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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF EBURNANE ALKALOIDS

III. APPLICATION OF DIFFERENT PHASE SYSTEMS

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SUMMARY

Methods developed for the separation of eburnane alkaloids have been applied for solving different analytical problems; plant extracts, mother liquors and pharmaceutical formulations have been analysed on both reversed-phase and silica packings. For the determination of apovincaminic acid ethyl ester (vinpocetine) in human blood plasma octylsilica was used as the stationary phase and acetonitrile–0.01 *M* aqueous ammonium carbonate as the eluent. For quantitation apovincamine can be used as an internal standard. The detection limit was found to be 2 ng/ml. To determine the corresponding acids of ester derivatives of eburnane alkaloids a reversed-phase ion-pair chromatographic method was developed.

INTRODUCTION

In earlier papers[1,2] the separation of eburnane alkaloids on both a reversed phase and silica microparticles was described, the selectivities and efficiencies of the different separation methods were compared and a few examples were given to demonstrate the capabilities of the methods. However, an unsatisfactory separation of the corresponding acids of eburnane alkaloid ester derivatives (vincaminic acid, epivincaminic acid and apovincaminic acid) were obtained because in their ionized form, these acids were only slightly retarded in reversed-phase chromatography, while on silica irreversible adsorption can occur. For solving this problem the application of reversed-phase ion-pair chromatography has been investigated.

As in the previous papers[1,2] only a general consideration of the application of the methods was given, in this paper the selection of optimal conditions for practical application is discussed.

EXPERIMENTAL

Varian Model 8500 (Varian Aerograph, Walnut Creek, CA, U.S.A.), Hewlett-Packard 1081/B (Hewlett-Packard, Avondale, PA, U.S.A.) and Liquochrom Model

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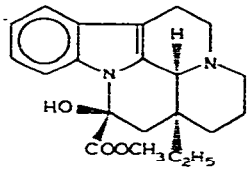
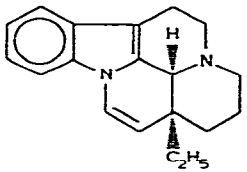
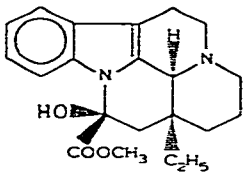
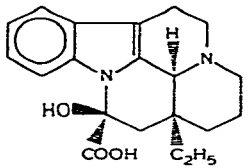
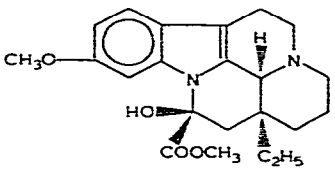
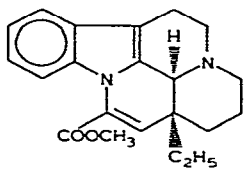
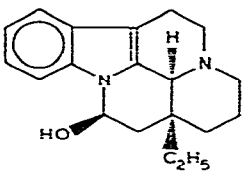
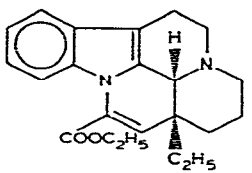
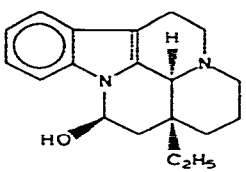
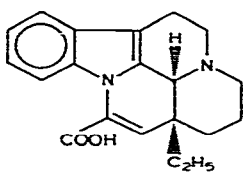
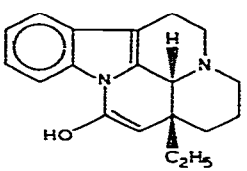
TABLE I

STRUCTURES OF COMPOUNDS INVESTIGATED

Compound	*Structure*	*Compound*	*Structure*
Vincamine		Vincamenine	
Epivincamine		Vincaminic acid	
Vincine		Apovincamine	
Vincanole		Apovincaminic acid ethyl ester	
Isovincanole		Apovincaminic acid	
Vincamone			

2010 (Labor MIM, Estergom, Hungary) high-performance liquid chromatographs were used. All instruments were equipped with Rheodyne 7010 and 7120 loop injection systems and variable-wavelength UV detectors (Variscan Model 635, Schoeffel Model 770 Liquodet Model 308).

