CHROMATOGRAPHIC SEPARATION OF 17-HYDROXYCORTICOSTEROIDS AND 17-KETOSTEROIDS

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A chromatographic system consisting of a partially esterified carboxylic acid type ion-exchange resin as the stationary phase and a mixture of alcohol and water as the mobile phase, has proved useful for the separation of estrogens[1,2], Δ^4-3-ketosteroids[3], $C_{21}O_2$-steroids[4] and 17-ketosteroids[5]. From studies on the behaviour of steroids under various chromatographic conditions, a chromatographic system has been developed whereby most of the 17-hydroxycorticosteroids and 17-ketosteroids commonly found in human urine can be separated and estimated.

Three kinds of eluents were used with a column of partially esterified Amberlite IRC-50. The first was a mixture of methanol, ethanol (99 %) and water (3:9:8 by volume, eluent A). The second was a mixture of ethanol (99.5 %), benzene, *n*-hexane and water (50:350:80:3.3 by volume, eluent B), and the third was a mixture of ethanol (99.5 %), benzene, cyclohexane and water (50:350:300:2.3 by volume, eluent C). The mixture of steroids was first separated using eluent A, and the steroids which were poorly resolved, if at all, with eluent A were separated with eluent B or C.

EXPERIMENTAL

Materials

Tetrahydrocortisol and tetrahydrocortisone were supplied by Dr. T. F. GALLAGHER and allotetrahydrocortisol was supplied by Dr. W. KLYNE. 6β-Hydroxy-11-deoxycortisol was supplied by Dr. H. IBAYASHI and tetrahydro-11-deoxycortisol was isolated from the urine of a patient with congenital adrenal hyperplasia. 11β-Hydroxyetiocholanolone and 11-ketoetiocholanolone were supplied by Dr. S. LIEBERMAN. 11β-Hydroxyandrosterone and 11-ketoandrosterone were supplied by Dr. J. ENDO. Other steroids used in the present study were available commercially. The potassium hydroxide, dinitrobenzene, ethanol (99.5 %), sulfuric acid and phenylhydrazine sulfate used for the ZIMMERMANN reaction and PORTER–SILBER reagent were of analytical grade. Methanol, ethanol (99 %), benzene, *n*-hexane and cyclohexane were distilled before use. A 10 % (w/v) aqueous solution of Hyamine 1622 was purchased from Sankyo Co., Ltd.

Ion exchange resin

Commercial Amberlite IRC-50 (A.G.) is 16–50 mesh and must be pulverized. The resin was converted to the sodium ion form by treatment with 5 volumes of 2 *N* sodium hydroxide and was then washed with deionized water. The resulting

sodium salt of the resin was pulverized in a ball mill without drying. The powdered resin was suspended in deionized water and preliminary fractionation was performed by the sedimentation method[6]. Then the resin was classified hydraulically and particles of 55–65 μ, 35–40 μ, and 30–35 μ were collected[7,8]. These were washed as described previously and were partially esterified[5]. Particles of 55–65 μ were refluxed with aqueous acidic alcohol D and those of 35–40 μ and 30–35 μ with acidic alcohol E for 60 h (see Table I). One liter of acidic alcohol was used with 50 ml in the H form of Amberlite IRC-50, and bumping of the mixture was prevented by adding two or three pieces of porcelain boiling stone (8 mm cube) the edges of which had been removed by grinding.

TABLE I

COMPOSITION OF AQUEOUS ACIDIC ALCOHOL

Aqueous acidic alcohol	*Composition*
D	Methanol–ethanol–2 *N* HCl (1:3:2 by volume)
E	Ethanol–conc. HCl (19:1 by volume)

Preparation of the chromatographic column

The column used with eluent A was prepared with partially esterified particles of 55–65 μ. These were washed with eluent A and then poured into a chromatographic tube with eluent A and packed under an air pressure of 1 atmosphere. After about 200 ml of the eluent had passed through the column, it was ready for use. The column used with eluent B or C was packed in the following manner.

