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Microdetermination of Adrenocortical Steroids by Double Isotope Method. I. Determination of Cortisol and Corticosterone in Blood as Thiosemicarbazone-^{35}S[1]

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A method for microdetermination of cortisol and corticosterone in blood by the isotope derivative dilution analysis was established by the use of thiosemicarbazide-^{35}S as the labeled reagent and ^{3}H-labeled corticosteroid for correcting the loss during extraction and separation procedures.

Cortisol and corticosterone are reacted with thiosemicarbazide-^{35}S in acetic acid–methanol at 65° for 90 min, and the thiosemicarbazones-^{35}S formed are extracted with dichloroethane. After addition of the carrier to the extract, it is submitted to repeated thin-layer chromatography at a low temperature of 10°, using solvent systems of chloroform–ethanol (9: 1, v/v) and benzene–acetone (1: 1), by which corticosteroids are separated from each other and from substances present in blood. The amount of isolated cortisol and corticosterone thiosemicarbazones are estimated from the radioactivity of ^{3}H and ^{35}S.

According to this method, the values of cortisol and corticosterone are in the range of 0.001—10.0 μg, recovery rate is *ca.* 100%, and relative standard deviation is 1.0—6.7%. The determination can be made with good accuracy and precision. Comparative examination of the same sample by this method and by fluorometry gave a slightly higher values from this method, and the relative standard deviation showed a large dispersion of 6.0—18.0%. However, the analytical precision and sensitivity of this method are better than fluorometry. Determination of 0.1—5.0 ml of sample plasma by this method showed relative standard deviation of 0.8—3.4%, with small dispersion, and accuracy of determination was found to be satisfactory.

Introduction

Determination of microquantities of cortisol and corticosterone in body fluid is extremely important for the test of adrenocortical and pituitary functions, and for endocrinological research. The amount of these steroids in blood is very small, the normal values for adults being 10—20 μg of cortisol (I) and 1—2 μg of corticosterone (II) per 100 ml of plasma so that if 1 ml of plasma is used as a sample, their concentration would be a very low 0.1—0.01 μg/ml. Determination of corticosteroids in blood had heretofore been made by colorimetry[3] and fluorometry.[4] Sensitivity of colorimetry is insufficient and, although sensitivity of fluorometry is good, the measurement is greatly interfered by the presence of fluorescent components, which have to be separated by complicated procedures, and the recovery rate becomes scattered. Corticosteroids like I and II, and their metabolites are separated from plasma or urine generally by extraction with an organic solvent and fractionated as a neutral fraction after washing the extract with alkali, with the result that almost all the liposoluble compounds in the sample will be contained in this fraction. For this reason, it becomes necessary to

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2) Location: *Kitashinjuku 3-20-1, Shinjuku-ku, Tokyo.*
3) C.C. Porter and R.H. Silber, *J. Biol. Chem.*, **185**, 201 (1950); J.D. Few, *J. Endocrinol.*, **22**, 31 (1961); G.R. Kingsley and G. Getchell, *Anal. Chem.*, **2**, 1 (1961).
4) R.H. Silber, R.D. Busch, and R. Oslapas, *Clin. Chem.*, **4**, 278 (1958); P. DeMoore, O. Steens, M. Raskin, and A. Hendrix, *Acta Endocrinol.*, **33**, 297 (1960); R.E. Peterson, *J. Biol. Chem.*, **225**, 25 (1957).

find a method of separation with high specificity in the determination of corticosteroids and their metabolites.

We attempted the simultaneous determination of microquantities of cortisol and corticosterone in blood by the double isotope derivative dilution method, using ^{35}S-labeled thiosemicarbazide as the labeling reagent and ^{3}H-labeled corticosteroids for correction of their loss during the analytical procedures. In the present series of work, fundamental conditions for this method were examined and a good method of this determination was established. Fluorometric determination was also carried out to compare with this new method.

