[7] and thin-layer chromatography.[8]

In the previous papers[9,10] of this series, colorimetric procedures for the determination of serum cholesterol were described, in which *p*-nitrophenylhydrazone of 4-en-3,6-dione steroid converted from cholesterol by the chromic acid oxidation was determined colorimetrically at 580 mμ in dimethylformamide (DMF) with alkali. Since DHEA also has the partial structure of 5-en-3β-ol, it was assumed that the same principle should be applicable to the determination of this steroid. By the oxidation procedure outlined above, androsterone and etiocholanolone may also be converted into the corresponding saturated compounds, androstane-3,17-dione and etiocholane-3,17-dione. In an earlier study[11] on the colorimetric determination of 4-en-3-one steroids with *p*-nitrophenylhydrazine based on the fact that only 4-en-3-one steroids such as progesterone and testosterone produced reddish purple colors showing maximum absorption at 540 mμ under the established procedure, the saturated 3,17-dione steroids such as mentioned above gave a maximum absorption at 520 mμ in a degree of about one-tenth that of

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*1 Part Ⅸ : Bunseki Kagaku, **14**, 125 (1964).

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4-en-3-one steroids and favourably no appreciable extinctions at 580 mμ. It has, furthermore, been shown that steroids having a ketogroup in other positions developed no color under the same conditions. These facts suggested the possibility of the specific determination of DHEA without preliminary fractionation of 17-one steroids accompanied in urine.

The present report describes a micro method for the determination of 0~50 μg. of urinary DHEA, which is based on these observations described above.

Reagents and Apparatus

Kiliani's oxidizing reagent : Prepared by dissolving 6.7 g. of CrO_3 in H_2O, adding 5.4 ml. of conc. H_2SO_4 and diluting the mixture to 25.0 ml. with H_2O.[10]

Methanol free from carbonyl compounds : Prepared with refluxing 2.0 L. of MeOH containing 0.2 g. of 2,4-dinitrophenylhydrazine and 0.1 ml. of conc. H_2SO_4 for 2 hr. and distilling. Should be stored in brown bottle.

p-Nitrophenylhydrazine reagent (PNPH) : In a 50 ml. volumetric flask, 10.0 mg. of *p*-nitrophenylhydrazine (m.p. 157~158°) is dissolved with carbonyl-free MeOH, adding 1.0 ml. of conc. HCl and make to volume with MeOH. Should be prepared daily.

1*N* Sodium hydroxide solution : Aqueous solution is prepared from conc. NaOH solution, removed precpitate of carbonate by filtration through a glass filter.

3% Sodium bisulfite solution : Aqueous solution should be prepared daily.

All other reagents and solvents were used with further purification.

Reaction vessels : Test tubes approximately 15×150 mm. fitted with 15/25 standard-taper ground-glass stopper were used. These could be readily attached to a reduced pressure still head fitted with 15/25 standard taper joint for distillation of aliquot of the sample to dryness.

Spectrophotometer : Absorption spectra and absorbances were measured on a Hitachi EPS 2U Recording Spectrophotometer and a Hitachi EPU 2A Photo-Electro Spectrometer, respectively.

Standard Procedure

Hydrolysis and Extraction

A 24 hr. specimen of urine is collected. A portion is taken which may be expected to contain between 40 and 50 μg. of DHEA (usually about 50 ml. of urine was used) and is acidified to pH 1.0 with 50% H_2SO_4 and brought to salt concentration of 20% with NaCl and extract once with the same volume of EtOAc. The organic phase is dried with 6 g. of anhydrous Na_2SO_4 for every 100 ml. of solution. The subsequent filtrate is then kept at 30° for 20 hr., extracted with 10 ml. of aqueous 10% KOH, twice with 10 ml. of H_2O. The washed solution is dried over Na_2SO_4 and evaporated to dryness. The dried residue is dissolved in 10 ml. of ethylene dichloride. Approximately 20 pellets of NaOH are added and the bottle is stoppered and shaken for 10 min. The solution is filtered through filter paper. A pair of 4 ml. of the ethylene dichloride solution is transferred into two test tubes marked A and B respectively and evaporated to the last trace of solvent in a water bath at 40° under reduced pressure.