The separations were performed on pre-packed columns: μBondapak C_{18} (300 × 3.9 mm I.D.) (Waters Assoc., Milford, MA, U.S.A.); Nucleosil 10 C_{18} and LiChrosorb RP-8 (250 × 4.6 mm I.D.) (Chrompack, Middelburg, The Netherlands); LiChrosorb SI-60, 5 μm (250 × 4.6 mm I.D.) (Pierce Eurochemie, Rotterdam, The Netherlands); and MicroPak SI-10, 10 μm (250 × 2.0 mm I.D.) (Varian Aerograph).

Chemicals and solvents were of analytical-reagent grade and were obtained from Reanal (Budapest, Hungary). Trioctylmethylammonium chloride was obtained from Fluka (Buchs, Switzerland).

The structures of the compounds investigated are shown in Table I.

RESULTS AND DISCUSSION

When eburnane alkaloids are investigated the following practical problems of their determination in plant extracts, production mother liquors, raw materials, different pharmaceutical formulations and in biological fluids have to be solved. To select the most appropriate separation method a phase system was chosen that permits first the elution of possible by-products and degradation products, which occur in relatively low concentrations.

When vincamine has to be determined in plant extracts (*Vinca minor*) the problem is to separate it from other eburnane alkaloids, mainly vincine. In Fig. 1, separations performed on a reversed-phase packing (Fig. 1A) and silica (Fig. 1B) are shown. It can be seen that separation on silica is more suitable, because a perfect separation from vincine and other related alkaloids can be achieved, whereas on a reversed-phase packing vincine is eluted with the virtually same retention as vincamine.

As mentioned previously[1,2], on octyl- or octadecylsilica vincanole is less retained than isovincanole[1], whereas on silica the order of elution of these two compounds is highly dependent on the composition of the mobile phase[2]. For the separation of isovincanole and other impurities (vincamone and vincamenine) from vincanole, adsorption chromatography using silica gel as the stationary phase and a two-component eluent (chloroform–ethanol) is suitable, as illustrated in Fig. 2, where the chromatogram of a mother liquor containing vincanole is shown.

When the problem is the analysis of different pharmaceutical formulations, the methods that can be used depend on whether the main aim of the investigation is stability indicating, assay or a content uniformity test. For the determination of the active ingredient content of a formulation, reversed-phase chromatography with octadecylsilica as the stationary phase and acetonitrile–aqueous ammonium carbonate as the eluent can be applied, as illustrated on the example of Cavinton tablets (containing 5 mg of apovincaminic acid ethyl ester) in Fig. 3. For quantitation vincamone is used as the internal standard.

In a content uniformity test, the determination can be solved by adsorption chromatography on a silica column. Fig. 4 shows the chromatogram obtained for Devincan tablets, and the results of the test are illustrated in Fig. 5 for Cavinton

