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STUDIES ON AMINOPHYLLINE DISPOSITION I. A RAPID AND SENSITIVE HPLC ASSAY FOR ETHYLENEDIAMINE IN PLASMA AND URINE

IAN A. COTGREAVE AND JOHN CALDWELL*

Department of Pharmacology, St. Mary's Hospital Medical School, London W2 1PG, England

ABSTRACT

A simple and rapid assay for ethylenediamine in plasma and urine is described. Ethylenediamine is treated with *m*-toluoyl chloride, yielding its *N,N'*-di(*m*-toluoyl) derivative, which is extracted into dichloromethane and assayed by reversed-phase high pressure liquid chromatography (HPLC) with u.v. detection. Quantitation is achieved with reference to the corresponding derivative of cadaverine as internal standard. The assay is reproducible, and the lower limit of detection is 0·05 μg ml^{-1}. Calibration curves in plasma and urine are linear over the concentration range 0·05–100 μg ml^{-1}. The assay has been applied to the analysis of ethylenediamine in plasma and urine following the administration of aminophylline orally and intravenously to a volunteer.

KEY WORDS Ethylenediamine HPLC assay Human pharmacokinetics

INTRODUCTION

Ethylenediamine (1,2-diaminoethane) is a strong aliphatic base widely used in the chemical and pharmaceutical industries. It finds application in the manufacture of caprolactam polymers, epoxy resins, dyestuffs and EDTA (ethylenediamine tetraacetic acid), and is also present in various cutting oils and wetting agents. Ethylenediamine is used as an excipient in the formulation of various topical pharmaceuticals, notably the triamcinolone acetonide-neomycin sulphate cream marketed by Squibb as Triadcortyl in the U.K. and Mycolog in the U.S.A., and to aid the dissolution of theophylline for oral and intravenous administration. The combination of theophylline with ethylenediamine is the widely used aminophylline, first described by Dessauer in 1908.[1]

Ethylenediamine has a variety of biological actions, most notably the induction of both immediate and delayed allergic hypersensitivity reactions.[2] It

*Addressee for reprints Dr. J. Caldwell, Department of Pharmacology, St. Mary's Hospital Medical School, London W2 1PG.

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enchances the rate and extent of metabolism of theophylline,[3] and *in vitro* shares many of the actions of the biogenic diamine putrescine (1, 4-diaminobutane). Recently, attention has been drawn to the effects of ethylenediamine on the central nervous system, acting as GABA mimetic and CNS depressant.[4] Although the acute toxicity of ethylenediamine assessed by LD_{50} or MLD tests is low, it is now clear that ethylenediamine represents a severe hazard to man when exposure occurs by skin contact or inhalation. With this in mind, regulatory agencies in various countries have imposed a TLV of 10 ppm.[5]

Ethylenediamine is a small, highly polar molecule, which has very little u.v. or visible absorbance and which gives a poor response in a flame ionization detector. Taken together these properties render its assay difficult and to date no method appropriate to its quantitative analysis in body fluids has been reported in the literature. Recently, Ishiguro *et al.*[6] have developed an HPLC assay for ethylenediamine in aminophylline preparations, depending upon its conversion to a 9,10-phenanthroquinone derivative. Ethylenediamine has been used as the internal standard in an assay for various biogenic di- and poly-amines in tissue extracts, this being achieved by HPLC of their dansyl derivatives.[7]

This paper describes an HPLC assay for ethylenediamine in plasma and urine, in which it is converted to its *N,N'*-di-(*m*-toluoyl)-derivative, developed from a method for the analysis of various polyamines in industrial effluents.[8]

The application of the assay to a preliminary study of the pharmacokinetics of ethylenediamine following oral and intravenous administration of aminophylline is described.

MATERIALS AND METHODS

Compounds

[U-^{14}C]-ethylenediamine dihydrochloride, radiochemical purity >99 per cent, sp. act. 25 mCi mmol^{-1}, was purchased from Amersham International, U.K. Ethylenediamine and cadaverine (1,5-diaminopentane), both as free bases >99 per cent pure were purchased from Sigma. *m*-toluoyl chloride, b.p. 86°, was purchased from Aldrich. HPLC grade dichloromethane and acetonitrile were purchased from Fisons, U.K. Aminophylline injection B.P. (250 mg 10 ml^{-1}; Antigen Ltd., Eire, Batch No. E104 52024) and aminophylline tablets B.P. (100 mg; Macarthy Ltd., Romford, U.K., Batch No. BN A007194A) were supplied by the Pharmacy of St. Mary's Hospital.