Colorimetric Procedure

To test tube A, 3.0 ml. of acetone is added and this tube is cooled in an ice-water bath to bring the temperature of solution to 0~2° and 0.1 ml. of the Kiliani's reagent is added. The solution is kept at 0~2° for 20 min. The excess of oxidant is destroyed by adding 2 ml. of freshly prepared 3% solution of $NaHSO_3$, adding 8 ml. of CCl_4, stopping the tube and is shaken throughly. The aqueous layer is drawn and discarded. The process of washing with 2 ml. of H_2O is repeated three times and is removed the solvent at the vaccum. Two ml. of dry CCl_4 is added and evaporated again on a water bath under reduced pressure for the removal of the last trace of acetone.

To each test tube A and B respectively, 1.0 ml. of PNPH reagent is added and mixed well. The solution is kept at 60° for 60 min. After cooling for several minutes, 0.5 ml. of 1*N* NaOH and 4 ml. of DMF to each tube with gentle shaking and is filtered through filter paper. The content of each tube is transferred to colorimeter cuvette (1 cm.) and read the per cent transmission or absorbance against reagent blank at 590 mμ, which is obtained from 3.0 ml. of acetone by the exactly same procedure described above.

The following correction formula is applied to allow for interfering chromogen and free 4-en-3-one steroids in the extract :

$$\text{Corrected extinction at } 590\ \text{m}\mu = E_{\text{A at } 590\ \text{m}\mu} - E_{\text{B at } 590\ \text{m}\mu}$$

The amount of DHEA contained in the extract is obtained by reading off the calibration curve using the corrected extinction values. The calibration is linear from 0 to 50 μg. (Fig. 2). If the amount of steroid is greater than 50 μg., then smaller aliquot of ethylene dichloride extract should be used. If equivalent amount of steroid is less than 5 μg., a larger aliquot of extract should be used.

Results and Discussion

A study on the determination procedure was made using 30~50 μg. of DHEA. It was drawn that almost same colorimetric procedure with that of serum cholesterol previously reported[10] should be applied also in this case except an additional filtration of color solution before photometry for occasional precipitation. The standard procedure on 50 μg. of DHEA gave an absorption curve (I) showing maximum absorption at 590 mμ where the blank solution against DMF gave an almost negligible absorption as shown on the curve (III) in Fig. 1. The colored solution given by the standard procedure showed reasonable stability so that the absorbance was kept steady for at least 120 min. The Lambert-Beer relationships were valid for these solutions containing 0~50 μg. of DHEA (Fig. 2).

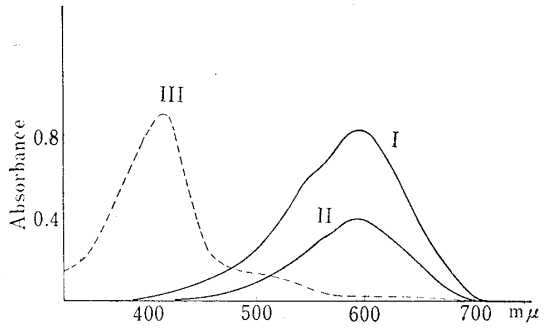


Fig. 1. Absorption Curves of the Colored Solution by the Standard Procedure

I : 50 μg. of dehydroepiandrosterone
II : urine extract
III : reagent blank against dimethyl formamide

