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56. Michiya Kimura, Toshihiro Nishina, and Takashi Sakamoto : Fundamental Studies on Clinical Chemistry. XI.*1 A New Micromethod for the Determination of General Ketosteroids using *p*-Nitrophenylhydrazine Reagent.

(*Faculty of Pharmaceutical Sciences, University of Hokkaido**2)

A highly sensitive spectrophotometric method for the determination of general ketosteroids was investigated. The development of chromophore with *p*-nitrophenylhydrazine reagent was achieved efficiently in alkaline dimethylformamide. Micro amounts (0~50 μg. for saturated ketosteroids; 0~25 μg. for Δ^4-3-ones) of steroids were determined by the standard procedure. One of the remarkable advantages is the possibility to estimate micro quantities of the saturated 3-ketosteroids such as cholestan-3-one, that has been almost impossible by the most published methods.

A discussion on the reactivities of different kinds of steroidal keto group for the method derived was also presented from the results of the study using forty oxosteroids.

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In the previous papers[1,2] of this series, it was shown that only Δ^4-3-ketosteroids such as progesterone and testosterone reacted rather specifically with the *p*-nitrophenylhydrazine-hydrochloric acid reagent to form hydrazones, which produced reddish purple colors showing absorption maximum at 540 mμ in an alkaline solution of dimethylformamide (DME). A specific method for the detrmination of Δ^4-3-ketosteroids (Method A) based upon this principle was presented subsequntly.[3] The larger molar ratio (160) of hydrochloric acid to *p*-nitrophenylhydrazine (PNPH) in the method A should be kept in order to show higher specificity for Δ^4-3-ketosteroids. When the ratio is reduced down to 2.0, on the contrary, the sensitivity was highly increased with a subsequent increase of the reagent blank losing the specificity for Δ^4-3-ketosteroids.

The present paper deals with a micro method for the determination of ordinary ketosteroids (Method B) derived from the above-mentioned finding, accompanied with an improvement on the purification of solvent used. A discussion on the comparison of these two methods A and B is also given with respect to the reactivity of different steroidal keto groups.

Experimental

Reagents and Apparatus——1. Ketosteroids : Steroids having carbonyl group in A- or B-ring in the cholestane series were prepared by the known methods and those proved to be pure on thin-layer chromatography and by elemental analysis were used; some of them were gifted from other laboratories, which are acknowledged elsewhere.

2. Carbonyl-free Ethanol : Reagent-grade alcohol (2.0 L.) with 2,4-dinitrophenylhydrazine (2.0 g.) and conc. H_2SO_4 (1.0 ml.) were refluxed and distilled. The same procedure was repeated once and redistilled.

3. HCl-Ethanol Solution : Reagent-grade conc. HCl (2.7 ml.) was made to 250 ml. with the carbonyl-free EtOH.

4. *p*-Nitrophenylhydrazine Reagent (PNPH) : After *p*-nitrophenylhydrazine (m.p. 157~158°; 20 mg.) was dissolved in a small amount of the carbonyl-free EtOH, the HCl-EtOH solution (2.0 ml.) was added and the solution was made to 50 ml. with the EtOH.

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*1 Part X : This Bulletin, **13**, 414 (1965).

*2 Nishi-5-chome, Kita-12-jo, Sapporo (木村道也, 仁科甫啓, 坂本 嵩).

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

2) *Idem* : This Bulletin, **12**, 521 (1964).

3) T. Nishina, Y. Sakai, M. Kimura : Steroids, **4**, 255 (1964).

5. 1*N* NaOH : Prepared as an aqueous solution from concentrated carbonate-free NaOH solution, removing precipitate by filtration through a glass filter.

6. Dimethylformamide (DMF) : Reagent-grade DMF was purified by distillation under reduced pressure in an all-glass apparatus.

7. Spectrophotometer : Hitachi Model EPU-2A and Hitachi Self-Recording Model EPS-2U Spectrophotometers were used.

Development of Method——1. Absorption Spectra : EtOH solutions (0.5 ml.) containing cholestan-3-one (50 μg.) and progesterone (50 μg.), respectively were treated under the standard procedure. Absorption spectra of these solutions showed maxima at 520 mμ and 540 mμ for cholestan-3-one and progesterone, respectively (Fig. 6).

2. Concentration of PNPH : Under the standard procedure varying with the concentration of PNPH, almost constant absorbance was observed within the range of 0.3 to 0.6 mg. per ml. The higher the concentration of PNPH, the more increased the reagent blank and 0.4 mg. per ml. was set as an optimum.