1. Reagent and Apparatus

1.1 Reagents

Cortisol[1,2-^{3}H]: Commercial cortisol[1,2-^{3}H] of Radiochemical Centre, Amersham, England (specific activity, 2.0 Ci/mmole) was purified by paper chromatography (PPC), using the solvent systems B_3, B_5, and C of Bush[5] at the time of use.

Corticosterone[1,2-^{3}H]: Commercial product (specific activity, 1.0 Ci/mmole) from the same source was used after the same purification as above.

Thiosemicarbazide[^{35}S]: Synthesized from $H_2{}^{35}SO_4$ by the method described in 3.1.1.

Silica Gel: Wakogel B-5F of Wako Pure Chemicals, Tokyo, was used.

Liquid Scintillator: Prepared from 4 g of PPO and 0.1 g of dimethyl-POPOP dissolved in 1 liter of toluene.

1.2 Apparatus

Liquid Scintillation Counter: Nihon Musen Medicophysical Laboratory Model LSC-501 was used. Paper Chromatoscanner: Nihon Musem Mediophysical Laboratory Model PCS-4 was used. Fluorometer: Hitachi Model 203. Ultraviolet Ray Detector: Osawa Shigaisen Kogyo Kenkyujo Model UV-LS-DI was used.

2. Analytical Procedure

2.1 Double Isotope Derivative Dilution Method

2.1.1. Determination of Cortisol and Corticosterone in Prepared Samples——Standard solutions containing cortisol and corticosterone (0.001—10 μg/ml MeOH) were prepared. To 1 ml of this solution, 1×10^5 dpm each of cortisol[1,2-^{3}H] and corticosterone[1,2-^{3}H] were added to make the sample solutions.

Step 1: To the sample solution, 100 μg of thiosemicarbazide-^{35}S (III) and 1 ml of 10% methanolic solution of acetic acid are added and the mixture is heated at 65° for 90 min. After addition of 2 ml of

sample
↓
Step 1: addition of cortisol[1,2-^{3}H] and corticosterone[1,2-^{3}H]
wash with petr. ether
extraction with CH_2Cl_2
↓
extract
↓
Step 2: thiosemicarbazide[^{35}S] (MeOH, AcOH, 65°, 90 min)
extraction with $ClCH_2CH_2Cl$
addition of carrier
cortisol thiosemicarbazone
corticosterone thiosemicarbazone
↓
thiosemicarbazones
↓
Step 3: TLC separation(silica gel)
1) $CHCl_3$–EtOH(9:1, v/v)
2) benzene–CH_3COCH_3 (1:1, v/v)
3) cyclohexane–iso-PrOH (7:1, v/v)
↓
counting of radioactivity

Chart 1

5) I.E. Bush, *Biochem. J.*, **50**, 370 (1952).

Na_2CO_3 solution (59.5 mg/ml), the mixture is extracted with three 5 ml portions of dichloroethane. The extract solution is washed consecutively with water, 1% thiosemicarbazide solution, and water.

Step 2: Detection of cortisol-^{3}H thiosemicarbazone-^{35}S (IV) and corticosterone-^{3}H thiosemicarbazone-^{35}S (V) by ultraviolet (UV) lamp through separation by thin-layer chromatography (TLC) cannot be made with the extract solution *per se* and, therefore, 20 μg each of the unlabeled thiosemicarbazones of I and II were added as a carrier, and repeated TLC was carried out with the solvent systems of $CHCl_3$–EtOH (9: 1, v/v), cyclohexane–iso-PrOH (7: 3, v/v), and benzene–acetone (1: 1, v/v), to effect their separation. Silica gel of the spots absorbing UV ray was scraped off.

Step 3: The silica gel scraped off from the TLC plate was filled in a column, eluted with dichloroethane–MeOH (1: 1, v/v), and the effluent was collected in a vial. The solvent was evaporated from the vial, 10 ml of liquid scintillator was added, and radioactivity of ^{3}H and ^{35}S was measured in a liquid scintillation counter. The amounts of the two corticosteroids were calculated from the formula given in 2.3.