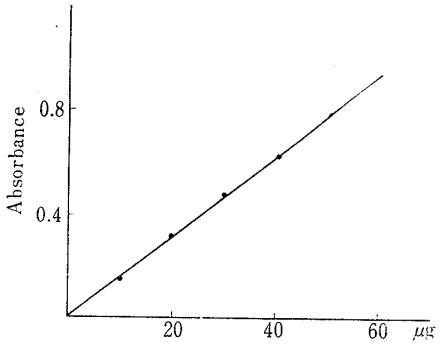


Fig. 2. Calibration Curve

Specificity for DHEA and Reactivity of Other Steroids (Table I)

Two kinds of colorimetric methods, the standard procedure (method A) and the direct coloration with PNPH reagent in alkaline DMF (method B), were carried out on each 50 μg. of the various types of steroid such as given in Table I. The results are summarized in this table which reveal that, of the main urinary 17-oxo steroids, DHEA (1) alone can give definite absorption at around 590 mμ so that the standard procedure may be specific for this steroid in the presence of androsterone (2), epiandrosterone (3), and etiocholanolone (4). On this procedure it can also be assumed from Table I that: (a) when the oxidation by chromic acid is lacking (method B), no such 17-one steroids as (1), (2), (3), and (4) can give any color; (b) by means of the oxidation, the saturated 3-hydroxy steroids such as (2), (3), and (4) are converted into the corresponding saturated 3,17-dione steroids; (c) since each of the diketosteroids such as androstane-3,17-dione (5) and 5β-androstane-3,17-dione (6) shows nearly same absorbance in both procedures (method A and B), no further oxidation undergo from the stage of these 3,17-diones.

The 5-en-3β-ol steroids such as (1), cholesterol (7), pregnenolone (8), and diosgenine (9) showed maximum absorptions at around 590 mμ in the standard procedure (method A.) While cholest-5-en-3-one (10) gave a maximum absorption at 585 mμ showing almost same extinction with that of (7) in the method A, the wavelength of the maximum absorption shifted to 540 mμ in the method B, in which cholest-4-en-3,6-dione (11) gave

Table I. Colorations given by Two Methods (A and B) for Various Types of Steroid

		λ_{max} mμ	E_{max}	E_{590}
Dehydroepiandrosterone (1)	A	590	0.807	0.807
	B	negative	—	—
Androsterone (2)	A	520	0.128	—
	B	negative	—	—
Epiandrosterone (3)	A	520	0.115	—
	B	negative	—	—
Etiocholanolone (4)	A	520	0.120	—
	B	negative	—	—
Androstane-3,17-dione (5)	A	520	0.118	—
	B	520	0.123	—
5β-Androstane-3,17-dione (6)	A	520	0.115	—
	B	520	0.134	—
Cholesterol (7)	A	585	0.820	0.820
	B	negative	—	—
Pregnenolone (8)	A	590	0.900	0.900
	B	negative	—	—
Diosgenine (9)	A	585	0.619	0.619
	B	negative	—	—
Cholest-5-en-3-one (10)	A	585	0.790	0.790
	B	540	0.575	0.313
Cholest-4-en-3,6-dione (11)	B	585	0.845	0.845
Androsterone acetate (12)	A	negative	—	—
	B	negative	—	—
Testosterone (13)	A	540	0.810	0.610
	B	540	0.835	0.625
Cholest-4-en-3-one (14)	A	540	0.670	0.530
	B	540	0.691	0.528
Androst-4-en-3,17-dione (15)	A	540	0.845	0.622
	B	540	0.850	0.656
Cholestan-3-one (16)	A	520	0.083	—
	B	520	0.100	—

a maximum absorption at 585 mμ. Accordingly, it might be assumed from these observations that the standard procedure was specific for 5-en-3β-ol steroids which converted into the corresponding 4-en-3,6-diones through the oxidized intermediate 5-en-3-one steroids under the conditions settled in this procedure, just as observed in the previous study[12] on the oxidation of cholesterol.