The esterified resin (30–35 μ and 35–40 μ particles) was washed successively with 10 volumes of ethanol (99 %), 10 volumes of a mixture of benzene, ethanol (99.5 %) and water (100:50:1 by volume) and 20 volumes of eluent B (35–40 μ particles) or C (30–35 μ particles). The washed resin was suspended in three volumes of the eluent used for chromatography, and the suspension was poured into the chromatographic tube and allowed to settle. After about 200 ml of the solvent had been passed through the column, it was ready for use.

Chromatographic separation of synthetic mixtures

When eluent A was used, the sample was dissolved in 0.45 ml of a mixture of methanol and ethanol (99 %, 1:3 by volume), and 0.35 ml of deionized water was mixed with the solution. This was then placed on the column and overlayered carefully with eluent A; elution was performed under an air pressure of 1 atm. When eluent B was used, the sample was first dissolved in 0.5 ml of a mixture of ethanol (99.5 %), benzene and water (30:210:1 by volume) and then 0.2 ml of a mixture of *n*-hexane and carbon tetrachloride (5:1 by volume) was added and mixed. This solution was applied to the column and overlayered carefully with eluent B and elution was performed with the same eluent. With eluent C, the sample was dissolved in 0.3 ml of a mixture of ethanol (99.5 %), benzene and water (50:350:1 by volume) and then 0.4 ml of a mixture of cyclohexane and carbon tetrachloride (5:1 by volume) was

mixed with the solution. This was applied to the column and overlayered carefully with eluent C, elution being performed with the same eluent. When a larger amount of the sample is available, a larger quantity of solvent may be used for dissolution and an aliquot of this solution may be applied to the column. The effluent was collected in fractions of 14, 20 or 21 drops in test tubes, using a drop count type automatic fraction collector. 17-Hydroxycorticosteroids were analyzed by adding two volumes of PORTER–SILBER reagent[9] to each fraction when eluent A was used. When eluent B or C was used, each fraction was allowed to evaporate at room temperature, and the residue was dissolved in 0.5 ml of 60 % ethanol and then 1.0 ml of PORTER–SILBER reagent was added to each fraction. The mixture was allowed to stand at room temperature for 15 h and the optical density was measured at 410 mμ using a Carl Zeiss PMQ-II spectrophotometer. PORTER–SILBER reagent was prepared by dissolving 50 mg of phenylhydrazine sulfate in a mixture of 21 ml of ethanol (99.5 %) and 39 ml of concentrated sulfuric acid. Concentrated sulfuric acid was prepared by mixing 158 ml of sulfuric acid with 37 ml of deionized water. Analysis of 17-ketosteroids was performed by the NATHANSON–WILSON modification of the HOLTORFF–KOCH method[10] or EPSTEIN's method[11] using aqueous Zimmermann reagent after evaporation of the eluent. The eluent was evaporated by placing the test tubes in a suitable rack and heating them in a boiling water bath for about 30 min. The recovery of steroids having a Δ^4-3-keto group was estimated from their ultraviolet absorption at 240 mμ.

