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Research Article

QUANTITATIVE PAPER CHROMATOGRAPHY OF CONJUGATED 17-KETOSTEROIDS IN PLASMA *

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The determination of urinary 17-ketosteroids has limited applications in the understanding and diagnosis of the nature of endocrine disorders associated with mild hirsutism and virilism in the female, because a very large number of these patients excretes 17-ketosteroids well within the normal range. Recent experience of Finkelstein, Forchielli and Dorfman (1) suggests that in these cases the blood levels and nature of the circulating androgens may be very important. Earlier indirect evidence (2–12) and the more recent isolation and partial identification of sulfates of dehydroisoandrosterone, androsterone, and etiocholanolone by Baulieu (13) indicate that 17-ketosteroids are present in plasma mainly in the form of esters. Direct estimation of these conjugated steroids in plasma offers distinct advantages: the alteration of the steroid molecule which occurs with certain types of hydrolysis as well as possible problems of incomplete hydrolysis can be circumvented. Also, information concerning the physiological significance of the steroid conjugating mechanisms may be gained through direct qualitative and quantitative estimation of these conjugates. The present paper describes a convenient method, using quantitative paper chromatography, for the estimation of dehydroisoandrosterone and androsterone sulfates (DHIA-SO_4 and Andro-SO_4) in plasma.

MATERIALS AND METHOD

All reagents and solvents used were of analytical grade, and all solvents, except absolute ethanol, were redistilled prior to use. Florisil was purified as described earlier (14). M-dinitrobenzene was purified by sublimation, using a modification of the "cold finger" technique, and stored in the dark at room temperature.

The sodium salts of dehydroisoandrosterone sulfate (DHIA-SO_4) and androsterone sulfate (Andro-SO_4) were obtained from Mann Research Laboratories, Inc., N. Y., and androsterone glucuronide was supplied through the courtesy of Dr. J. J. Schneider, Jefferson Medical College, Philadelphia, Pa.

Alkaline M-dinitrobenzene reagent (DNB). Potassium hydroxide pellets were dissolved in absolute ethanol by shaking gently. The solution was filtered through a sintered glass funnel under vacuum and adjusted to 3 N with hydrochloric acid prior to use. The DNB reagent was prepared immediately prior to use by mixing the 3 N absolute ethanolic potassium hydroxide with twice its volume of 2 per cent DNB (wt/vol) in absolute ethanol.

Collection of plasma. Blood from fasting subjects was drawn into heparinized syringes between 8:30 and 9:30 a.m. The blood was then transferred to a glass centrifuge bottle containing 1 ml of heparin per 100 ml of blood and centrifuged at 2,500 rpm for 30 minutes. The plasma was removed with a syringe equipped with a long spinal needle and transferred to a clean glass vessel.

Extraction of steroid conjugates. All analyses were run in triplicate. Fifteen-ml aliquots [1] of the plasma were placed in three 250-ml Erlenmeyer flasks and 45 ml of absolute ethanol was added to each flask. The flasks were swirled several times and placed in an ice bath for 10 to 15 minutes. After filtering the mixture, under vacuum, through two discs of Whatman no. 1 filter paper, the flasks were rinsed three times with 10 ml of absolute ethanol, and these washings were then used to wash the precipitate. The filtrate was transferred quantitatively to a 1 L round-bottomed flask with further washings of absolute ethanol and evaporated to near dryness (1 to 2 drops) on a flash evaporator (Laboratory Glass and Instruments Corp., model FE-2) under vacuum, using dry ice in ethylene glycol for refrigeration. Secondary butanol (15 ml) was then added to effect a partial separation of steroid conjugates from inorganic salts. The steroid conjugates, which dissolve quantitatively in secondary butanol, along with small amounts of inorganic salts were transferred to a 300 ml round-bottomed flask and the transfer completed with three 5-ml washings with secondary butanol. The secondary butanol solution was then evaporated to dryness on the flash evaporator and the extract transferred quantitatively to a test tube with five washings of absolute ethanol and dried at 45° to 50° C with a gentle stream of air.

Preliminary purification by column chromatography. Glass columns (1 cm in diameter, 11 cm in height) equipped with a 100 ml reservoir and a ground-glass stop-

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[1] Since completion of this work, the method has been adapted to 10-ml aliquots.