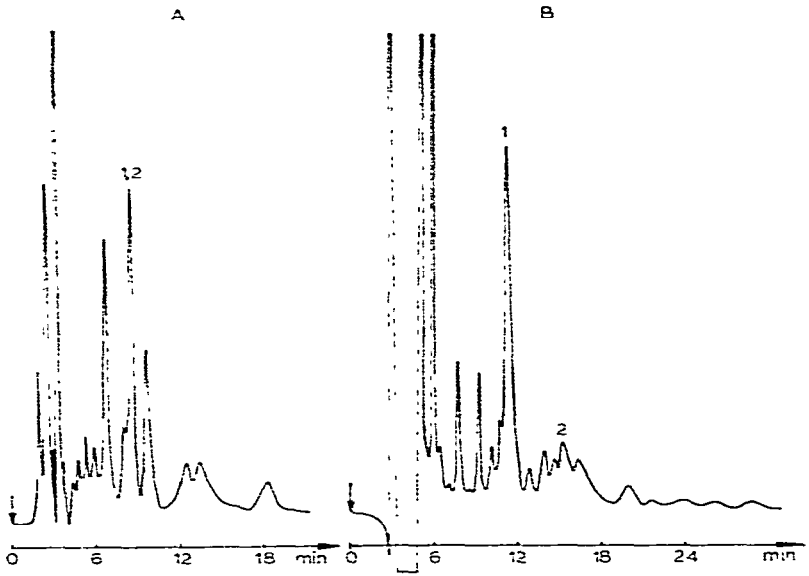


Fig. 1. Determination of vincamine in *Vinca minor* plant extract. (A) Instrument, Liquochrom Model 2010; column, μBondapak C_{18} (300 × 3.9 mm I.D.); eluent, acetonitrile–aqueous 0.01 *M* ammonium carbonate (6:4); flow-rate, 1 ml/min; detection at 280 nm. (B) Instrument, Varian 8500; column, LiChrosorb Si-60, 5 μm (250 × 4.6 mm I.D.); eluent, hexane–chloroform–methanol (8:1:1); flow-rate, 1 ml/min; detection at 280 nm. Compounds: 1 = vincamine; 2 = vincine.

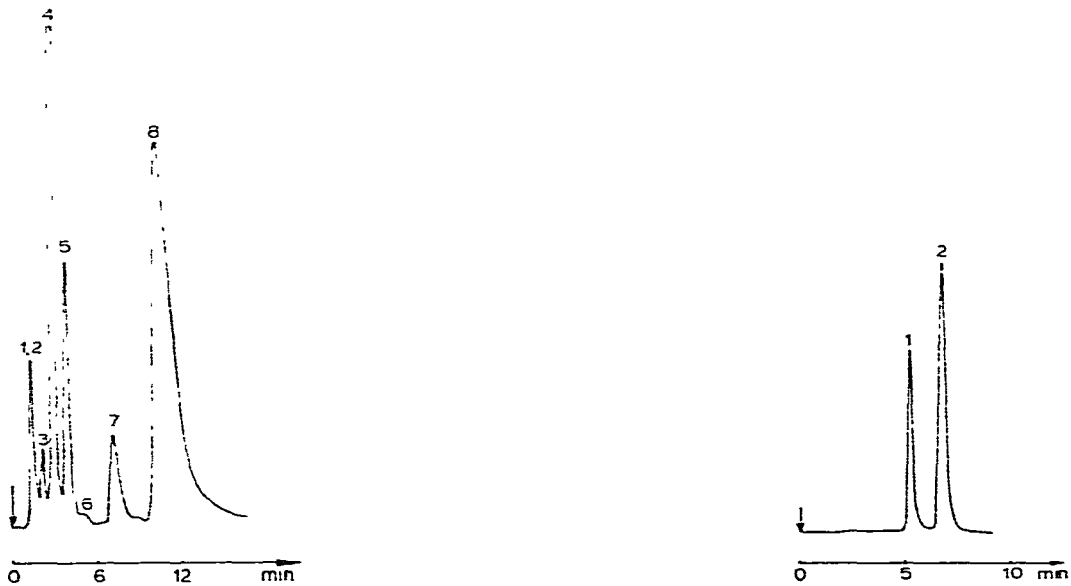


Fig. 2. Separation of vincanole and isovincanole in production mother liquor. Instrument, Varian 8500; column, MicroPak SI-10 (250 × 2 mm I.D.); eluent; chloroform–ethanol (95:5); flow-rate, 20 ml/h; detection at 280 nm. Compounds: 1 = toluene; 3 = vincamone; 4 = vincamenine; 7 = isovincanole; 8 = vincanole; 2, 5, 6 = unknowns.

Fig. 3. Determination of vinpocetine in Cavinton tablets. Instrument: Hewlett-Packard 1081/3; column, Nucleosil 10 C_{18} (250 × 4.6 mm I.D.); eluent, acetonitrile–aqueous 0.01 *M* ammonium carbonate (9:1); flow-rate, 1.5 ml/min; detection at 280 nm. Compounds: 1 = vincamone (internal standard), 2 = apovincaminic acid ethyl ester (vinpocetine).