2.1.2. Determination of Cortisol and Corticosterone in Plasma——A definite quantity (1×10^5 dpm) of cortisol-^{3}H and corticosterone-^{3}H is added to 1 ml of plasma as an indicator for correction of their loss during the separatory determination procedure, a brief scheme of which is given in Chart 1, and this mixture is washed with petroleum ether to remove lipid. Corticosteroids are then extracted three times with 5 ml each of dichloromethane and the combined extract is washed consecutively with cold 0.1N NaOH solution, 0.1N H_2SO_4, and water to remove phenolic compounds, and lower and medium fatty acids. The crude extract thereby obtained is submitted to the procedures outlined as Step 1 to 3, and the quantities of I and II are calculated.

2.2 Fluorometry

Determination of Cortisol and Corticosterone in Plasma——One ml of plasma, which was added 1×10^5 dpm each of cortisol-^{3}H and corticosterone-^{3}H, was washed three times with 1 ml each of petroleum ether, then extracted with three 5 ml portions of dichloromethane. The extract was washed consecutively with cold 0.1N NaOH solution, 0.1N H_2SO_4, and water to obtain an extract containing corticosteroids. TLC is repeated twice with $CHCl_3$–EtOH (9: 1) and benzene–acetone (1: 1), the silica gel in the area of I and II are scraped off and eluted with 5 ml of dichloromethane–MeOH (2: 1). The solvent is evaporated from 4.5 ml of the extracts, 4 ml of H_2SO_4–EtOH (65: 35) reagent is added and, after 20 min at 45° for I and 10 min for II, fluorescence intensity of the solution is measured at 525 nm, with an excitation wavelength of 470 nm. Calibration curves are prepared with the standard solutions (0.001—10.0 μg/ml) of I or II, respectively. The amounts of the two corticosteroids are calculated from these calibration curves. Radioactivity of ^{3}H is measured with remaining 0.5 ml of the above extracts, and recovery rate for TLC separation is corrected from the radioactivity.

2.3 Calculations

$$C_p = \frac{S_p}{H_p} \times \frac{H_s}{S_s} \times \frac{I_p}{I_s} \times C_s$$

where C_p: Amount (in μg) of cortisol (I) or corticosterone (II) in the sample.
C_s: Amount (in μg) of I or II added to the standard sample.
I_p: dpm of ^{3}H-labeled I or II added to the sample.
I_s: dpm of ^{3}H-labeled I or II added to the standard sample.
S_p and Hp: dpm of ^{35}S and ^{3}H in the sample after the analytical procedures.
S_s and Hs: dpm of ^{35}S and ^{3}H in the standard sample after analytical procedures.

It has been found the examination of analytical conditions (3.2) that the molar ratio of thiosemicarbazide binding with the steroid is always constant and the values of determination can be calculated from the above formula without requiring the calibration curves.

3. Experimental

3.1 Synthesis of Reagents

3.1.1. Synthesis of Thiosemicarbazide-^{35}S[6]——By the route shown in Chart 2, 0.1M $BaCl_2$ solution is added to 20 mCi of $H_2{}^{35}SO_4$[7] and the precipitated $Ba^{35}SO_4$ is collected and dried thoroughly. A mixture of this dried precipitate and 50 mg of carbon is heated in a platinum crucible to 800° for 30 min to be reduced to $Ba^{35}S$. Without isolating $Ba^{35}S$, 5 ml of 10N H_3PO_4 is added to it under a reduced pressure, using a manifold, to evolve $H_2{}^{35}S$ which is absorbed in 4 ml of KOH solution (52.5 mg/ml) under ice cooling for 48 hr to from $K_2{}^{35}S$. This solution is heated at 80°, and 100, 50, and 50 mg of CNBr are added every 10 min, and then heated at 80° for 1 hr. The solution is neutralized with 1N HCl and evaporated to dryness under a reduced pressure. The residue is extracted with three 10 ml portions of hot ethanol and the solvent is evaporated from the extract to obtain $KNC^{35}S$. A mixture of 145 mg of $KNC^{35}S$, 0.1 ml of water, 45 mg of

