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Gas chromatography nitrogen phosphorous detection (GC-NPD) assay of tofisopam in human plasma for pharmacokinetic evaluation

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Abstract

Tofisopam (1-(3,4-dimethoxyphenyl)-4-methyl-5-ethyl-7,8-dimethoxy-5H-2,3-benzodiazepine) has been shown to be an effective anxiolytic agent in the wide-ranging clinical practice. A high sensitive gas chromatography nitrogen phosphorous detection (GC-NPD) bioanalytical method was developed and validated for the purpose of pharmacokinetic study of tofisopam. A liquid–liquid extraction method was used for the sample preparation. The mean recovery for tofisopam was 69.8% and the inter- and intra-day precision values were well below the 15% limit established for bioanalytical methods. A similar compound, girizopam was used as internal standard. The assay was linear in the 5–500 ng/ml range corresponding to therapeutically relevant plasma levels. The concentrations of the compound were measured in the plasma samples of 12 healthy male volunteers and the pharmacokinetic parameters were determined from the plasma concentration–time data. A rapid absorption and distribution, relatively short biological half-life and considerable inter-individual variation in the plasma concentration levels of parent compound were the main characteristics of the pharmacokinetics of tofisopam. According to these results, the new (GC-NPD) bioanalytical method proved to be capable of measuring concentration of tofisopam in human plasma and was successfully applied in a single dose pharmacokinetic study.

Keywords: Gas chromatography nitrogen phosphorous detection (GC-NPD); Tofisopam; Pharmacokinetics; Single dose clinical study

1. Introduction

Tofisopam (1-(3,4-dimethoxyphenyl)-4-methyl-5-ethyl-7,8-dimethoxy-5H-2,3-benzodiazepine; Fig. 1a) synthesized by EGIS Pharmaceuticals Ltd. (Budapest, Hungary) differs from the traditional 1,4-benzodiazepines regarding the positions of the nitrogen atoms. Tofisopam molecules in solution exist in two conformations as it was confirmed by NMR studies [1]. The ratio of conformers, which can be separated by liquid chromatography, is a function of time, temperature and solvent [2]. In gas phase only one conformer is present (one gas chromatographic peak).

Tofisopam does not bind to the GABA receptors in the brain, and does not replace labelled benzodiazepines from their binding sites [3]. It has been postulated that tofisopam modulates the benzodiazepine-GABA chloride channel ionophore complex [4].

Tofisopam is an orally active anxiolytic agent [5,6]. It has favourably influenced the signs of vegetative dysregulation due to a variety of reasons such as alcohol withdrawal [7], menopause [8], mood and anxiety disorders [9], neurological diseases, irritable colon syndrome [10]. Contrary to the classical 1,4-benzodiazepines, tofisopam does not cause impairment in the cognitive [11,12] and psychomotor task [13,14], and dependence has not been observed even in long-term therapy.

For the determination of tofisopam in biological fluids (among them human plasma) various techniques have been described. Thin layer chromatography, reversed and normal phase liquid chromatography, gas liquid chromatography with mass spectrometry or nitrogen phosphorous detection (NPD) were shown to be suitable for the analysis [15–17], but none

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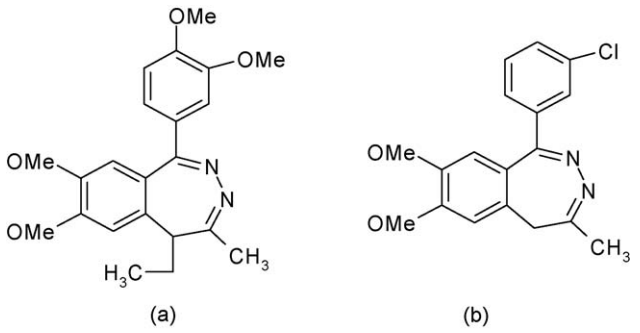

Fig. 1. Chemical structure of investigated compounds: (a) tofisopam; (b) girizopam (internal standard).

of the GC assays were validated according to requirements of a pharmacokinetic study and GLP regulations. The aim of the present study was to develop and validate a gas chromatographic bioanalytical method for the determination of tofisopam in human plasma for pharmacokinetic study purpose.

The applicability of the new GC-NPD method in a single dose pharmacokinetic study of tofisopam is demonstrated in this paper.