Analysis of steroids in human urine

Urine (1/5 or 2/5 of a day's urine) was hydrolyzed at pH 4.7 with limpet β-glucuronidase[12] (100 units per one ml of urine) for 40 h at 37°, and extracted twice with an equal volume of ethyl acetate. The combined ethyl acetate extract was washed twice with 1/10 volume of concentrated sodium carbonate solution[13], once with 1/15 volume of 0.5 % acetic acid in water and once with 1/15 volume of water. The washed ethyl acetate extract was evaporated to dryness in a rotary evaporator below 40°. The dry residue was transferred with 15 ml of methanol to a separatory funnel and 20 ml of *n*-hexane and 1.5 ml of water were added to it. Then it was shaken for 5 min to extract less polar lipids into the *n*-hexane phase. The methanol layer was separated and the *n*-hexane layer was washed with 10 ml of a mixture of methanol and water (9:1 by volume). The combined methanol layer was evaporated to dryness and transferred quantitatively with 5 ml of 60 % aqueous ethanol to a small column (0.7 × 2.0 cm) of partially esterified Amberlite IRC-50 (60–80 μ in the wet sodium ion form) and overlayered carefully with 10 ml of 70 % aqueous ethanol. The filtrate from this column was divided into two equal parts and each was evaporated to dryness in a rotary evaporator. The first was analyzed by a one step elution using eluent A (Fig. 4). The other was first separated using eluent A (21 drops per fraction), and the steroids which were poorly or not resolved by the first chromatographic separation were pooled and separated with eluent B or C (Figs. 5–8). The fractions containing tetrahydrocortisol, tetrahydrocortisone and cortisol were determined by reaction of an aliquot (1/10 volume) of fraction 61 to 80 with PORTER–SILBER reagent. Other fractions containing $C_{19}O_3$-17-ketosteroids could be determined from the position of the fractions containing the above mentioned three 17-hydroxycorticosteroids.

RESULTS AND DISCUSSION

As shown in Figs. 1–3, eight 17-hydroxycorticosteroids and eight 17-ketosteroids could be separated systematically by using three solvent systems. They were first separated with eluent A. Tetrahydrocortisol, tetrahydrocortisone, cortisol and 6β-hydroxy-11-deoxycortisol, which were eluted together forming a single peak, were then separated by eluent B. Tetrahydro-11-deoxycortisol, 11-deoxycortisol, 11β-hydroxyetiocholanolone and 11-ketoetiocholanolone, which were not completely separated from one another, were then separated using eluent C. Separation of dehydroepiandrosterone, 11-ketoandrosterone and 11β-hydroxyandrosterone was also possible with eluent C. The unique feature of the chromatographic system described above is that partially esterified carboxylic acid type resin was used as the polar and the less polar stationary phase relative to the mobile phase. With aqueous alcohol as the mobile phase, the resin phase became less polar relative to the mobile phase and the reverse was true when a mixture of organic solvents containing less alcohol and some water was used as the mobile phase.

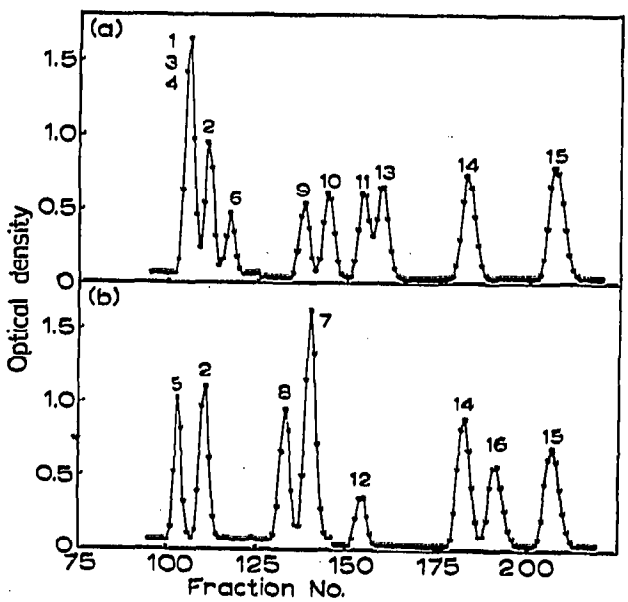


Fig. 1. Elution of 17-hydroxycorticosteroids and 17-ketosteroids. Elution patterns a and b were obtained in separate experiments performed under the same conditions. The size of the column was 0.6 × 136 cm, the mobile phase was eluent A and the temperature of the column was 33°. The effluent was collected in fractions of 14 drops and the flow rate was 4 fractions per hour. For numbers, see Table II.