TABLE I

Column chromatography elution scheme

Eluting solvent	Effluent	Fraction no.	17-Ketosteroid content
	ml		
Benzene	20		
Benzene:ethyl acetate (1:1, vol/vol)	10	I	Free 17-ketosteroid, if any
Ethyl acetate	10		
Ethyl acetate:absolute ethanol (9:1, vol/vol)	25		
Ethyl acetate:absolute ethanol (1:9, vol/vol)	25	II	17-Ketosteroid sulfates
Absolute ethanol	20	III	No steroid; impurities only
Absolute ethanol:water (9:1, vol/vol)	10		
Absolute ethanol:water (1:1, vol/vol)	10	IV	17-Ketosteroid glucuronides
Absolute ethanol:water (1:9, vol/vol)	10		
Water	20	V	No steroid content

cock and plugged with a small quantity of glass wool were packed with 4.5 g of activated Florisil in benzene. During the addition of Florisil, the column was tapped with a rubber hammer to assure the escape of air bubbles. The benzene was allowed to run out until the lower edge of its meniscus just touched the surface of the Florisil. The crude extract was transferred quantitatively to the column with three washings (2 drops of absolute ethanol, 3 drops of benzene) and rinsed three times with benzene. The elution was carried out with benzene, benzene-ethyl acetate, ethyl acetate, ethyl acetate-ethanol, ethanol, ethanol-water, and water as shown in Table I. Fraction II, the sulfate fraction, and fraction IV, the glucuronide fraction, were evaporated to dryness under vacuum and the residue transferred quantitatively to test tubes with five 1-ml washings of ethyl acetate: methanol (2:1 vol/vol) and re-evaporated.

Florisil was chosen as the supporting medium in preference to alumina, Celite or silica gel because it gave better separation and recovery of the sulfates and glucuronides.

Paper chromatography. Standards and unknowns were applied to 1-inch strips of Whatman no. 2 chromatography paper. The strips were cut from the same sheet (46 × 57 cm) after a starting line had been pencilled 3 inches from one end. This procedure insured that all compounds would be applied to the same face of the paper and run in the same direction. A mixture of standards containing DHIA-SO_4 and Andro-SO_4 in absolute ethanol was pipetted into test tubes at various concentrations in triplicate and dried. The dried standards and plasma eluates were applied to the strips by rinsing the test tubes three times with 4 drops of ethyl acetate: methanol (2:1, vol/vol), and the compounds concentrated at the starting line by the method of Bush (15), using the same mixture. The strips were air-dried and placed in a paper-lined tank containing Schneider and Lewbart's system no. 2 (16). After equilibration for 3 hours, the mobile phase was added and allowed to run off the strips. The running time varied from 18 to 24 hours. The strips were air-dried, and were then ready for quantification.

Quantification. In quantitative paper chromatography, standardization of all steps involved is mandatory to insure proper reproducibility of the results. The Zimmermann reagent must be freshly prepared, the staining procedure adhered to rigorously, and the drying and heating times, as well as the temperature of the chromatography oven, must be strictly controlled. The humidity of the atmosphere should not exceed 40 per cent.

Separate solutions of KOH and DNB were freshly prepared in absolute ethanol. KOH was filtered through sintered glass under vacuum and adjusted to 3 N with HCl immediately prior to use. The paper strips were stained in groups of four. Fourteen ml of 2 per cent DNB and 7 ml of 3 N KOH were mixed and placed in a porcelain moistener. Each strip was run over the surface of the roller twice, dried horizontally over a fan for 90 seconds, and hung in the chromatography oven at 45° C for 3 minutes. The rolling and horizontal drying assure an even application of the Zimmermann reagent and uniformity of background throughout the entire length of the strips. After heating, the strips were stored between two larger blank strips previously treated with the reagent (17) for 1 hour, the time required for stabilization of the color.