2. Materials and methods

2.1. Materials and reagents

Tofisopam and internal standard girizopam were obtained from EGIS Pharmaceuticals Ltd. (Budapest, Hungary). Methanol and ethyl acetate from Scharlau Chemie SA (Barcelona, Spain), *n*-hexane and dichloromethane were purchased from Merck (Darmstadt, Germany). Sodium carbonate and sodium bicarbonate were obtained from Reanal Ltd. (Budapest, Hungary). Stock solutions and other aqueous solutions were prepared using Milli-Q ultra pure water (Millipore, Bedford, USA) after proper quality control by UV-spectrophotometer and built in conductivity test.

2.2. Sample extraction

Fifty microliters of internal standard solution (5000 ng/ml) was added to 1 ml of plasma sample in a 10 ml ground stoppered glass extraction tube. After homogenisation 2 ml 0.1 M carbonate buffer (pH 9.2) and 4 ml dichloromethane/hexane, 1/1 (v/v) mixture were added. Sodium carbonate solution was prepared by dissolving 10.6 g (0.1 M) sodium carbonate in 1 l MilliQ water and sodium bicarbonate solution was prepared by dissolving 840 mg (0.1 M) sodium bicarbonate in 100 ml MilliQ water. Samples were manually extracted by shaking for about 1 min. After it, the tubes were centrifuged at 3000 rpm for 10 min, and then the organic phase was transferred into clean 10 ml ground stoppered tubes. The organic phase was evaporated to dryness under nitrogen stream at around 40 °C. The residue was dissolved in 50 μl ethyl acetate, vortexed and then pipetted into inserts of sample vials and placed in the autosampler.

2.3. Chromatographic system

Chromatographic analysis was carried out using HP 5890 Series II gas chromatograph equipped with a HP 7673 autosampler and a nitrogen-phosphorous detector. The separation was performed on a Supelco SPB-1™ (25 m × 0.32 mm × 0.25 μm) capillary column connected to a HP retention gap (5 m × 0.32 mm). The injector and detector were operated at 270 °C and 280 °C, respectively. Four microliters of the processed samples were injected in split mode (split flow: 20 ml/min). Helium (He) was used as carrier gas and its pressure program was as follows: 340 kPa (0.05 min), 500 kPa/min: 150 kPa (13.57 min). The oven temperature was programmed as follows: 260 °C for 10 min, increasing to 280 °C (40 °C/min) and holding for 3.5 min. For the detection He was used as make up gas at a flow rate of 30 ml/min, airflow was 100 ml/min and H_2 flow was set of 3.8 ml/min. The total run time was 14 min.

2.4. Method validation

Recovery of tofisopam was tested at 400, 50 and 12.5 ng/ml concentration levels and it was calculated by comparing the peak area values obtained in the test of intra-day repeatability to the peak area values of reference standard solutions (100%). Recovery of the internal standard was also determined at 250 ng/ml. The linearity of the calibration was tested by the analysis of six different calibration series. Calibration curves were constructed by fitting a straight line using the least squares method and $1/y^2$ as weighting factor on the calibration points. For the calibration the peak area ratio values were plotted in the function of tofisopam concentration. Lower limit of quantitation (LLOQ) was defined as the lowest concentration of the calibration curve that can be determined with not higher than ±20% accuracy and precision values. Lower limit of detection (LOD) is defined as the lowest solute concentration for which the signal to noise ratio is at least 3:1. For the determination of inter-day precision and accuracy 6 calibration and QC series were analysed on six different days. The intra-day precision and accuracy were tested with analysis of five parallel samples at 400 ng/ml, 50 ng/ml and 12.5 ng/ml concentrations levels.