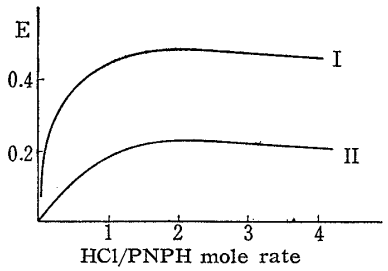

Fig. 1. Effects of Concentration of Hydrochloric Acid upon the Reaction of *p*-Nitrophenylhydrazine with 30 μg. of Ketosteroids

I : cholestan-3-one
II : pregnenolone

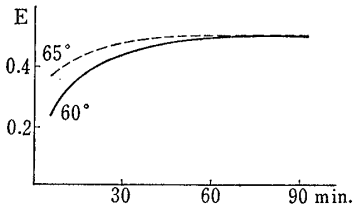

Fig. 2. Effect of Temperature on Absorbance at 520 mμ. from 30 μg. of Cholestan-3-one

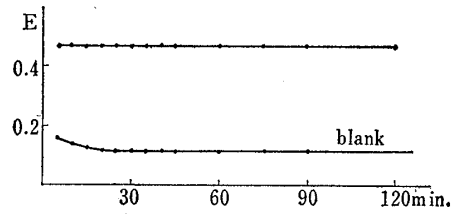

Fig. 3. Stability of the Colored Solution produced from 30 μg. of Cholestan-3-one by the Standard Procedure

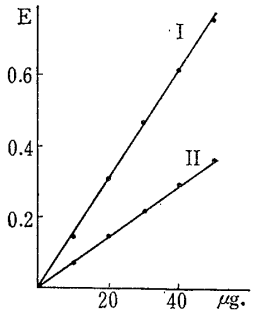

Fig. 4. Calibration Curves by the Method B

I : cholestan-3-one
II : pregnenolone

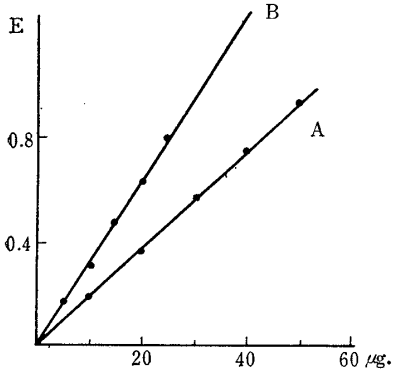

Fig. 5. Calibration Curves of Progesterone by the Methods A and B

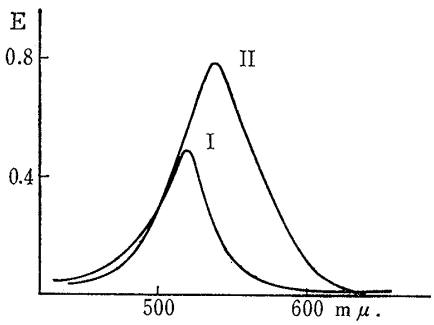

Fig. 6. Absorption Curves of Colored Solution by the Standard Procedure

I : 30 μg. cholestan-3-one
II : 25 μg. progesterone

Table I. Absorption Maxima and Apparent Molar Extinction Coefficients of the Colored Solution produced from Various Oxosteroids by the Method A and B