6) Lorentz Eldjarn, *Acta Chem. Scand.*, **7**, 343 (1953); Louis L. Bambas, U.S. Patent 2450406 (1948).
7) Commercial Product of Japan Atomic Energy Research Institute (chemical form, $H_2{}^{35}SO_4 \cdot HCl$ solution; radioactivity, 45 mCi/ml; specific activity, Carrier Free).

$N_2H_4 \cdot H_2O$, and 100 mg of $N_2H_4 \cdot H_2SO_4$ is warmed, 0.5 ml of Methylcellosolve is added, and the mixture is stirred thoroughly. This is centrifuged, the supernatant is refluxed at 135° for 2 hr, and the crystals that precipitate out are collected. Recrystallization from ethanol gives the labeled thiosemicarbazide, $NH_2NHC^{35}SNH_2$, mp 183°, with specific activity of 50 mCi/mmole; chemical yield, 81.80%; radiochemical yield, 75.80%.

$$H_2{}^{35}SO_4 \xrightarrow{BaCl_2} Ba^{35}SO_4 \xrightarrow[800°,\ 30\,min]{charcoal} Ba^{35}S \xrightarrow{H_3PO_4} H_2{}^{35}S \xrightarrow[ice\ cooling\ 48\,hr]{KOH}$$

$$K_2{}^{35}S \xrightarrow{CNBr} KNC^{35}S \xrightarrow[methyl\ cellosolve\ 135°,\ 2\,hr]{N_2H_4 \cdot H_2O \text{ and } N_2H_4 \cdot H_2SO_4} H_2NHN\text{-}C(={}^{35}S)\text{-}NH_2 \quad (III)$$

Chart 2

3.1.2. Synthesis of the Carriers——Cortisol Thiosemicarbazone (IV): A mixture of 50 mg of cortisol, 100 mg of thiosemicarbazide, 5 ml of glacial acetic acid, and 30 ml of methanol is refluxed at 65° for 90 min, neutralized with Na_2CO_3 solution, and extracted with three 20 ml portions of dichloroethane. IV was obtained by evaporation of the solvent from this extract.

Corticosterone Thiosemicarbazone (V): The same procedure as above was carried out with 50 mg of corticosterone.

3.2 Examination of Analytical Conditions

3.2.1. Reaction Time——In the procedure outlined in 2.1.1, the time for reaction of I or II with III was varied between 15 and 300 min, and the relationship between the reaction time and formation of IV and V was examined. As shown in Fig. 1-a, the formation of thiosemicarbazone becomes approximately constant at about 100% after 60 min but considering the safety margin, the reaction time was set for 90 min in the procedure outlined in 2.1.1.

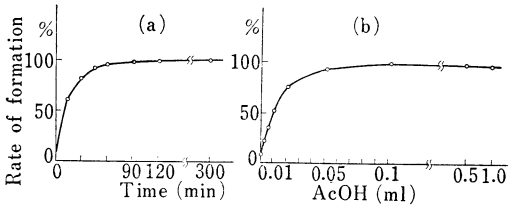

Fig. 1. Effect of Reaction Time and Amount of Acetic Acid on Formation of Cortisol[1,2-^{3}H]thiosemicarbazone[^{35}S]

3.2.2. Amount of Glacial Acetic Acid to be Added[8]——The amount of glacial acetic acid to be added as a protonic catalyst in the procedure outlined in 2.1.1 was varied from 0 to 1 ml and the amount of thiosemicarbazone formed was measured. As shown in Fig. 1-b, amount of thiosemicarbazone formed was maximum when acetic acid was added in 0.1 ml, and this quantity was adopted for the procedure. In practice, for the sake of convenience, glacial acetic acid was made into 10% methanolic solution and its 1 ml was used. It was confirmed from UV and infrared (IR) spectra, elemental analytical values, TLC, and specific radioactivity measurement that, under the above reaction conditions, thiosemicarbazide-^{35}S reacted with the two corticosteroids quantitatively and solely with the carbonyl group in C–3 position to form the respective monothiosemicarbazone.