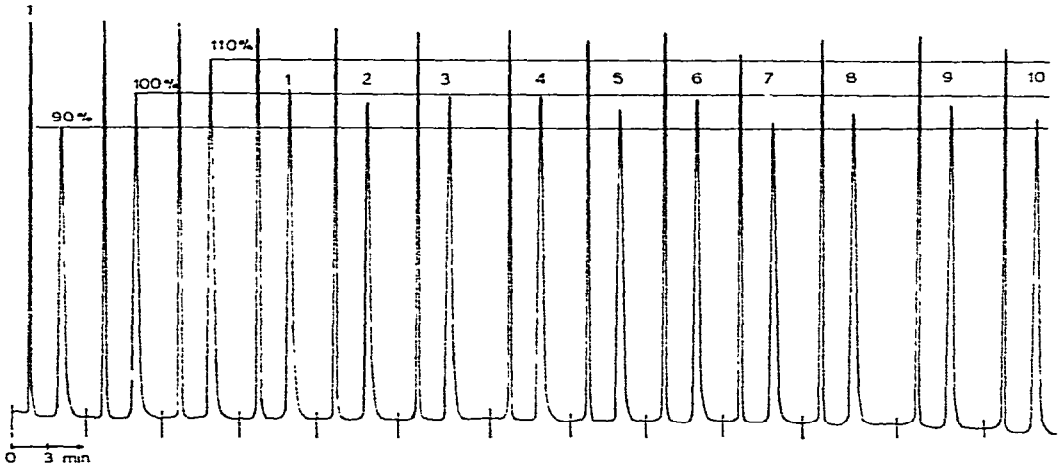


Fig. 4. Content uniformity test on Devincan tablets. Instrument: Varian 8500; column, MicroPak SI-10; eluent, chloroform–ethanol (95:5); flow-rate, 30 ml/h; detection at 275 nm. Compounds: 1 = diazepam (internal standard); 2 = vincamine.

tablets. It can be seen that all the tests can be carried out within a reasonable time, providing a satisfactory separation of the active ingredients and internal standards.

The relative standard deviation of the method calculated on seven parallel runs was 1.17% for Devincan tablets and 1.26% for Cavinton tablets. The detector re-

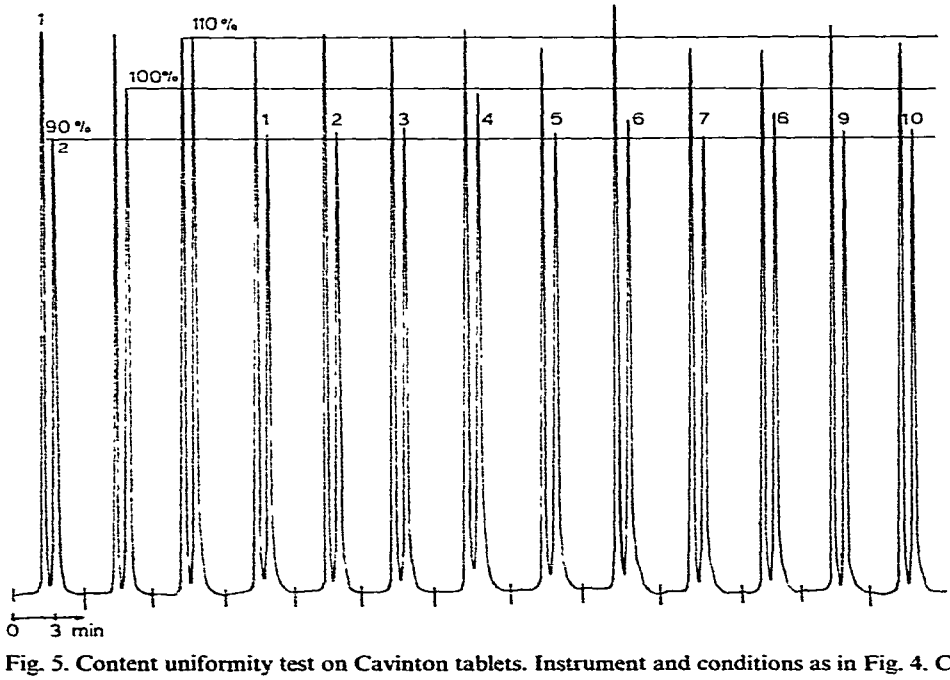


Fig. 5. Content uniformity test on Cavinton tablets. Instrument and conditions as in Fig. 4. Compounds. 1 = diazepam (internal standard); 2 = apovincaminic acid ethyl ester.

sponse is linear over the ranges 0.20–10 μg of vincamine and of 0.05–2.5 μg of apovincaminic acid ethyl ester.