Since, contrary to the 5-en-3-one steroids, the isomeric 4-en-3-one steroids such as testosterone (13), cholest-4-en-3-one (14) and androst-4-en-3,17-dione (15) gave scarcely any different wavelengths and extinctions at their maximum absorptions in both methods (A and B), no further oxidation to 4-en-3,6-diones from these isomers might be occured in the standard procedure (method A). However, it should be noted that 4-en-3-one steroids gave some absorbance at 590 mμ in this procedure. Although most of them are excreted usually in the form of glucuronide in urine, the correction formula described above should, therefore, be applied to the samples from biological origins.

Steroid having a substituted 3-hydroxyl group such as androsterone acetate (12) was stable for oxidation so that no color was available in the standard procedure, just as observed on cholesterol esters in the previous paper.[10]

Application of Standard Procedure to Urinary DHEA

Most of urinary 17-one steroids are androsterone, DHEA and etiocholanolone which are excreted in the forms of glucuronide and/or sulfate conjugate. Since DHEA is excreted mostly as a sulfate in urine, the hydrolysis and subsequent extraction in advance are necessary for the determination. The usual hydrolysis of sulfate as well

12) T. Nishina, M. Kimura: Yakugaku Zasshi, 85, 390 (1964).

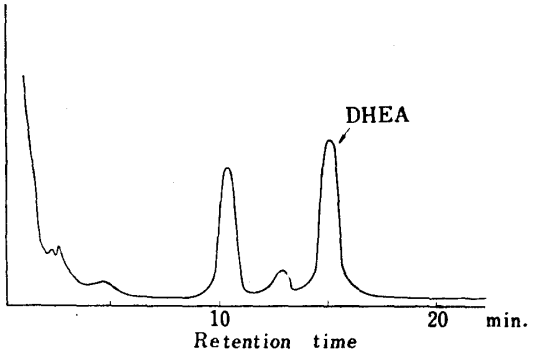
as glucuronide conjugate has been carried out commonly by using a strong acid at elevated temperatures and the consequent destruction of steroids has occured in some extent entailing considerable errors. Lieberman[13] and Nes[14] developed a novel method of solvolysis specific for sulfate conjugates in the presence of glucuronides, which was followed up by Eik-Nes[15] with the consequent recommendation. Since 4-en-3-one steroids which can come into reaction merely with PNPH reagent alone as mentioned above, are excreted in urine mostly as their glucuronides, this method of solvolysis seems to be particularly suitable for the determination of urinary DHEA.

Table II. Colorations given by Two Methods (A and B) for Urine Samples obtained through Various Purification Procedure

Before solvolysis \ After solvolysis		No treatment	Treatment with $ClCH_2 \cdot CH_2Cl$ and NaOH
Without extraction	A	0.900	0.572
	B	0.578	0.048
After extraction with CCl_4	A	0.319	0.262
	B	0.280	0.043
" $CHCl_3$	A	—	0.445
	B	—	0.069
" benzene		emulsion	—

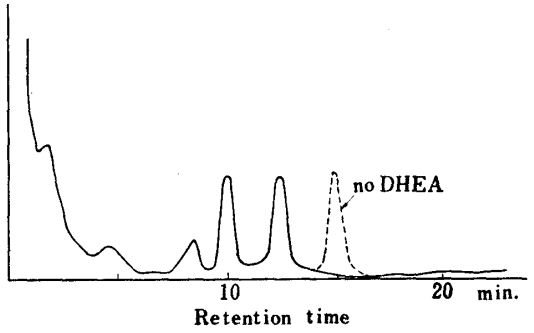
A : by the standard procedure.
B : by the direct coloration with PNPH reagent and alkaline DMF.