Compound	A			B		
	λ_{max} mμ	ε′ [b]	Index [a]	λ_{max} mμ	ε′ [b]	Index [a]
1) Cholestan-1-one [c]	negative	—	—	520	1600	5
2) Cholestan-2-one [d]	520	7900	26	520	30100	99
3) Cholestan-3-one [e]	520	6000	20	520	37400	123
4) Cholestan-3-one [f]	520	5400	18	520	37600	123
5) Cholestan-4-one [g]	520	2300	8	520	5600	18
6) Cholestan-3β-ol-6-one acetate [h]	520	2900	9	520	12200	40
7) Cholestan-7-one [i]	negative	—	—	520	19000	62
8) Cholestan-3β-ol-7-one acetate [j]	〃	—	—	520	13700	45
9) 5α-Pregnan-11-one	〃	—	—	negative	—	—
10) 3α,17α,20-Trihydroxy-5β-pregnan-11-one	〃	—	—	〃	—	—
11) Hecogenin	〃	—	—	520	5200	17
12) 12-Ketopregnane-3,20-diol 3-acetate	520	11600	38	520	44000	144
13) 3β-Hydroxypregn-16-en-12,20-dione acetate	520	5200	17	520	20000	66
14) 3β,20-Dihydroxy-14β,17α-pregnan-15-one 3-acetate	negative	—	—	520	5000	18
15) 3β-Hydroxyandrostan-16-one acetate	520	1300	4	520	7500	24
16) Androsterone	negative	—	—	520	4500	15
17) Pregnenolone	〃	—	—	520	10700	35
18) Cholest-2-en-1-one [k]	〃	—	—	540	1000	3
19) Cholest-1-en-3-one [l]	540	15000	49	540	36700	120
20) Cholest-4-en-3-one [m]	540	30500	100	540	44500	146
21) Cholest-4-en-6-one [n]	540	2500	8	540	8200	27
22) Cholest-5-en-7-one [i]	negative	—	—	540	7100	23
23) 3β,20-Dihydroxypregn-9(11)-en-12-one diacetate	540	2300	7	540	4900	16
24) Progesterone	540	30200	99	540	49800	163
25) Corticosterone	540	32900	108	540	53500	175
26) Desoxycorticosterone	540	29000	95	540	51600	169
27) Hydrocortisone	540	33300	109	540	45100	148
28) Cortisone	540	26600	87	540	43900	144
29) Prednisolone	565	15600	51	565	25600	84
30) Prednisone	565	15700	51	565	27600	90
31) Cholest-4,6-dien-3-one [o]	570	44600	146	570	57300	192
32) Cholest-3,5-dien-7-one [i]	570	1100	4	570	20000	66
33) 7-Ketocholesteryl acetate [p]	negative	—	—	555	3500	11
34) Cholestane-3,6-dione [q]	520	2100	7	520	46100	151
35) Cholest-4-ene-3,6-dione [r]	590	37900	124	620	40500	131
36) Cholest-4-ene-3,6-dione 6-enol ethyl ether [s]	590	41000	134	620	46100	151
37) 3β-Hydroxy-14β,17α-pregnane-15,20-dione acetate	520	1030	3	520	8900	29
38) Cholest-3-en-2-one-3-ol tosylate [d]	negative	—	—	570	21400	70
39) 2α-Bromocholestan-3-one [t]	520	1300	4	540	19200	63
40) 4-Br-pregnane-17α,21-diol 3,11,20-trione 21-acetate	540	6900	23	540	30600	110

[a] The relative absorbance at maximum was represented as cholest-4-en-3-one index, when the observed value with an equimolar solution of this ketosteroid by the method A was taken as 100.
[b] $\varepsilon' = E/l \times c$, where E: optical density, l: light path of cell, c: concentration on moles per liter.
[c] C.W. Shoppee, S.K. Roy, B.S. Goodrich: J. Chem. Soc., **1961**, 583.
[d] L. Ruzicka, P.A. Plattner, M. Ferrer: Helv., **27**, 524 (1944). [e] W.F. Bruce: Org. Synth. Coll. Vol., **2**, 139.
[f] H. Grasshof: Z. physiol. Chem., **223**, 249 (1934).
[g] N.L. Allinger, M.A. Darooge, R.B. Hermann: J. Org. Chem., **26**, 3628 (1961).
[h] H.C. Brown, C.P. Carg: J. Am. Chem. Soc., **83**, 2951 (1961). [i] A. Nickon, J.F. Bagli: *Ibid.*, **83**, 1498 (1961).
[j] O. Wintersteiner: *Ibid.*, **65**, 1503 (1943).
[k] C. Djerassi, D.H. Williams, B. Berkoz: J. Org. Chem., **27**, 2205 (1962).
[l] G.F.H. Green, A.G. Long: J. Chem. Soc., **1961**, 2532. [m] L.F. Fieser: Org. Synth., **35**, 43 (1955).
[n] J. Broome, B.R. Brown, A. Roberts, A.M.S. White: J. Chem. Soc., **1960**, 1406.
[o] E.J. Agnello, G.D. Laubach: J. Am. Chem. Soc., **82**, 4293 (1960).
[p] L.F. Fieser: *Ibid.*, **75**, 4386 (1953). [q] A. Windaus: Ber., **39**, 2249 (1906).
[r] Reference 1). [s] A. Windaus: Ber., **40**, 257 (1907).
[t] L.F. Fieser: J. Am. Chem. Soc., **75**, 1706 (1953).

3. Concentration of HCl : Under the standard procedure varying with the molar ratio of HCl to PNPH, maximum absorption was obtained at the ratio of 2.0 as shown in Fig. 1. Sulfuric acid revealed the same result.

4. Effect of Temperature upon Time of Reaction : The standard procedure was carried out at different temperatures such as 60°, 65°, 70° and 75° in different periods ranging to 120 min. and the developments of the reactions are shown in Fig. 2. Reagent-blank was increased at higher temperatures and for longer periods. For practical purpose, 60 min. at 65° was selected as an optimum.