*N,N'-Di(*m*-toluoyl)-ethylenediamine*

Ethylenediamine (2 g, 33 mmol) was dissolved in 10 per cent aqueous sodium carbonate (20 ml) and *m*-toluoyl chloride (11 g, 71 mmol) added dropwise over 30 min with stirring at room temperature. After 4 h further stirring, the reaction mixture was extracted with dichloromethane (100 ml). The extract was dried over anhyd. Na_2SO_4 and the solvent removed by rotary evaporation. The white

solid residue was recrystallized twice from methanol, giving the title compound as white crystals, m.p. 167°.

Chemical-ionization mass spectrometry (isobutane reagent gas) gave a molecular ion at *m/z* 296, and the electron impact mass spectrum (70 eV) showed a molecular ion at *m/z* 296 with diagnostic ions at *m/z* 162, 148, 134, 119 (base peak) and 91. The proton NMR spectrum (250 MHz in $CDCl_3$, internal standard TMS) showed absorptions at δ (ppm) 2·34 (s, 6, CH_3 Ar), 3·60 (m, 4, R-(CH_2)$_2$-R), 7·26–7·64 (m, 8, *meta* Ar*H*), 7·48 (broad s, 2, R-CON*H*-R).

*N,N'-Di-(*m*-toluoyl)-cadaverine (N,N'-di(*m*-toluoyl)-1,5-diaminopentane)*

Cadaverine (2 g, 19 mmol) was treated with *m*-toluoyl chloride (6·6 g, 42 mmol) as described above for the corresponding derivative of ethylenediamine. The product was obtained initially as an oil which solidified upon storage at −20° for 72 h, and recrystallized from ethanol to give white crystals, m.p. 120°. Chemical ionization-mass spectrometry (isobutane reagent gas) gave a molecular ion at *m/z* 338, and the electron impact mass spectrum (70 eV) showed a molecular ion at *m/z* 338, with prominent diagnostic ions at *m/z* 219, 204, 290, 176, 162, 148, 134, 119 (base peak) and 91. The proton NMR spectrum (250 MHz in $CDCl_3$, internal standard TMS) showed absorptions at δ (ppm) 1·44–1·52 (m,2, α-CH_2), 1·53–1·75 (m,4, β-CH_2), 2·37 (s, 6, CH_3 Ar), 3·44–3·52 (m, 4, γ-CH_2) 6·29 (broad s, 2, R-CON*H*-R) 7·26–7·58 (m, 8, *meta* Ar*H*).

Mass spectrometry

Mass spectra were obtained with the VG ZAB IF instrument of the University of London Intercollegiate Service.

Nuclear magnetic resonance (NMR) spectroscopy

Proton NMR spectra were obtained with the Bruker WM 250 instrument of the University of London Intercollegiate Service.

High pressure liquid chromatography (HPLC)

This used a Rheodyne 7120 valve loop injector an HPLC Technology (Wilmslow, Cheshire, U.K.) RR 015 pump and an Altex 154 u.v. detector equipped with a 254 nm filter. The column was of stainless steel, 100 × 5 mm i.d. packed with ODS-Hypersil 5μ (Shandon Southern Products, Runcorn, Cheshire U.K.). The mobile phase was 40 per cent aqueous acetonitrile, flow rate 0·9 ml min^{-1}. In this system, the *N,N'*-di(*m*-toluoyl)-derivatives of ethylenediamine and cadaverine had retention times 5·3 min and 7·2 min respectively.

Assay of ethylenediamine in plasma

Plasma (1 ml) was spiked with 2 μg cadaverine (20 μl of a 100 μg ml^{-1} solution) as internal standard, 1 ml 10 per cent aqueous trichloroacetic acid added and the whole shaken gently for 20 min and centrifuged at 1000 *g* for 5 min. One millilitre of the supernatant was mixed with 1 ml 10 per cent aqueous

sodium carbonate, 5 mg *m*-toluoyl chloride added (50 µl of a 100 mg ml^{-1} solution in acetone) and the whole shaken gently overnight. The reaction mixture was extracted with 10 ml dichloromethane, the layers separated and the organic phase evaporated to dryness with a stream of air, heating to 40°. The residue was taken up in 50 µl acetonitrile and 10 µl aliquots injected on to the HPLC column.