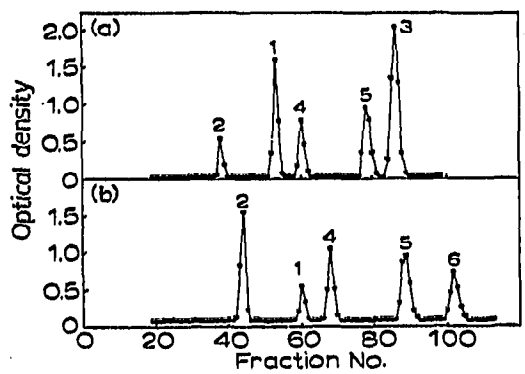


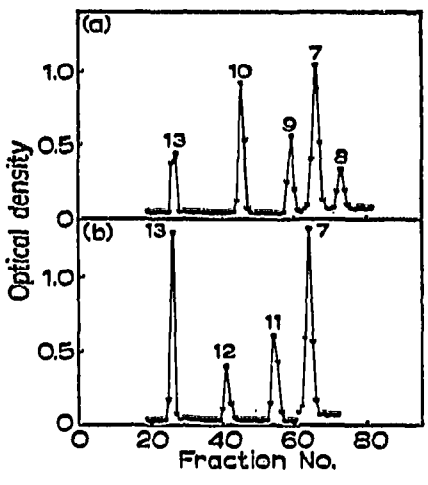
Fig. 2. Elution of 17-hydroxycorticosteroids. Elution patterns a and b were obtained in separate experiments performed under the same conditions. The size of the column was 0.5 × 71 cm, the mobile phase was eluent B and the temperature of the column was 21°. The effluent was collected in fractions of 20 drops and the flow rate was 1.5 fractions per hour. For numbers, see Table II.

The first system permitted the separation of steroids with widely different polarities by a one step elution and also provided good separation of the 5α- and 5β-isomers of 3α-hydroxysteroids. The second system was well suited for the separation of steroids of similar polarity, although separation of 5α- and 5β-isomers of 3α-hydroxysteroids was less satisfactory. Studies on the chromatographic conditions

TABLE II

STEROID NOMENCLATURE

No. in figures	*Chemical name*	*Trivial name*
1	11β,17α,21-Trihydroxypregn-4-ene-3,20-dione	Cortisol
2	17α,21-Dihydroxypregn-4-ene-3,11,20-trione	Cortisone
3	3α,11β,17α,21-Tetrahydroxy-5β-pregnan-20-one	Tetrahydrocortisol
4	3α,17α,21-Trihydroxy-5β-pregnane-11,20-dione	Tetrahydrocortisone
5	6β,17α,21-Trihydroxypregn-4-ene-3,20-dione	6β-Hydroxy-11-deoxycortisol
6	3α,11β,17α,21-Tetrahydroxy-5α-pregnan-20-one	Allotetrahydrocortisol
7	17α,21-Dihydroxypregn-4-ene-3,20-dione	11-Deoxycortisol
8	3α,17α,21-Trihydroxy-5β-pregnan-20-one	Tetrahydro-11-deoxycortisol
9	3α,11β-Dihydroxy-5β-androstan-17-one	11β-Hydroxyetiocholanolone
10	3α-Hydroxy-5β-androstane-11,17-dione	11-Ketoetiocholanolone
11	3α,11β-Dihydroxy-5α-androstan-17-one	11β-Hydroxyandrosterone
12	3α-Hydroxy-5α-androstane-11,17-dione	11-Ketoandrosterone
13	3β-Hydroxyandrost-5-en-17-one	Dehydroepiandrosterone
14	3α-Hydroxy-5β-androstan-17-one	Etiocholanolone
15	3α-Hydroxy-5α-androstan-17-one	Androsterone
16	3β-Hydroxy-5α-androstan-17-one	Epiandrosterone