3.2.3. Extraction Solvent——Extraction rate was measured by the use of 8 kinds of extraction solvent, dichloromethane, dichloroethane, chloroform, ethyl acetate, methyl acetate, tetrachloroethane, carbon tetrachloride, and trichloroethylene. From the results of this measurement, it was decided to use dichloromethane for the extraction of corticosteroids from plasma and dichloroethane for the extraction of corticosteroid thiosemicarbazones.

3.3 Thin-Layer Chromatography of Corticosteroids and Related Steroids

Natural and synthetic adrenocortical steroids like cortisone (VI), 11-deoxycortisol (VII), deoxycorticosterone (VIII), dexamethasone (IX), paramethasone (X), and prednisolone (XI) have chemical structure similar to those of I and II, and it would be difficult to separate them from each other by TLC after they have been derived to the thiosemicarbazone-^{35}S by the procedure outlined in 2.1. As shown in Table I, their *Rf* values are fairly close to each other. In addition, all these steroids collect in the neutral fraction obtained by solvent extraction. For this reason, they are likely to affect the determination values of a microquantity of cortisol and corticosterone unless their separation can be made completely. Therefore, development conditions for TLC were examined for the separation of cortisol-^{3}H thiosemicarbazone-^{35}S and corticosterone-^{3}H thiosemicarbazone-^{35}S from each other, and from thiosemicarbazones of substances present in blood and which are found in this fraction. This examination of TLC was made by using a silica gel containing fluorescent pigment.

8) N.L. Wendler, Huang Minlon, and M. Tishler, *J. Am. Chem. Soc.*, 73, 3818 (1951).

TABLE I. *Rf* Values of Thiosemicarbazones of Corticosteroid and Related Compounds by TLC

Compounds	Solvent system[a)]			
	a	b	c	d
Cortisol	0.28	0.40	0.12	0.39
Corticosterone	0.41	0.46	0.28	0.30
Aldosterone	0.33	0.40	0.25	0.19
Cortisone	0.39	0.45	0.30	0.42
11-Deoxycortisol	0.48	0.52	0.42	0.52
Deoxycorticosterone	o.57	0.69	0.44	0.49
Progesterone	0.60	0.70	0.53	0.59
Dexamethasone	0.37	0.46	0.30	0.42
Paramethasone	0.31	0.45	0.31	0.24
Prednisolone	0.31	0.38	0.30	0.28
Glucose	0.52	0.61	0.45	0.48
Acetone	0.56	0.53	0.38	0.55

a) solvent system: a, $CHCl_3$–EtOH (9:1) b, benzene–CH_3COCH_3 (1:1) c, cyclohexane–iso-PrOH (7:3) d, AcOEt
developing temp. 10°
TLC plate: silica gel, 0.25 mm in thickness

3.3.1. Developing Temperature——TLC was carried out on corticosteroids I to XI and progesterone (XII) as the related steroid, and the plates were developed at various temperatures. As shown in Fig. 2-a,b, it was found that the *Rf* values of corticosteroids except VII, VIII, and XII are at around 0.3 when developed at room temperature (*ca.* 25°) and their mutual separation would be impossible or quite difficult. When the developing temperature is lowered to 20, 15, 10, 5, or 0°, the *Rf* values vary widely, and the steroids VI to XII show *Rf* values far removed from those of I and II, making their separation possible. This fact proves that the *Rf* values in TLC depends greatly on the temperature of development and, therefore, development at around 10° was adopted in the procedures outlined in 2.1.