Assay of ethylenediamine in urine

Preliminary studies in which urine samples were examined by the above procedure revealed the presence of large amounts of interfering substances and the assay was therefore modified. Urine (1 ml) was spiked with 5 µg cadaverine (50 µl of a 100 µg ml^{-1} solution in blank urine) 1 ml 5M-NaOH added and the whole shaken with diethyl ether (10 ml). The layers were separated by centrifugation and the ether discarded. One millilitre aliquots of the aqueous phase were then treated with *m*-toluoyl chloride and assayed as described above for plasma. Control experiments confirmed that neither ethylenediamine nor cadaverine were extracted into ether under these conditions.

Human volunteer studies

A single male volunteer, age 26 yrs, wt. 74 kg, who gave his informed consent, participated in the investigation, which was approved by the Ethical Committee of St. Mary's Hospital and Medical School. He abstained from all foods containing methylxanthines for 72 h prior to, and during each study.

Oral administration

The subject took 43 mg ethylenediamine in the form of 300 mg aminophylline, taken as 3 × 100 mg aminophylline tablets B.P., with water at 9 a.m. on an empty stomach. Food was withheld for 3 h. Blood samples (5 ml) were taken from a vein in the antecubital fossa before dosing and at regular intervals up to 6 h. The 0–24 h urine was also collected.

Intravenous administration

Thirty-six milligrams of ethylenediamine in the form of 250 mg aminophylline, (made by mixing a 10 ml ampoule of aminophylline injection 25 mg ml^{-1} B.P. with 100 ml sterile isotonic saline) was infused into a vein in the right antecubital fossa, over a period of 8 min. Blood samples were withdrawn from a polyethylene cannula placed in the left median cephalic vein, immediately prior to, during, and at the termination of the infusion, and thence at regular intervals up to 6 h. The 0–24 h urine was also collected.

Storage of samples

Blood was placed upon collection in tubes containing lithium heparin as anticoagulant and plasma separated by centrifugation at 1000 *g* for 5 min. Plasma and urine were stored at −20° until analysis. Control experiments

showed that there was no deterioration upon storage under these conditions for at least 1 month.

Assay of ethylenediamine content of administered aminophylline

Four aminophylline tablets from the batch used in this study were individually dissolved with shaking in 100 ml portions of distilled water. Two ampoules of aminophylline injection from the batch used were diluted to 100 ml with distilled water. Aliquots of these solutions were diluted to approx 10 μg ml^{-1} ethylenediamine based on the stated B.P. content of the tablets and injection ampoules and these diluted solutions assayed as described for plasma. The found content was within 2 per cent of the B.P. values, and the dose of ethylenediamine was thus 14·4 mg 100 mg^{-1} aminophylline tablet and 35·6 mg 250 mg^{-1} ampoule of aminophylline injection.

Pharmacokinetic analysis

The decline in plasma concentration of ethylenediamine after the intravenous injections of aminophylline was analysed according to a two-compartment open model using least-squares regression and the method of residuals.[9] Terms were corrected for the duration of the infusion according to Loo and Reigelman.[10]

RESULTS

*Separation of N,N'-di(*m*-toluoyl)-derivatives of ethylenediamine and cadaverine*

The HPLC system described afforded rapid, base-line separation of the *N,N'*-di(*m*-toluoyl)-derivatives of ethylenediamine and cadaverine, and authentic samples of these had retention times 5·3 and 7·2 min respectively. HPLC traces of extracts of blank plasma and urine treated with *m*-toluoyl chloride as described (a) with no additions (b) with ethylenediamine added and (c) with ethylenediamine and cadaverine added are shown in Figures 1 and 2 respectively, which illustrate the base-line separation of ethylenediamine and the internal standard.

It will be seen that blank plasma contains a small amount of a compound identical upon chromatography with the derivative of ethylenediamine (see below).