As was mentioned earlier[1,2], none of the methods are suitable for the separation and determination of the corresponding acids of vincaminic and apovincaminic acid esters, which are the possible degradation products of vincamine and apovincaminic acid ethyl ester (vinpocetine) in aqueous solution. In the reversed-phase chromatographic systems used a very weak retention of these acids was observed, while these acids are irreversibly adsorbed on to the silica surface when less polar eluent mixtures were used for their separation. For this reason, when a stability-indicating method was elaborated the polar nature of the acids was decreased by ion-pair formation using conditions such that the separation of other compounds, that do not have acidic character, can also be carried out. We found that octadecylsilica as the stationary phase and acetonitrile-aqueous ammonium carbonate (7:3) as the eluent, containing a small amount of trioctylmethylammonium chloride as an ion-pair reagent, is suitable for stability assay. This is illustrated in Fig. 6, where the separation

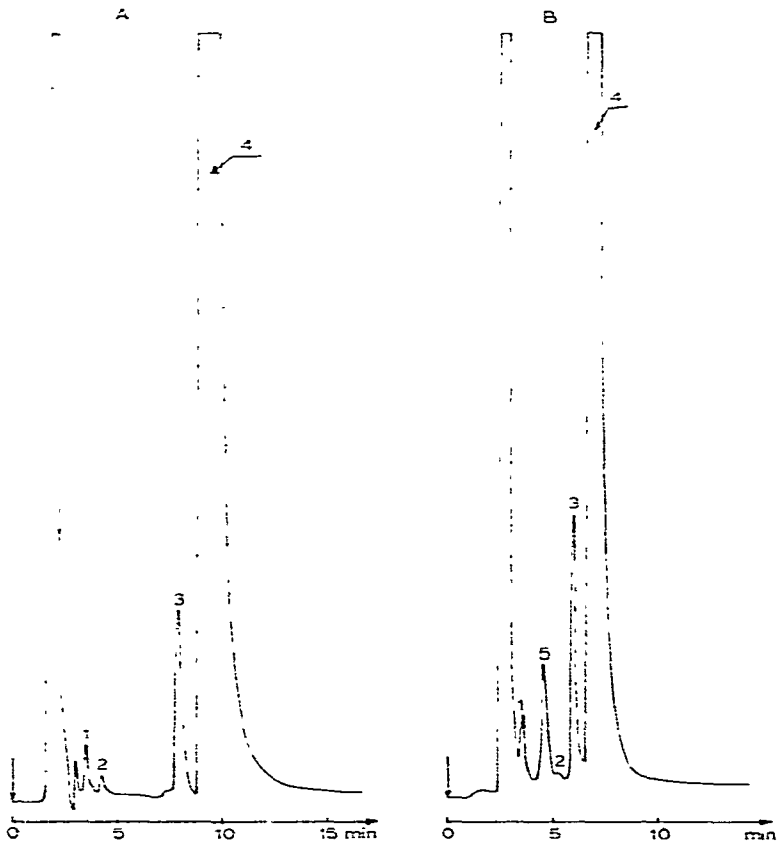


Fig. 6. Separation of vincaminic acid in heat-loaded Devincan injection (keeping the ampoules at 75°C for 24 h). Instrument, Liquochrom Model 2010; column, Nucleosil 10 C_{18}; eluent, (A) acetonitrile–aqueous 0.01 *M* ammonium carbonate (7:3) and (B) acetonitrile–aqueous 0.01 *M* ammonium carbonate (7:3) containing 0.001 *M* trioctylmethylammonium chloride; flow-rate, 1 ml/min; detection at 280 nm. Compounds: 1, 2 = ingredients; 3 = epivincamine; 4 = vincamine; 5 = vincaminic acid.

of a heat-loaded (*i.e.*, keeping the ampoules at 75°C for 24 h) Devincan injection sample is shown in the presence and absence of the ion-pair reagent.

As can be seen in Fig. 6, in the presence of an ion-pair reagent vincaminic acid can be determined without significant disturbance of the selective determination of any other impurities, although the retention of these compounds decreases slightly.

The reversed-phase chromatographic separation of vinpocetine offers a good possibility for its determination in human blood plasma samples. This is illustrated in Fig. 7, using apovincamine as an internal standard.