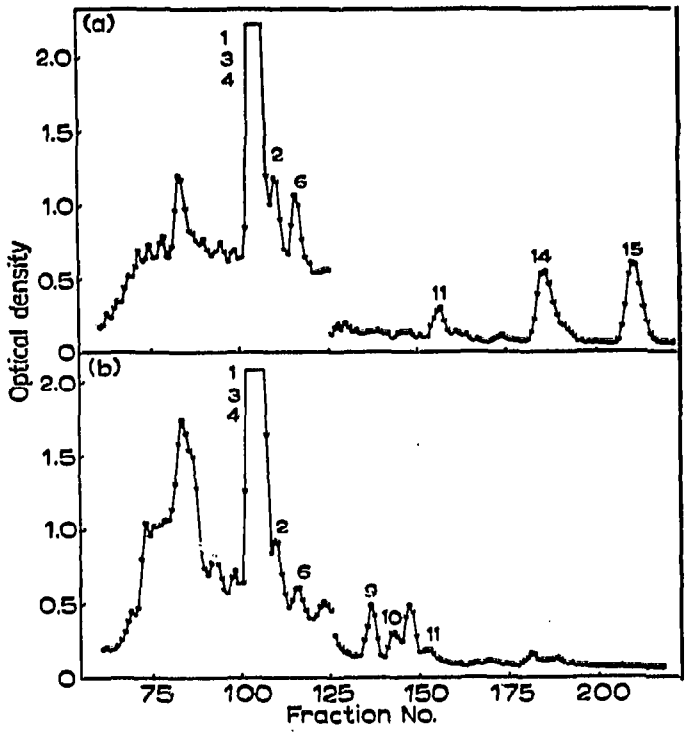
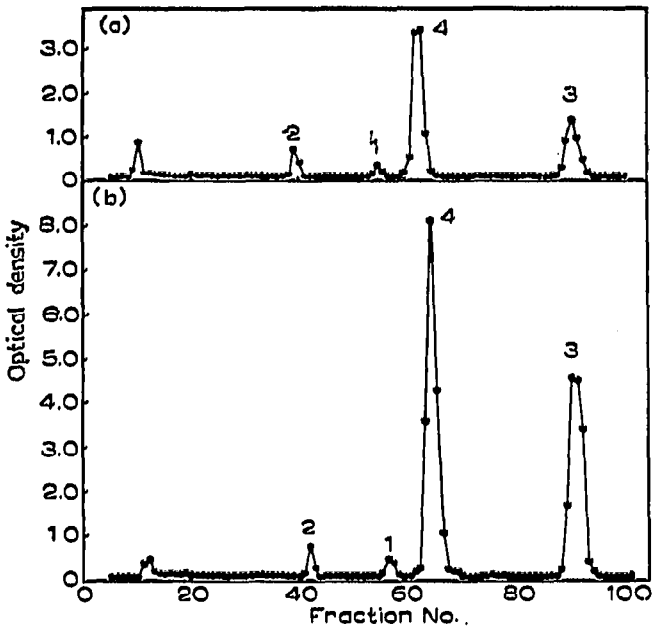


Fig. 3. Elution of 17-hydroxycorticosteroids and 17-ketosteroids. Elution patterns a and b were obtained in separate experiments performed under the same conditions. The size of the column was 0.5 × 69 cm, the mobile phase was eluent C and the temperature of the column was 21°. The effluent was collected in fractions of 20 drops and the flow rate was 2 fractions per hour. For numbers, see Table II.

Fig. 4. Elution of the neutral fraction of β-glucuronidase hydrolysate of human urine performed under the conditions described in Fig. 1. (a) Normal male, 39 years old, 1/5 day's urine. (b) Cushing's syndrome, female, 37 years old, 1/10 day's urine. For numbers, see Table II.