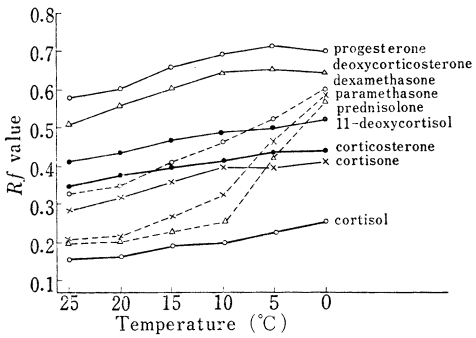

Fig. 2-a. Relation between Developing Temperature and *Rf* Values

TLC plate: silica gel, 0.25 mm in thickness
solvent system: cyclohexane–iso-PrOH (7:3)

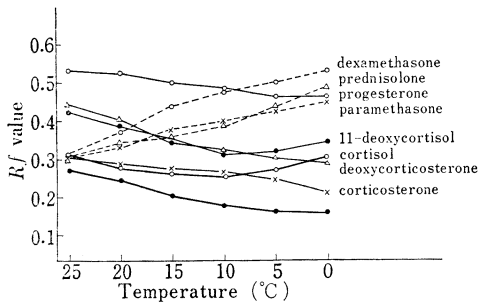

Fig. 2-b. Relation between Developing Temperature and *Rf* Values

TLC plate: silica gel, 0.25 mm in thickness
solvent system: $CHCl_3$–EtOH (9:1)

3.3.2. Developing Solvent——Examinations were made on the developing solvent systems that would effect mutual separation of the labeled thiosemicarbazones of ^{3}H-labeled IV and V, and from the thiosemicarbazones of coexisting substances. As shown in Table I, this separation would be difficult by the use of one kind of a solvent system. It was found that a repeated development at 10°, using the solvent systems of chloroform–ethanol (9: 1), cyclohexane–iso-propanol (7: 3), benzene–acetone (1: 1), and ethyl acetate effected a complete separation. It was considered that some of the substances present in blood and having a carbonyl group, like glucose, α-keto acid, and acetone, would affect the determination values by this method but repeated TLC at a low temperature of around 10° was proved to be effective in separating these blood substances.

Table IIa. Recovery of Cortisol from Prepared Sample[a)]

Taken (μg)	Found mean (μg)	Recovery (%)	Relative standard deviation (%)
0.0010	0.0011	105.00	6.69
0.0197	0.0202	102.79	1.66
0.0394	0.0401	101.80	1.33
0.1044	0.1046	100.26	1.53
0.1972	0.1991	100.96	1.88
0.3944	0.3958	100.35	1.38
1.0440	1.0491	100.49	1.28
5.0320	5.0247	99.86	1.07
10.0640	10.1084	100.44	1.38

a) Containing each 0.001—10 μg cortisol and corticosterone/ml MeOH solution.

Table IIb. Recovery of Corticosterone from Prepared Sample[a)]

Taken (μg)	Found mean (μg)	Recovery (%)	Relative standard deviation (%)
0.0010	0.0011	104.73	5.37
0.0247	0.0253	102.83	2.15
0.0493	0.0504	102.23	1.76
0.0986	0.0998	101.21	1.77
0.1956	0.1967	100.56	1.50
0.4930	0.4968	100.77	1.63
0.9860	0.9903	100.43	1.33
4.9300	4.9538	100.48	1.16
9.8600	9.8708	100.10	1.21

a) Containing each 0.001—10 μg corticosterone and cortisol/ml MeOH solution.