Calibration curves

Calibration curves of the peak height ratios of the ethylenediamine derivative to that of the internal standard vs. ethylenediamine concentration were constructed by spiking blank plasma and urine over the concentration range 0·55–100 μg ml^{-1} and 1–100 μg ml^{-1} respectively. Figure 3 shows calibration curves over the ranges 0·05–1 μg ml^{-1} from plasma and 1–10 μg ml^{-1} in urine. The instrument response was in both cases linear, with regression coefficients

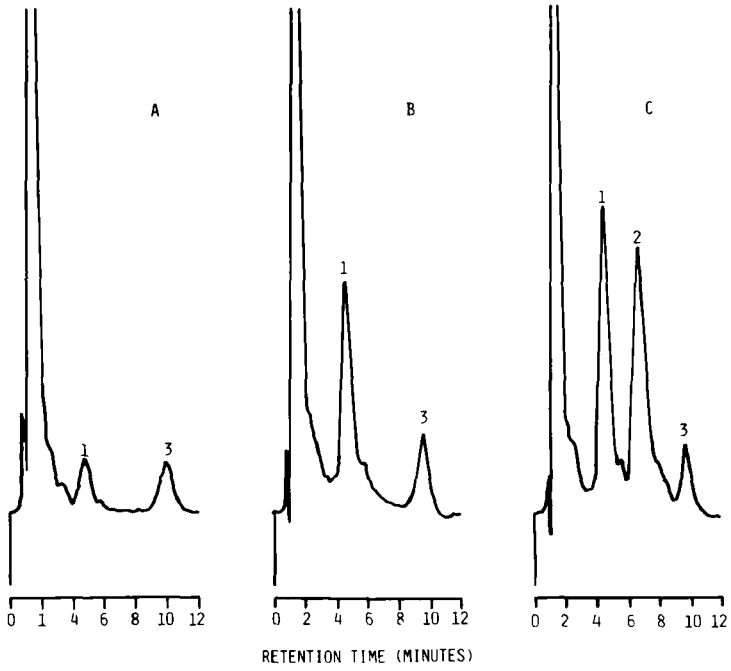

Figure 1. HPLC traces from the analysis of ethylenediamine in plasma, as its *N,N'*-di(*m*-toluoyl)-derivative. Trace A—blank plasma; Trace B—blank plasma spiked with 0·5 μg ml^{-1} ethylenediamine; Trace C—blank plasma spiked with 0·75 μg ml^{-1} ethylenediamine and 2 μg ml^{-1} cadaverine. Peak 1, *N,N'*-di(*m*-toluoyl)-ethylenediamine, peak 2, *N,N'*-di(*m*-toluoyl)-cadaverine, peak 3, *m*-toluic acid. Detector sensitivity 0·04 AUFS, other details as in text

always >0·995. It will be noted that in neither case does the line pass through the origin, suggesting the presence of small amounts of ethylenediamine either occurring endogenously in plasma and urine or in the solutions used during the derivatization procedure. The background of ethylenediamine remained even when all solutions were made using double distilled deonized water.

Sensitivity and reproducibility

The minimum concentrations of ethylenediamine which may be reliably assayed by this method are 0·05 μg ml^{-1} in plasma and 0·5 μg ml^{-1} urine, in both cases limited by the background of ethylenediamine. The reproducibility of assay on a given day in plasma was ±7 per cent 0·1 μg ml^{-1} and ± 3 per cent at 5 μg ml^{-1}, and in urine ± 5 per cent at 1 and 10 μg ml^{-1} ($n = 4$, in all cases). Day-to-day variability of the calibration curves was never greater than ±5 per cent at all concentrations ($n = 4$).

Absolute recovery

The absolute recovery of ethylenediamine as its *N,N'*-di(*m*-toluoyl) derivative through this assay was 79 per cent ± 6 per cent at 0·1 μg ml^{-1} and 64 per cent