Quantification was carried out in a Spinco model RB Analytrol equipped with a B_2 cam and two 550-mμ interference filters. With the B_2 cam, the Analytrol records a linear plot of the optical densities along the strip being scanned. To determine the optimal wave length for

scanning, the Zimmermann-positive area on a stained strip was cut out, placed in the cell holder of a Beckman DU spectrophotometer, and read against a blank, which had been cut from an area of background from the same strip. It was consistently found that the peak absorption occurred at 565 mμ. The 550-mμ interference filters used provide the closest approximation to this commercially available at present. That the readings at this wave length follow Beers' law is illustrated by the standard curves (Figure 1).

The Zimmermann-positive spots appeared on these strips as light violet areas against a brownish background which varied with the alkalinity of the chromatographic system. To insure optimal scanning, each strip was placed in a carrier of clear heavy plastic which centered the strip over the slit opening. A typical scanning curve is shown in Figure 2.

Standard curves. A standard curve was drawn next. It has been noted that triplicate determinations of the standards and plasma extracts have resulted in better standard curves and more reproducible results. Therefore, the plasma was initially divided into three aliquots, each extracted and chromatographed separately as described above. The standard mixture was chromatographed on paper, in triplicate, at concentrations of 20, 40, 60 and 100 μg for DHIA-SO_4 and at 5, 10, 15 and 25 μg for Andro-SO_4. Using four concentrations assured proper standard curves for quantification of the plasma extract, and all plasma aliquots tested thus far contained the sulfates in amounts within the limits of the above standard concentrations.

The values for each standard strip were calculated as above, and the means of the triplicates of each concentration determined. The individual values and the means were next plotted on graph paper, and the standard curve was drawn. The plasma values were then calculated by reading them from this curve.

RESULTS

A. Recovery of added standards. A number of experiments was done in which DHIA-SO_4 and/or Andro-SO_4 were added to the plasma aliquots. The standards were dissolved in absolute ethanol and added to the plasma before its precipitation with absolute ethanol. The protocol given above was followed, and the results are listed in Table II.

B. Characterization of dehydroisoandrosterone and androsterone sulfates. The compound in the dehydroisoandrosterone sulfate zone from paper chromatograms was eluted using ethyl acetate: methanol (2:1, vol/vol) and evaporated to dryness. It was then dissolved in 10 ml of 0.5 M acetate buffer (sodium acetate: acetic acid, pH 4.8) and incubated for 24 hours at 38° C with 10,000