On the other hand, considerable absorbances by the method B were observed on the extract obtained after solvolysis of urine sample (Table II) and it was assumed consequently to be unfavourable to employ the standard procedure without preliminary purification. In anticipation of the prevention from the interference due to these chromogens which were assumed to be urinary pigments and free 4-en-3-one steroids, two procedures were attempted : (1) the shake-out of free 4-en-3-one steroids with such an organic solvent as carbon tetrachloride, chloroform or benzene from the urine samples before solvolysis; (2) the elimination of urinary chromogens by shaking the ethylene



A. Solvolysates under Standard Procedure.

The steroids obtained by solvolysis were converted into their trimethylsilyl ethers for gas chromatography.
Column conditions : 2.25 m.×4 mm. column; 2% CNSi(XF 1150) on 100/120 Gas chrom P; 206°; N_2 30 ml./min. at 2 kg./cm²; detector 260°; flash heater 240°.



B. After Acid-hydrolysis of Non-solvolysates

Fractions resisting solvolysis in the standard procedure was hydrolysed by conc. HCl as usual and trimethylsilylated.

Fig. 3. Gas Chromatographic Tracing of Human Male Urine following Solvolysis

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14) W. R. Nes, L. Segal, B. Segal : *Ibid.*, **235**, 3108 (1960).
15) J. C. DePaoli, E. Nishizawa, K. B. Eik-Nes : J. Clin. Endocr. & Metab., **23**, 81 (1963).

dichloride solution of the solvolysates with pellets of NaOH, which was developed successfully by Drector.[16] It was evident from the data of Table II that the solvent extraction before solvolysis was practically ineffective because of the formation of stable emulsions and that free 4-en-3-one steroids which have been recognized to be hardly extractable by use of strong alkali[17] were scarcely responsible for the blank value (by the method B). Although treatment with NaOH pellets in ethylene dichloride, on the contrary, seemed to be quite effective, there appeared still some blank value ($E \leq 0.05$) so that the correction formula described above should be used for the calculation.

According to the standard procedure thus obtained, the sample from 20 ml. of urine gave an identical absorption curve with that of DHEA showing maximum absorption at 590 mμ (Fig. 1). Gas chromatography (Fig. 3) of the extraction residue showed that the solvolysis of DHEA sulfate was complete as has been studied by Eik-Nes.[15]

Recovery Test

A series of definite amounts of DHEA was added to the urine samples obtained by the extraction procedure described above and the subsequent determination was carried out by the standard procedure. The results of this recovery test are summarized in Table III showing the mean recovery of 95.3%.

Table III. Recoveries of Added Dehydroepiandrosterone

Sample	DHEA added (μg.)	Found (μg.)	Recovery (%)	Sample	DHEA added (μg.)	Found (μg.)	Recovery (%)
1.	0	31.2	—	2.	0	19.2	—
	10	40.8	94.9		10	28.7	95.0
	20	50.7	97.5		20	37.1	89.5
	30	61.0	99.4		30	47.8	95.3
						mean	95.3

Conclusion

As in the case of cholesterol,[9,10] steroids having partial structure of 5-en-3β-ol such as DHEA, pregnenolone and diosgenin can also principally be determined by the colorimetric method in the standard procedure. By means of the combination of the solvolysis that is highly specific for sulfate conjugates in the presence of glucuronides, the standard colorimetric procedure with correction formula may be suitable for the specific determination of the amounts between 0~50 μg. of urinary DHEA sulfate in the presence of other ketosteroids such as androsterone, etiocholanolone, testosterone and androstenedione. The stability of the coloration is larger than usual methods.

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Summary

A new micro method for the colorimetric determination of dehydroepiandrosterone (DHEA) was studied and the standard procedure for the amounts of 0~50 μg. of the steroid was established. The principle of this method is based on the observation that DHEA in acetone is oxidized to androst-4-en-3,6,17-trione by the Kiliani's reagent and its *p*-nitrophenylhydrazone produced a stable color in an alkaline solution of dimethylformamide showing an absorption maximum at 590 mμ.

By means of the combination of the solvolysis with sulfuric acid in ethyl acetate, the urinary DHEA may quite specifically be determined in the presence of other steroids.

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