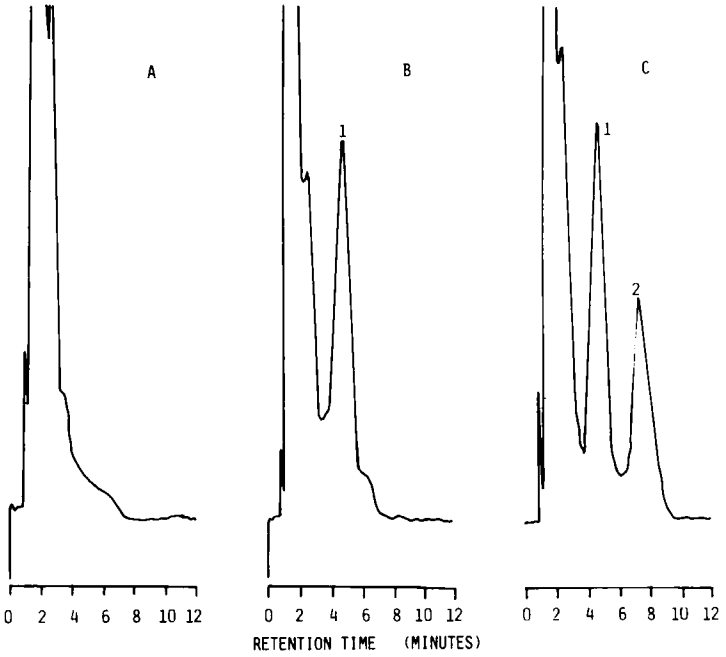

Figure 2. HPLC traces from the analysis of ethylenediamine in urine, as its *N,N'*-di(*m*-toluoyl)-derivative. Trace A—blank urine; Trace B—blank urine spiked with 2·5 μg ml^{-1} ethylenediamine; Trace C—blank urine spiked with 2·5 μg ml^{-1} ethylenediamine and 5 μg ml cadaverine. Peak 1, *N,N'*-di(*m*-toluoyl)-ethylenediamine, peak 2, *N,N'*-di(*m*-toluoyl)cadaverine. Detector sensitivity 0·08 AUFS, other details as in text

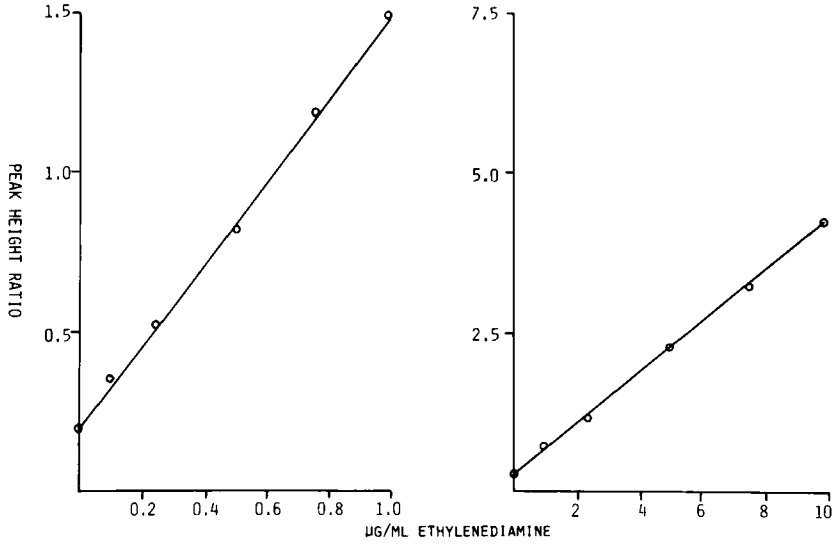

Figure 3. Calibration curves for the assay of ethylenediamine in plasma (left-hand graph) and urine (right-hand graph). The ratios of the peak heights of the *N,N'*-di(*m*-toluoyl)-derivatives of ethylenediamine and cadaverine are plotted againts ethylenediamine concentration, $r = 0·995$ for plasma, $r = 0·996$ for urine

at 2 per cent at $1\,\mu g\,ml^{-1}$ ($n = 4$, in each case) established by the use of ^{14}C-ethylenediamine.

Plasma concentrations of ethylenediamine after the administration of aminophylline

Plasma concentrations of ethylenediamine after the administration of 43 mg orally and 35 mg by intravenous infusion, both in the form of aminophylline, are shown in Figure 4. After intravenous infusion, a peak level of $2{\cdot}4\,\mu g\,ml^{-1}$ was achieved at the termination of the infusion, and plasma concentrations fell rapidly thereafter in a biphasic fashion. Ethylenediamine could not be detected i.e. levels less than $0{\cdot}05\,\mu g\,ml^{-1}$, in any blood sample taken after 180 min following the end of the infusion. Pharmacokinetic parameters describing the behaviour of ethylenediamine in plasma are listed in Table 1. After oral adminstration, ethylenediamine was present at low levels in the samples taken from 10–180 min but later, samples contained only background amounts. These data are illustrated in Figure 4. Only limited pharmacokinetic analysis could therefore, be performed, but the area under the plasma level–time curve was estimated to be $29\,\mu g\,ml^{-1}$. min from the available data.