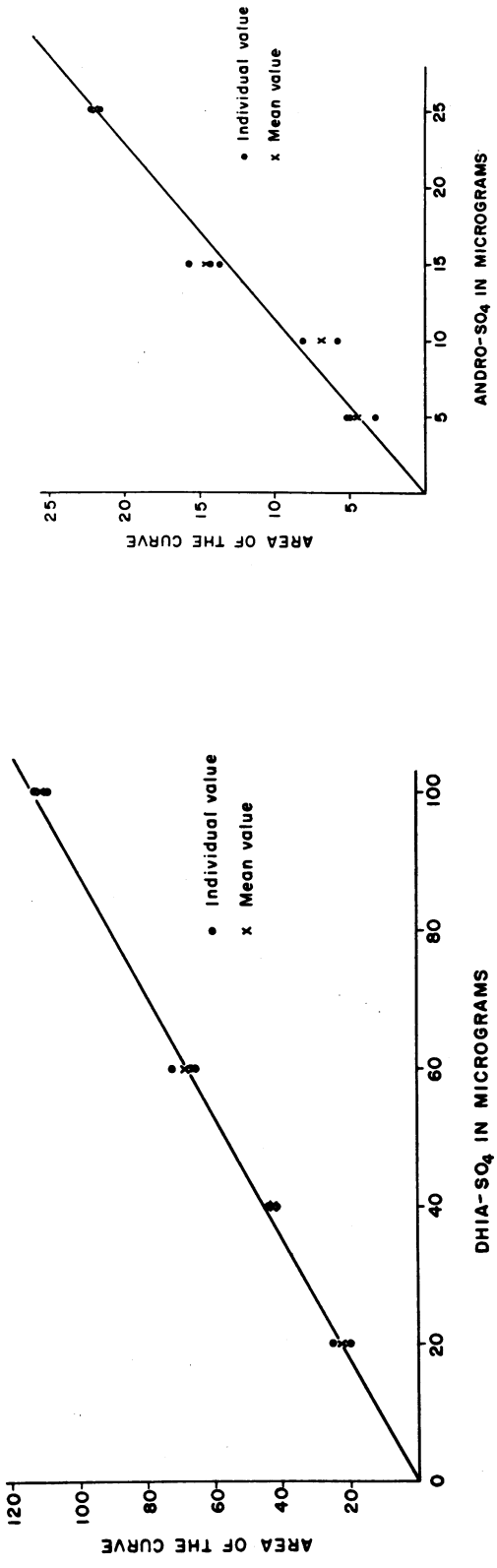

FIG. 1. STANDARD CURVES FOR DEHYDROISOANDROSTERONE SULFATE (LEFT) AND ANDROSTERONE SULFATE (RIGHT).

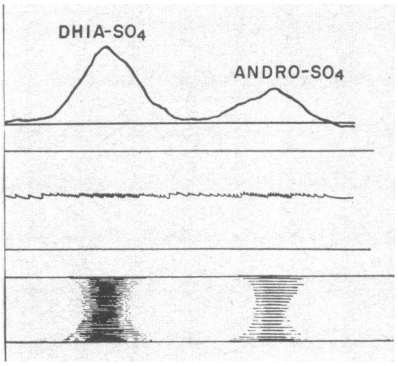

FIG. 2. SCANNING CURVE OF PAPER CHROMATOGRAM. The upper panel is a recording trace; the middle panel is an integrator trace; the lower panel is a facsimile of a paper chromatogram.

U Ketodase (β-glucuronidase). The incubation mixture was extracted with 100 ml ether : ethyl acetate (2:1). The organic layer was washed twice with 10 ml of 1 N NaOH and once with 10 ml of water. The washings were combined with the incubation mixture, which was then brought to pH 1 with dilute hydrochloric acid and continuously extracted with ether for 36 hours.

Aliquots of extracts after β-glucuronidase and HCl hydrolysis were run for 4 and 16 hours on a Whatman no. 2 sheet in 1-inch lanes along with free dehydroisoandrosterone in a system consisting of ligroin : methanol : water (100:96:4, vol/vol) (L/96) designed to separate free 11-deoxy-17-ketosteroids (18). The paper strips were developed using alkaline DNB. The β-glucuronidase hydrolysis product did not show the presence of any 17-ketosteroids, indicating the absence of glucuronides. The HCl hydrolysis product indicated the presence of a 17-ketosteroid which agreed in its color reaction and rate of migration on paper, in the above mentioned system, with dehydroisoandrosterone. A second aliquot of the HCl hydrolysis product was acetylated using acetic anhydride and pyridine and run for 4 hours in a 1 inch lane of Whatman no. 2 paper along with dehydroisoandrosterone acetate in the same system. On staining with alkaline DNB, it was found to be identical in color reaction and rate of migration with dehydroisoandrosterone acetate.

Approximately 40 μg of the HCl hydrolysis product was refluxed for 6 hours with a mixture of 20 ml of acetone, 30 ml of benzene and 0.5 g of aluminum isoporpoxide and the mixture washed twice with 25 ml 1 N H_2SO_4 (19). The organic layer was washed with water, dried over sodium sulfate and evaporated to dryness. It was then run for 4 hours on a 2-inch lane of Whatman no. 2 paper in L/96 along with Δ^4-androstene-3-17-dione. The 2-inch lane was cut into four ½-inch strips: one strip was developed with alkaline DNB while another was tested for NaOH fluorescence. The Oppenauer oxidation product had the same NaOH fluorescence reaction as a Δ^4-3-ketone, the same color with alkaline DNB and rate of migration as Δ^4-androstene-3-17-dione. The steroid zone of the other ½-inch strips was eluted and one fraction was subjected to acetylation, the other to chromic acid oxidation. These two reactions left the steroid molecule unchanged, as revealed by paper chromatography. The various steps in the identification of dehydroisoandrosterone sulfate are given in Figure 3.

The characterization of androsterone sulfate was carried out along the same lines as that of dehydroisoandrosterone although not as extensively, owing to small amounts of available material. The following experimental evidence for proof of identity was found: *1*) failure of hydrolysis with β-glucuronidase; *2*) formation of free androsterone by hydrolysis with HCl at pH 1, identified by paper chromatography; and *3*) acetylation of the androsterone thus produced yielded androsterone acetate which agreed with an authentic sample of androsterone acetate. Further proof of the identity of the sulfate moiety with both DHIA-SO_4 and Andro-SO_4 was obtained with the rhodizonate reaction as described by Schneider and Lewbart (20).