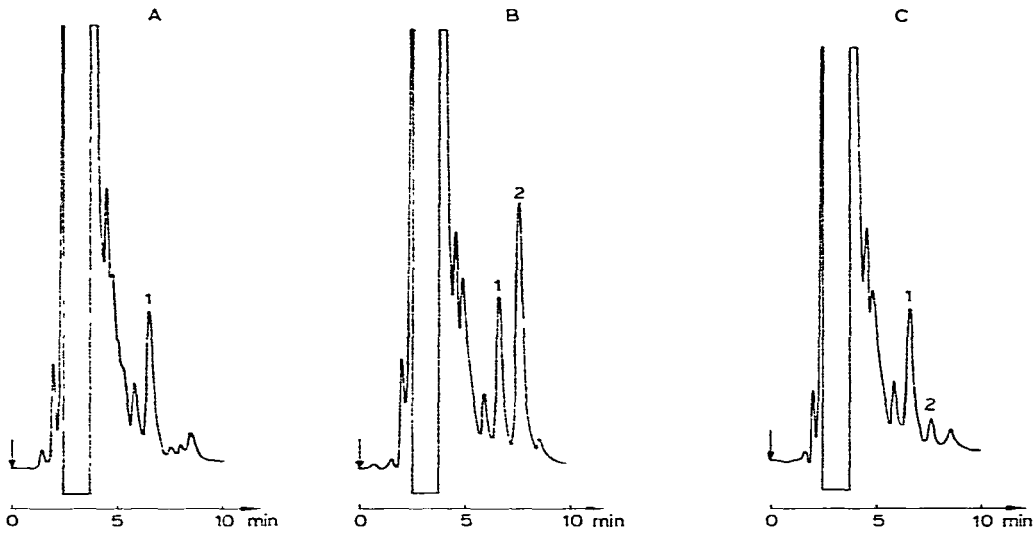


Fig. 7. Determination of vinpocetine in human blood plasma samples. Instrument, Hewlett-Packard 1081/B; column, LiChrosorb RP-8 (250 × 4.6 mm I.D.); eluent, acetonitrile–aqueous 0.01 *M* ammonium carbonate (8:2); flow-rate, 1 ml/min; detection at 254 nm. (A) blank plasma; (B) 5 min after; (C) 60 min after administration of Cavinton injection. Compounds: 1 = apovincamine (internal standard); 2 = vinpocetine. *Sample preparation*[3]: 10 µl (corresponding to 100 ng of apovincamine) of internal standard solution is added to 1.0 ml of human plasma containing sodium citrate as anticoagulant; 0.1 ml of aqueous 1% sodium fluoride, 1 ml of 0.2 *M* glycine buffer solution (pH ≈ 11) and 5 ml of diethyl ether are added and the solution is shaken for 15 min. After centrifugation at 3500 *g* for 10 min at 4°C the organic layer is separated and washed with 1 ml of 0.1 *N* hydrochloric acid; 1 ml of glycine buffer solution is added to the aqueous phase, which is then extracted with 2 ml diethyl ether as above. The organic phases are collected and evaporated to dryness at room temperature. The residue is dissolved in 500 µl of eluent and 100 µl of this solution are injected on to the column. A reference solution is prepared similarly 10 µl of internal standard solution and 2–100 ng of vinpocetine being added to 1 ml of blank plasma; the sample preparation procedure is the same as described above. The recovery of vinpocetine was 71.2 ± 2.8%.

Using this method, a human pharmacokinetic study of Cavinton preparations was carried out; the results will be published separately[4]. The detection limit was 2 ng of vinpocetine in 1 ml of blood.

CONCLUSIONS

When we summarized the main aims of our study, the practical aspects were emphasized. For solving the different analytical problems we have tried both the

reversed-phase ion-suppression and normal-phase chromatographic separation of eburnane alkaloids, and selected the most suitable one. In the separation and determination of more polar acids of ester derivatives of eburnane alkaloids by reversed-phase ion-pair chromatography, only one separation problem remains unsolved, namely the enantiomeric separation of eburnane alkaloids. A new separation system has been developed in our laboratory for this purpose, and will be published separately[5].

ACKNOWLEDGEMENTS

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