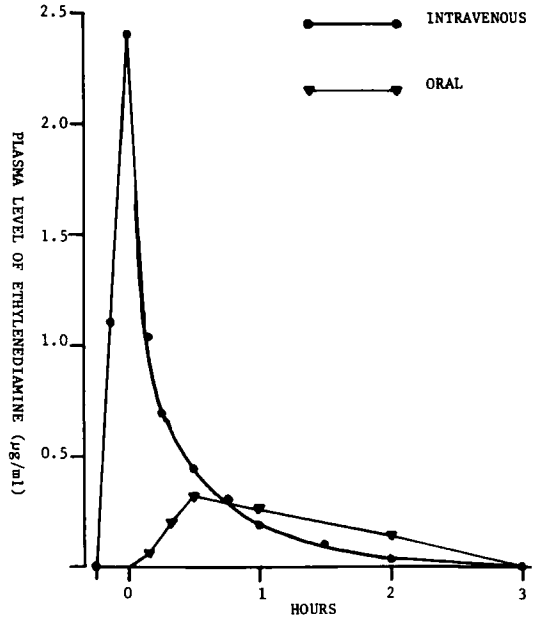

Figure 4. Plasma concentration–time curves for ethylenediamine after the administration of 36 mg (as 250 mg aminophylline) intravenously, and 43 mg (as 300 mg aminophylline) orally, to a volunteer

Urinary excretion of ethylenediamine

Recovery of ethylenediamine in the 0–24 h urine totalled 13 per cent of dose after intravenous infusion and 3 per cent after oral dosage.

Table 1. Pharmacokinetic parameters describing the disposition of ethylenediamine following the intravenous infusion of 36 mg as 250 mg aminophylline B.P. to a single volunteer

Parameter	Value
A ($\mu g\,ml^{-1}$)	1·40
α (min^{-1})	0·165
$T_{\frac{1}{2}}\alpha$ (min)	4·0
B ($\mu g\,ml^{-1}$)	1·0
β (min^{-1})	0·025
$T_{\frac{1}{2}}\beta$ (min)	28·0
V_p ($ml\,kg^{-1}$)	124·0
V_c ($ml\,kg^{-1}$)	190·0
Cl ($ml\,min^{-1}\,kg^{-1}$)	7·4
AUC ($\mu g\,ml^{-1}$. min)	63·0
K_e (min^{-1})	0·038

DISCUSSION

The analytical method presented in this paper is the first reported procedure for the assay of ethylenediamine in body fluids. By virtue of the simple, direct derivatization step performed in the body fluid, the need to extract ethylenediamine is avoided, and the favourable solubility characteristics of the derivative allow its facile one-step extraction into organic solvent. The derivative is amenable to simple and rapid reversed phase HPLC. Calibration curves were linear and highly reproducible over a wide range of concentrations and the method is sufficiently sensitive for the assay of levels as low as 50 $ng\,ml^{-1}$.

The applicability of the assay to pharmacokinetic studies of ethylenediamine in man is described. When given intravenously, the plasma levels of ethylenediamine fall with time in a bi-exponential fashion and the data have been analysed according to a two-compartment open model. However, after oral administration, the plasma levels of ethylenediamine were low, indicating the poor biovailability (approximately 35 per cent) of this compound. Recovery of unchanged drug in the urine totalled 13 per cent after intravenous and 3 per cent after oral administration.

The analytical method is being applied to further studies of ethylenediamine disposition in animals and man. In addition, the method is also suitable for the assay of ethylenediamine in pharmaceutical products and may be appropriate for such assays in samples of environmental interest. In view of the use of the biogenic diamine cadaverine as internal standard, it is also suggested that this procedure may provide the basis for rapid and sensitive assays for polyamines of biological interest.

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