5. Stability of Colored Solution : The standard procedure with 30 μg. of cholestan-3-one gave the result as shown in Fig. 3. As reagent-blank practically diminished after about 20 min., it was recognized to be suitable to read the absorbance after elapsed time of 30 min.

6. Calibration Curve : The linear relationship between the optical density and the concentration of ketosteroid was obtained in a range of 10 to 50 μg. Curves of cholestan-3-one and pregnenolone are shown in Fig. 4.

Standard Procedure (Method B)——Add 0.5 ml. of PNPH reagent to 0.5 ml. of ethanolic solution containing 0～50 μg. of ketosteroid (0～25 μg. in the case of Δ^4-3-ketosteroid) and keep the mixture at 65±1° for 60 min. After cooling for several minutes, add 4.0 ml. of DMF and 0.5 ml. of 1*N* NaOH. After the elapsed time of 30 min. read the optical density at the wave length of maximum absorption against reagent-blank which is prepared through the same procedure with regard to 0.5 ml. of ethanol without ketosteroid.

Parallel Test between Method A[3] and Standard Method (Method B)——Amounts of 30～50 μg. were determined individually by the two methods with regard to forty ketosteroids. Table I presents the results observed on the wave lengths of absorption maximum and the apparent molar extinction coefficients ($\varepsilon' = E/l \times c$) at the corresponding λ_{max}.

Discussion

Ketosteroids appeared in the field of clinical chemistry are roughly divided into two groups with regard to the active carbonyl function. One of them has a keto group in A-ring of the steroidal structure (chiefly Δ^4-3-one type and some saturated 3-one type) and the other one in the opposite side of the molecule——mostly in D-ring (17-ketosteroids) and some in side chain. Isonicotinic acid hydrazide[4] and 2,4-dinitrophenylhydrazine[5] have generally been utilized for the determination of the former group as summarized comparatively in Table II and Zimmermann reaction[6] using *m*-dinitrobenzene for that of the latter one, particularly for 17-ketosteroids. It should, however, be pointed out that these methods often serve only to give an estimate of

Table II. Comparison with Other Methods for Determination of Unsaturated 3-Ketosteroid

Method	λ_{max} mμ	ε'	Working temperature (°C)	Time (min.)
UV (Δ^4-3-one)	240	17000	—	—
Isonicotinic acid hydrazide[4]	380	11000	30	60
2,4-Dinitrophenylhydrazine[5]	460	22000	20	5
4-Aminoantipyrine[10]	354	9200	25	20～285
Method A[3] (Δ^4-3-one)	540	30500	50	60
Standard Procedure (Method B)	540	44500	65	60

the total amount of carbonyl functions, whether they are in the steroid nucleus or not, and that the reagent-blanks of the hydrazine methods are unsuitably tended to interfere the estimation of test solutions. A specific method for the determinaton of Δ^4-3-ketosteroids (method A)[3] presented by the authors has an advantage in this respect. The enormously higher ratio (160) of hydrochloric acid to PNPH is responsible

4) E. J. Umberger : Anal. Chem., **27**, 768 (1958).
5) A. G. Gornall, M. P. MacDonald : J. Biol. Chem., **201**, 279 (1953).
6) W. Zimmermann : Z. Physiol. Chem., **233**, 257 (1935).

for the substrate specificity of the method A. When the ratio was reduced down to 2.0, on the contrary, the specificity for Δ^4-3-ketosteroids was lost away, although the sensitivity became much higher with increase of reagent-blank. This would explain the increased reactivity of the PNPH reagent in such lower concentration of hydrochloric acid for the carbonyl group, so that even traces of carbonyl impurities in alcohol could be reacted enough to produce color and the unfavorably higher blank value was thus shown. The purification of alcohol described in the experimental part was fully effective to introduce a new micro method for the estimation of general ketosteroids presented in this paper.

It should be noted that the specificity for ketosteroids would, therefore, not be expected in the present procedure (method B). On the other hand, the relatively higher sensitivity of this method is useful for the micro estimation of the ketosteroids as shown in Figs. 4 and 5. Tne increased stability of the color produced is also shown in Fig. 3. One of the remarkable advantages is the possibilty to estimate colorimetrically micro amounts of the saturated 3-ketosteroids such as cholestan-3-one as shown in Fig. 4, that has been almost impossible by means of the most published methods. It seems most reasonable to conclude that the method B presented here could be expected to be utilized suitably for the estimation of isolated ketosteroids such as those separated on thin-layer chromatography and for the quantitative analysis of the medicinal steroid preparations.