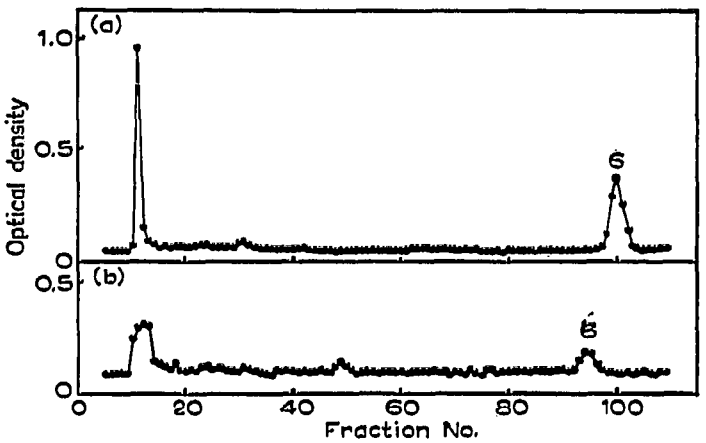
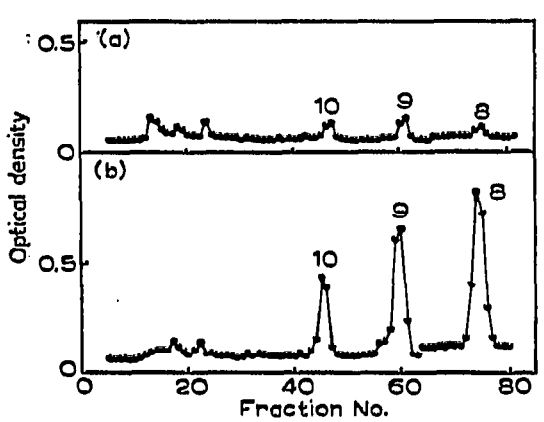


Fig. 5. Elution of the pooled fraction containing cortisol, cortisone, tetrahydrocortisol and tetrahydrocortisone performed under the conditions described in Fig. 2. a and b, as in Fig. 4. For numbers, see Table II.

Fig. 6. Elution of the allotetrahydrocortisol fraction performed under the conditions described in Fig. 2. a and b, as in Fig. 4. For numbers, see Table II.



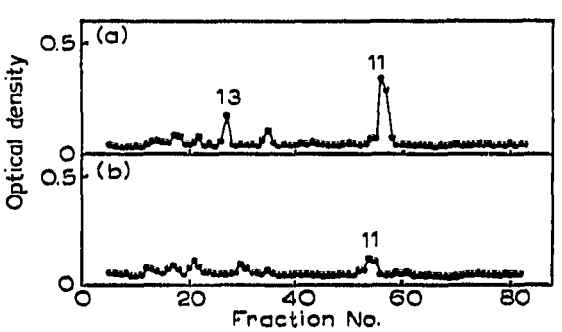


Fig. 7. Elution of the pooled fraction containing tetrahydro-11-deoxycortisol, 11-deoxycortisol, 11β-hydroxyetiocholanolone and 11-ketoetiocholanolone performed under the conditions described in Fig. 3. a and b, as in Fig. 4. For numbers, see Table II.

Fig. 8. Elution of the pooled fraction containing 11β-hydroxyandrosterone, 11-ketoandrosterone and dehydroepiandrosterone performed under the conditions described in Fig. 3. a and b, as in Fig. 4. For numbers, see Table II.

of the second system yielded the following rules for the preparation of the eluents.

(1) The water content of the eluent should be as high as possible, otherwise the peak width becomes excessively broad.