2.5. Pharmacokinetic study

2.5.1. Study design and sample collection

This was a single-centre, open-label pharmacokinetic study. The Hungarian National Institute for Pharmacy and the Local Ethical Committee of Hungarian Railways Hospital (Budapest, Hungary) approved the study protocol. The study was conducted in accordance with the Declaration of Helsinki (Edinburgh Amendment 2000) and each volunteer provided informed consent before entering the study. Twelve healthy Caucasian non-smoking male volunteers, ranging from 18 to 40 years of age, and body mass index within 19–26 kg/m^2 were enrolled in the study. Medical history, physical examination, ECG and clinical laboratory tests including HIV, hepatitis B and C tests and

urine drug screen within 2 weeks prior to inclusion to the study was evaluated for declaration the healthy status and a follow-up visit was performed 2–7 days after the study drug intake. The volunteers were not allowed to use any medication within 14 days before and during the study, only single doses of 500 mg paracetamol was permitted during the restriction period. The volunteers were not allowed to drink alcohol within 48 h preceding the treatment as well as during the entire study. Ingestion of beverages containing xanthine and chocolate was prohibited 48 h preceding and following drug administration. The volunteers had to avoid excessive physical exercises all over the study.

Eligible subjects were admitted to the study site on the day before dose administration, and they stayed there up to 24 h following the drug administration. Before drug administration, blood drawing ("0" time-sample), and safety measurements – blood pressure (BP) and heart rate (HR) – were performed. The subjects were given two tablets (2 × 50 mg tofisopam) between 8 and 9 a.m. after an overnight fast. Safety (BP, HR) measurements were performed 1.5 and 24 h after the single dose of tofisopam. Blood samples (7.5 ml) for tofisopam analysis were collected in the morning just before the tofisopam dose (0) and 0.25 h, 0.5 h, 0.75 h, 1 h, 1.33 h, 1.66 h, 2 h, 2.5 h, 3 h, 3.5 h, 4 h, 5 h, 6 h, 8 h, 10 h, 12 h, 16 h, 24 h, 36 h, 48 h after the tofisopam administration. Blood samples were collected either by venipuncture or through an indwelling plastic cannula inserted into a vein of the upper arm into tubes (S-Monovettes, Sarstedt, Nuembrecht, Germany) containing anticoagulant (K-EDTA). Blood samples were centrifuged at 3000 rpm at room temperature and plasma was separated within 15 min after venipuncture. The separated plasma was aspirated off and filled into test tubes for plasma samples. Each tube was labelled with study code, subject identification (treatment number and initials), date and time of sampling and signature of the person taking the blood. The plasma samples were stored at −20 °C or colder.

2.5.2. Pharmacokinetic analysis

The pharmacokinetic parameters were determined from the plasma concentration–time data using model independent methods and two compartment model using Kinetika™ Ver. 4.0.2 validated pharmacokinetic software package (Inna Phase Corp., Philadelphia, PA, USA).

2.5.3. Pharmacokinetic parameters calculated by model independent methods

The maximum plasma concentration (C_{max}) and the time to reach the maximum plasma concentration (t_{max}) were determined from the observed values. The area under the plasma concentration–time curve from 0 to the last measurable time point (AUC_{0-t}) was calculated by trapezoidal rule. The area under the plasma concentration–time curve from time 0 extrapolated to infinity was calculated according to the Eq. (1), where C_t and β represent the observed plasma concentration at the last measurable sampling time and the elimination rate constant, respectively. The elimination rate constant was determined as negative of the slope at the log-linear terminal part of the plasma concentration–time curve using linear regression.

$$AUC_{0-\infty} = AUC_{0-t} + \frac{C_t}{\beta} \quad (1)$$

The mean residence time (MRT) was calculated using Eq. (2), where AUMC is the area under the first moment curve.

$$MRT = \frac{AUMC}{AUC_{0-\infty}} \quad (2)$$

2.5.4. Pharmacokinetic parameters calculated by compartment model

Plasma concentration (C_p) data after the single dose tofisopam were fitted to the Eq. (3) using the Powell algorithm.

$$C_p = Ce^{-k_a t} + Ae^{-\alpha t} + Be^{-\beta t} \quad (3)$$

The concentrations were interpreted as arising from the two compartment open model, when k_a, α and β are the hybrid rate constants of absorption, distribution and elimination segment, respectively, and C, A and B are intercept of the mono-exponential k_a, α and β line with ordinate, respectively. The hybrid rate constants were determined by curve-feathering methods. The half-life of distribution and elimination were calculated by Eqs. (4) and (5), respectively.

$$t_{1/2}^{\alpha} = \frac{0.693}{\alpha} \quad (4)$$

$$t_{1/2}^{\beta} = \frac{0.693}{\beta} \quad (5)$$

The t_{max} and the lag time (t_{lag}) were calculated according the Eqs. (6) and (7) where k_a and β are the absorption and elimination rate constants, respectively.