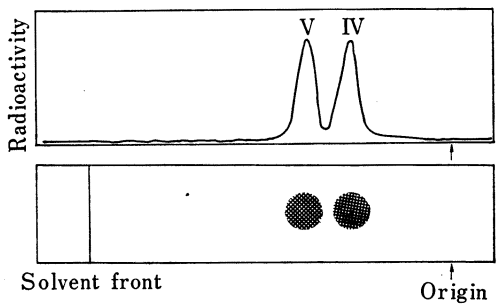

Fig. 3. Autoradiogram and Radioscannogram of Cortisol[1,2-^{3}H] thiosemicarbazone[^{35}S] (IV) and Corticosterone[1,2-^{3}H] thiosemicarbazone [^{35}S] (V)

TLC plate: silica gel, 0.25 mm in thickness
solvent system: $CHCl_3$–EtOH (9:1)

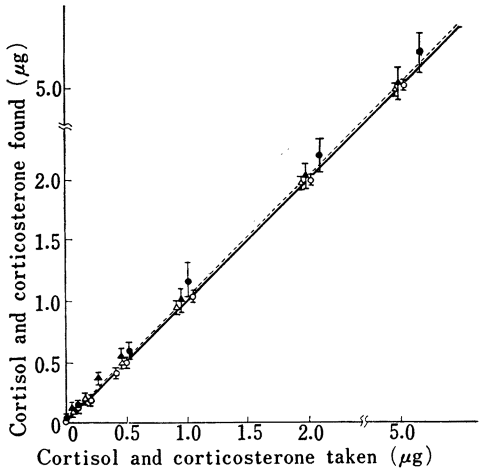

Fig. 4. Comparison of Determination of Cortisol and Corticosterone in Prepared Samples by Double Isotope Derivative Dilution Method and Fluorometric Method

—○—: cortisol, ---△---: corticosterone (isotope derivative method)
● : cortisol, ▲ : corticosterone (fluorometric method)

Table III. Determination of Cortisol and Corticosterone Added to Peripheral Plasma[a)]

Added steroid to plasma 1 ml (μg)	Found mean (μg)	Correction for initial concentration (μg)	Recovery of added steroid (%)	Relative standard deviation (%)
Cortisol				
5.1340	5.3829	5.1278	99.88	1.30
1.0268	1.2869	1.0318	100.49	1.29
0.5134	0.7658	0.5107	99.47	1.74
0.1027	0.3601	0.1050	102.24	1.59
0.0103	0.2654	0.0105	101.94	2.07
0.0010	0.2561	0.0010	103.15	2.59
0	0.2551			
Corticosterone				
4.9814	4.9933	4.9776	99.92	1.25
0.9963	1.0088	0.9931	99.68	1.55
0.4981	0.5172	0.5015	100.68	1.73
0.0996	0.1165	0.1008	101.21	2.41
0.0100	0.0260	0.0103	103.23	2.32
0.0010	0.0167	0.0010	104.07	3.47
0	0.0157			

a) Obtained from human male blood collected at 4 p.m.

3.3.3. Radiochemical Purity of Thiosemicarbazones——The radiochemical purity of the ^{3}H-labeled IV and V thiosemicarbazone-^{35}S separated by repeated TLC was confirmed from the agreement of the position of the spot detected by an UV lamp and that in the autoradiogram, and its peak in radioscannogram, and constant values of $^{3}H/^{35}S$ radioactivity ratio of the spot area in repeated TLC, as shown in Fig. 3.

3.4 Result of Determination

The values of cortisol and corticosterone in prepared samples were presented in Table IIa,b. According to this method, the values of I and II in prepared samples are in the range of 0.001—10 μg, recovery rate is approximately 100%, and relative standard deviation is 1—6.7%.

The values of cortisol and corticosterone added to 1 ml of plasma were presented in Table III. According to this method, recovery rate of added stercid (0.001—5 μg), which was calculated from the determined values corrected for the blank value (mean value with no steroid added), is approximately 100%, and relative standard deviation is 1.2—3.5%, and this method was found to determine I and II in plasma with good accuracy and precision.