(2) The ratio of benzene to ethanol in the eluent should be high for clear separation of steroids with a hydroxyl group(s) from those with an oxo group(s) in place of the former. With an eluent with a high ethanol–benzene ratio, *e.g.* a mixture of ethanol–benzene–cyclohexane–water (90:30:360:4 by volume), 11β-hydroxyetiocholanolone was eluted faster than 11-ketoetiocholanolone. Differences in the elution pattern and/or resolving power were observed with different lots of Amberlite IRC-50, particularly when eluent A was used as the eluent. The greatest change with different lots of resin was in the position of cortisone relative to those of cortisol and allotetrahydrocortisol. With some lots of Amberlite IRC-50, the temperature of the column had to be kept at 33° for cortisone to be eluted just between cortisol and allotetrahydrocortisol, while with other lots, the optimum temperature for this was 29°. The elution patterns shown in Figs. 1 and 4 were obtained with a column prepared from resin bought in February, 1964. The elution pattern of the steroids obtained with a column prepared from a resin bought in July, 1965 was less satisfactory, and use of a finer resin (45–50 μ) was necessary for satisfactory resolution. The recovery of steroids with a Δ^4-3-keto group, shown in Table III, was satisfactory and the elution pattern was reproducible with eluent A, when the temperature of the column was kept constant. With eluents B and C, the elution volume of steroids was not so constant as it was with eluent A, and in some cases it varied about 10%. Although the optimum load was 50 to 500 $\mu g/cm^2$ for each component, milligram amounts of steroids could be chromatographed as was shown in the case of the analysis of 17-hydroxycorticosteroids extracted from the urine of a patient with Cushing's syndrome (Figs. 4 and 5).

TABLE III

PER CENT RECOVERY OF Δ^4-3-KETOSTEROIDS FROM CHROMATOGRAPHIC COLUMN

Steroid	*Per cent recovery with eluent*		
	A	*B*	*C*
Cortisol	97.6 ± 2.6	83.7 ± 5.4	
Cortisone	102.4 ± 4.2	85.0 ± 6.0	
6β-Hydroxy-11-deoxycortisol	100.4 ± 4.5	82.5 ± 8.0	
11-Deoxycortisol	100.2 ± 4.2		82.3 ± 4.9

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SUMMARY

Eight 17-hydroxycorticosteroids and eight 17-ketosteroids were systematically separated from one another with chromatographic systems using partially esterified Amberlite IRC-50 as the stationary phase. The steroids were first separated using aqueous alcohol as the mobile phase (eluent A); these steroids which were only poorly resolved, if at all, were then separated from each other using eluents containing a little water (eluents B and C).

This method was applied to the analysis of urinary steroids, and elution patterns of a neutral fraction extracted from a β-glucuronidase hydrolysate of human urine are described.

REFERENCES

1 T. SEKI, *Nature*, 181 (1958) 768.
2 K. MATSUMOTO AND T. SEKI, *Endocrinol. Japon.*, 10 (1963) 136.
3 T. SEKI, *J. Chromatog.*, 2 (1959) 667.
4 K. MATSUMOTO AND T. SEKI, *Endocrinol. Japon.*, 9 (1962) 201.
5 T. SEKI AND K. MATSUMOTO, *J. Chromatog.*, 10 (1963) 400.
6 P. DECKER AND H. HOLLER, *J. Chromatog.*, 7 (1962) 392.
7 P. B. HAMILTON, *Anal. Chem.*, 30 (1958) 914.
8 B. VASSILIOU AND R. KUNIN, *Anal. Chem.*, 35 (1963) 1328.
9 R. H. SILBER AND C. C. PORTER in D. GLICK (Editors), *Methods of Biochemical Analysis*, Vol. 4, Interscience, New York, 1957, p. 139.
10 L. L. ENGEL in D. GLICK (Editor), *Methods of Biochemical Analysis*, Vol 1, Interscience, New York, p. 479.
11 E. EPSTEIN, *Clin. Chim. Acta*, 7 (1962) 735.
12 G. A. LEVVY, A. J. HAY AND C. A. MARSH, *Biochem. J.*, 65 (1957) 203.
13 J. B. BROWN, *Biochem. J.*, 60 (1955) 185.