As to the intensive coloration of hyrazones and the simultaneous degradation of excess of *p*-nitrophenylhydrazine in alkaline DMF, that play important parts in the methods A and B, the discussion will be presented in the following paper.

Reactivities of Different Steroidal Keto Groups for Methods A and B

Forty ketosteroids were estimated by both methods with the comparative results as shown in Table I.

1. In common with the two methods and independently of the position of keto group in a steroid structure, saturated ketosteroids (1~17) gave λ_{max} at 520 mμ; α,β-unsaturated ketosteroids (18~28) at 540 mμ; crossed dienone steroids such as prednisolone (29) and prednisone (30) at 565 mμ; more conjugated ketostsroids such as cholest-4,6-dien-3-one (31) and cholest-3,5-dien-7-one (32) at 570 mμ.

2. In the case of the method B almost quantitative formation of hydrazones were found on both of Δ^4- and saturated 3-ketosteroids, whereas that of 20-ketosteroid such as pregnenolone was estimated at about 30%, when the respective result (ε') obtained was referred to the individual molar extinction coefficient (ε) of the specimens of *p*-nitrophenylhydrazone.[1,3]

3. Saturated ketosteroids could hardly or slighty produce color in the method A.

4. As a rule, α,β-unsaturated ketone is considered to be less active than the corresponding saturated one, so that the formation of its hydrazone is rather difficult. In the method B, α,β-unsaturated ketosteroids, except for Δ^4-3-one, thus gave lower optical densities than those of the corresponding saturated ones. In the method A, on the other hand, Δ^4-3-ketosteroids gave exceptionally higher absorbances, contrary to the negative reactions of their isomers such as Δ^2-1-keto- (18) and Δ^5-7-ketosteroid (22) and to the lower activities of those such as Δ^1-3-keto- (19), Δ^4-6-keto- (21), $\Delta^{9(11)}$-12-ketosteroid (23). Thus the remarkable specificity of the method A for Δ^4-3-ketosteroids was clearly indicated. It is also of interest that Δ^1-3-ketosteroid (19) gave only a half of the reactivity shown by Δ^4-3-keto isomer (20).

5. No appreciable difference in reacivity was observed on a pair of conformational isomer, cholestan-3-one (3) and coprostan-3-one (4).

6. The method B could make more clear the relation between the reactivities of different carbonyl groups with their locations on steroid structure. The following order was observed as to the activities of saturated ketosteroids :

$$3>2>7\sim6\sim20>16>12\sim15\sim4>17>1>11$$

Wheelor[7] studied on the reactivities of ketosteroids for the Girard T reagent and estimated their velocities as well as the dissociation constants of their Girard T hydrazones. The order was observed on a reciprocal of dissociation constants as follows :

$$\Delta^3\text{-}3,3>6>17\sim20>7\sim12\sim11.$$

It has been clarified that the hydrazone is generally formed *via* following steps[8] :

$$\underset{\text{A}}{>C=O} + H_2N\text{-}NH\cdot R \xrightarrow[\text{slow}]{} \underset{\text{B}}{>C\langle^{OH}_{NH\cdot NH\cdot R}} \xrightarrow[\text{fast}]{} \underset{\text{C}}{>C\text{-}N\text{-}NH\cdot R}$$

and the step from A to B is rate determining. It has also been well known that the ketal is formed in a similar mechanism with that revealed on the formation of the intermediate B from A. Djerassi, *et al.*[9] studied semi-quantitatively the ketal formation of saturated ketones and aldehydes using optical rotatory dispersion method and observed the following order :

$$3\gg16\sim2\sim4>12\sim17\sim1$$

as to the facilities on steroidal ketones, with the subsequent interpretation of steric hindrances such as ring size. The above-mentioned order found on the method B would substantially be consistent with those observed in these papers.

7. Although the carbonyl function of 12-ketosteroids such as hecogenin (11) is generally regarded as rather inactive, it is of particular interst that 12-ketopregnane-3,20-diol 3-acetate (12) having no spirostane structure in the molecule was observed to have some remarkable activity for the method presented in this paper.

Details of such kinds of reactivity as described above should be discussed more definitely on the basis of the kinetic studies.

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7) O. H. Wheelor : Tetrahedron, **18**, 477 (1962).
8) R. B. Martin : J. Phys. Chem., **68**, 1369 (1964).
9) C. Djerassi, L. A. Mitscher, B. J. Mitscher : J. Am. Chem. Soc., **81**, 947 (1959).
10) E. P. Schulz, M. A. Diaz, G. Lopez, L. M. Guerrero, H. Barrera, A. L. Pereda, A. Aguilera : Anal. Chem., **36**, 1624 (1964).