The same prepared sample was submitted to determination by our isotope derivative method and fluorometric method,[4)] and the values from these two methods were comperes. As shown in Fig. 4, the values obtained from these two methods were approximately parallel, the values obtained from the isotope

Table IV. Determination of Cortisol and Corticosterone in Peripheral Plasma by Double Isotope Derivative Dilution Method and Fluorometric Method

Plasma (ml)	Cortisol[a)]				Corticosterone[a)]			
	Double isotope		Fluorometric		Double isotope		Fluorometric	
	Mean	Relative standard deviation (%)	Mean	Relative standard deviation (%)	Mean	Relative standard deviation (%)	Mean	Relative standard deviation (%)
0.1	25.90	2.38	31.50	16.28	1.63	3.38	—	—
0.5	25.86	1.61	27.38	5.86	1.56	2.03	2.42	18.21
1.0	25.71	1.49	26.16	5.46	1.55	1.26	1.71	5.18
2.0	25.34	1.10	27.01	5.59	1.53	1.31	1.65	5.48
5.0	25.20	0.83	25.75	4.14	1.51	1.81	1.58	4.76

a) μg/100 ml plasma

derivative method were less dispersed, and both the accuracy and precision were better than those of the latter.

In Table IV, result of determination of I and II in human male peripheral plasma by this method was compared with fluorometric method. The values obtained from these two methods were approximately identical, when the sample plasma used is more than 1 ml. Therefore, it would become possible carry out determination of I and II in plasma using the isotope derivative method. According to this method, cortisol is determined with good accuracy and precision, with a relative standard deviation of 1—2%, when the amount of plasma used was more than 0.1 ml. Corticosterone is determined with the same accuracy and precision, when the amount of plasma used was more than 0.5 ml, relative standard deviation being 3.4%, when 0.1 ml of plasma was used.

3.5 Comparison with Fluorometry

The existing method of fluorometry[4] consisted of adding sulfuric acid–ethanol reagent to samples of cortisol and corticosterone, and measuring the fluorescence produced at 525 nm, but the correction for losses in the steps of extraction and separation by TLC is difficult. Therefore, the values were corrected for about 10% of losses due to extraction and separation by the procedure outlined in 2.2.

In the case of plasma, as shown in Table IV, the values obtained from these two methods were approximately identical, though the values came out higher in fluorometric method, when the sample plasma used was small, and with large dispersion and poor in precision. In contrsat, the isotope derivative method was more than 10 times as sensitive as the fluorometric method, and gives values with little dispersion.

4. Conclusion and Discussion

The double isotope derivative analysis was found to have several advantageous points as a method for the determination of cortisol and corticosterone; (1) lossduring the extraction and separation steps can be corrected because ^{3}H-labeled corticosteroid is preliminarily added as an indicator for correction of lossduring the analytical procedure; (2) detection of thiosemicarbazones in the separation process is easy because of the use of silica gel thin layer with a fluorescent dye and a short wavelength UV lamp, utilizing the UV absorption of thiosemicarbazones; (3) this method is approximately 10 times more sensitive than the fluorometry; (4) higher precision than the existing colorimetry and fluorometry because determination dose not have to be made at or near the limit of analytical sensitivity.

In Table IV, it is seen that the values of corticosterone are 1.63 and 1.56 μg/100 ml plasma when the sample plasma taken was 0.1 and 0.5 ml, respectively, and their relative standard deviations are 3.38 and 2.03%, respectively. The value for cortisol is 25.90 μg/100 ml plasma when 0.1 ml of plasma was used as sample, and its relative standard deviation is 2.38%. The values came out higher in the isotope derivative method, when the sample plasma used was small. Consequently, this method can determine a microquantity like 0.001 μg of corticosteroids with good accuracy and precision, and the amount of the sample plasma required is around 0.5 ml for cortisol and 1.0 ml for corticosterone.

Ratio of various corticosteroids present in blood and urine varies greatly according to various endocrine diseases and the values are characteristic to each disease. For this reason, separatory determination of corticosteroids becomes necessary even from the point of correct grasping of the condition of the disease. By further consideration of the method for separating corticosteroids from each other and from the coexisting elements, simultaneous determination of corticosteroids in blood, urine, and tissues by the present method of determination will become possible.