C. Plasma values. DHIA-SO_4 and Andro-SO_4 values were determined in the plasma of normal males between the ages of 20 and 40. These

TABLE II

Recoveries of standards added to the plasma aliquot

DHIA-SO_4		Andro-SO_4	
Added	Recovered	Added	Recovered
μg	%	μg	%
20	100	20	99
		20	91
		15	84
80	100	20	100
		15	100
40	94		

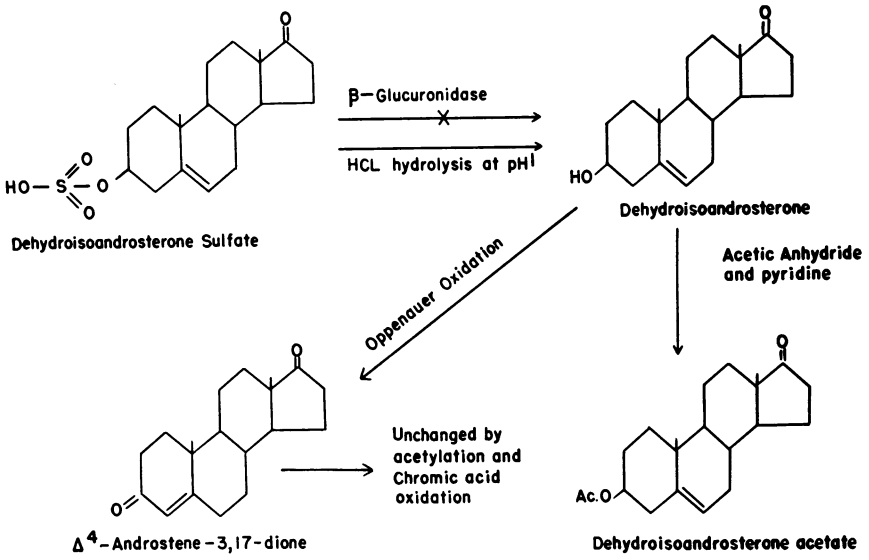

FIG. 3. IDENTIFICATION OF DEHYDROISOANDROSTERONE SULFATE.

values ranged from 155 to 298 μg per 100 ml for DHIA-SO_4 and from 29 to 92 μg per 100 ml for Andro-SO_4 (Table III).

Four plasma samples from normal females in this same age group have also been analyzed. Although this is a very small group, the DHIA-SO_4 levels (range, 85 to 166 μg per 100 ml) seem to be lower than those found in the males, while the Andro-SO_4 levels (range, < 15 to 45 μg per 100 ml) have generally been within the same range as those of the males (Table III).

Plasma drawn preoperatively from a patient with the Stein-Leventhal syndrome was studied. The DHIA-SO_4 level was 342 μg per 100 ml plasma, well above that of any of the normal males studied, while the Andro-SO_4 level was 70 μg per 100 ml. On the other hand, a patient with virilizing Cushing's syndrome secondary to an adrenal tumor (proven at operation), had 185 μg DHIA-SO_4 per 100 ml plasma and 552 μg Andro-SO_4 per 100 ml. This value for DHIA-SO_4 is at the lower level of the male range, and may not be significant; however, the Andro-SO_4 level is about seven times the normal range, and seems highly significant. This latter plasma also contained a third conjugate which has been tentatively identified as etiocholanolone sulfate and is the only plasma analyzed thus far that has been found to contain this particular conjugate in amounts detectable by the present method.

DISCUSSION

The present paper offers essentially two new aspects to the study of 17-ketosteroids in plasma: the direct estimation of these moieties in their conjugated form and the use of quantitative paper chromatography as a routine method.

In approaching the more difficult problem of extraction and purification of the steroid esters from plasma, as compared with a similar procedure following hydrolysis, it was hoped that possible losses incurred during acid treatment could be avoided. It is well known that complete hydrolysis is not achieved with currently available methods, and that dehydroisoandrosterone, par-

TABLE III

Plasma values

DHIA-SO_4		Andro-SO_4	
Subject*		Subject	
	μg %		μg %
WH	176	IM	73
LM	288	OM	64
EM	195	RS	34
T	298	T	51
JE	244	DW	92
SS	162	SS	29
WH	155	WH	39
SC	127	SC	<15
RP	166	RP	<15
MW	85	MW	45
AB	109	AB	25

* SC, RP, MW and AB are females.

ticularly, is partially destroyed by contact with strong mineral acids. These facts must be weighed against the more cumbersome quantitative recovery of such highly polar substances as the 17-ketosteroid sulfates. The figures obtained when the 17-ketosteroid sulfates were added to plasma, as well as the absence of Zimmermann reactive material following the extraction procedure, demonstrate the effectiveness of the present quantitative method. It is also interesting to note that our values for dehydroisoandrosterone are somewhat higher than those reported by other investigators using methods which employ various hydrolytic procedures.

In the limited number of subjects studied thus far, 17-ketosteroids were recovered solely as sulfates. The presence of 1 to 2 μg of androsterone per 100 ml of plasma after enzyme hydrolysis (glucuronidase) has been reported by others (3, 21). Since androsterone glucuronide added to the 15-ml samples of plasma has been adequately recovered, it is evident that glucuronides can be detected by the present method, but the small amounts of endogenous androsterone glucuronide (e.g., < 0.5 μg per 100 ml) present in samples of such size are not detectable. Minimal amounts also may be circulating as free alcohols as proposed by Cohn, Bondy and Castiglione (22), or perhaps as esters of other acids. The lower limit of sensitivity of the present method (15 ml of plasma) is 15 μg of steroid conjugates per 100 ml; any smaller amount would not be detected. However, it is apparent that the ratio of sulfates to glucuronides found in urine (16) is not present in plasma. This suggests either a more rapid clearance of glucuronides than of sulfates (21), or a renal conjugating mechanism by which glucuronic acid displaces the sulfate radical. The formation of glucuronides of free steroids in the kidney (23) is another possibility.

The direct quantification of the Zimmermann material on paper deserves further comment. This was first proposed by Oertel (24) and Bush and Willoughby (25), using a slightly different apparatus. The main advantage of such a method is that all problems encountered during elution of compounds are avoided. Some of the problems frequently encountered are: introduction of impurities from paper and solvents, loss of material, or the overlooking of "unexpected" spots when areas adjacent to reference standards are cut out without staining pilot strips. (Omission of pilot strips may become necessary when working with very small amounts of steroids.) Also, inadequate separation of compounds with closely related Rf values may lead to over- or underestimation through arbitrary selection of the areas to be eluted. Finally, the solvents used in the present system of paper chromatography seemed to interfere seriously with the Zimmermann reaction when quantification was attempted after elution, making this technique impractical, if not impossible.

Direct quantification through planimetric scanning procedures, as practiced here, also has its disadvantages, one of which is the rigorous time schedule which must be observed while staining and reading the strips. The greatest drawback, however, is its limited reproducibility, particularly when tailing or spreading of spots is excessive. This can be limited by careful application of the samples to paper and, as shown above, by triplicate readings. In our opinion, then, direct quantification should be given serious consideration, particularly when paper chromatography is part of a clinical method.

SUMMARY

A method for the direct estimation of dehydroisoandrosterone sulfate and androsterone sulfate in plasma has been presented. The procedure includes extraction, column and paper chromatography, and direct quantification on paper by planimetric evaluation. It has been shown that the criteria for recovery reproducibility and specificity of this method have been fulfilled. Plasma samples from normal men and women of reproductive age have been studied, as well as a limited number of samples obtained from female patients with various virilizing disorders. The normal values seem to be somewhat higher than those obtained by other investigators with a method involving hydrolytic procedures. This, we believe, can be explained by the greater molecular weight of the steroid esters as compared with the free alcohol, the greater Zimmermann chromogenicity of androsterone sulfate as compared with androsterone on a molar basis, and a greater recovery, since the loss occurring during hydrolysis was avoided. Free steroids or glucuronides of steroids were not detected with the present method. The reason for this has been discussed.

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