$$t_{max} = \frac{2.303}{k_a - \beta} \log \frac{k_a}{\beta} \quad (6)$$

$$t_{lag} = \frac{\log C - \log B}{(k_a/2.303) - (\beta/2.303)} \quad (7)$$

The AUC and AUMC were calculated according to Eqs. (8) and (9), respectively.

$$AUC = \sum C_p dt \quad (8)$$

$$AUMC = \sum C_p dt \times t \quad (9)$$

Apparent volume of distribution (V_d) was determined by Eq. (10) assuming complete absorption ($F = 1$).

$$V_d = \frac{FD}{AUC\beta} \quad (10)$$

Apparent oral clearance (Cl_{oral}) was calculated by dividing administered dose (D) by AUC according Eq. (11). In the calculation of this data complete absorption ($F = 1$) was assumed.

$$Cl_{oral} = \frac{FD}{AUC} \quad (11)$$

The mean residence time (MRT) was calculated according to Eq. (12).

$$\mathrm{MRT} = \frac{\mathrm{AUMC}}{\mathrm{AUC} - (\mathrm{lag} + 1/k_a)} \quad (12)$$

2.5.5. Statistical analysis

Only observed data were evaluated, no specific procedures were carried out due to missing data. Calculations of the pharmacokinetic variables were based on data sets with complete relevant data. The statistical analyses of safety parameters were performed according to the intention to treat (ITT) principle, i.e. all recruited study volunteers were included in the analyses. Descriptive statistics for statistical analysis was used for safety, demographic and pharmacokinetic parameters.

3. Results and discussion

3.1. Bioanalytical method and method validation

The concentration of tofisopam in plasma was determined by gas chromatography—nitrogen phosphorous detection (GC-NPD) method. Nitrogen content of the target compound allowed the use a NP detection, which enabled the development of a selective and sensitive assay. Girizopam (Fig. 1b), a structurally similar compound of EGIS Pharmaceuticals was used as internal standard for quantitative determination. Girizopam fulfilled the requirements of an internal standard in the sense of similar extraction and chromatographic behaviour and as compound of EGIS it was readily available. The peak area ratio of tofisopam and the internal standard was used for quantitation. A liquid–liquid extraction method was elaborated to recover and concentrate the compounds of interest from human plasma. Tofisopam was extracted from plasma at basic pH with hexane–dichloromethane (50:50, v/v) and analysed directly without derivatization. Typical chromatograms of blank plasma, plasma sample obtained from volunteer 05 after 0.5 h of drug administration and plasma spiked with tofisopam (150 ng/ml) are shown in Fig. 2. There were no interfering endogenous components in the retention time window of tofisopam as it was revealed by the comparison of the chromatograms of blank plasma and plasma spiked at LLOQ (5 ng/ml) (Fig. 3). Validation of the method (recovery, accuracy, system suitability, stability in plasma and solutes, intra- and inter-day precision, etc.) was carried out in accordance with the latest international requirements. The efficiency of the extraction procedure was evaluated by means of the recovery, which was for tofisopam and internal standard 69.8% and 71.4%, respectively. For all the calibration samples analysed, the accuracy value never exceeded the acceptable limits (15% or 20% at the 5 ng/ml) and the correlation coefficient of the individual calibrators were always higher than 0.995. The assay was linear in the 5–500 ng/ml range corresponding to therapeutically relevant plasma levels. The inter- and intra-day precision values of the assay were well below the 15% limit established for bioanalytical methods and are shown in the Table 1. LOD for tofisopam was 2 ng/ml, therefore this method can be well used to characterize the pharmacokinetics of tofisopam.

Table 1
Reproducibility of measurement of tofisopam in human plasma samples

Theoretical concentration (ng/ml)	Mean concentration found ± S.D. (ng/ml)		CV (%)	
	Within-day ($n = 5$)	Day-to-day ($n = 16$)	Within-day	Day-to-day
12.5	12.23 ± 0.70	13.19 ± 0.62	5.69	4.68
50	50.69 ± 3.49	51.30 ± 2.54	6.89	4.95
400	445.04 ± 8.36	422.38 ± 27.69	1.88	5.60

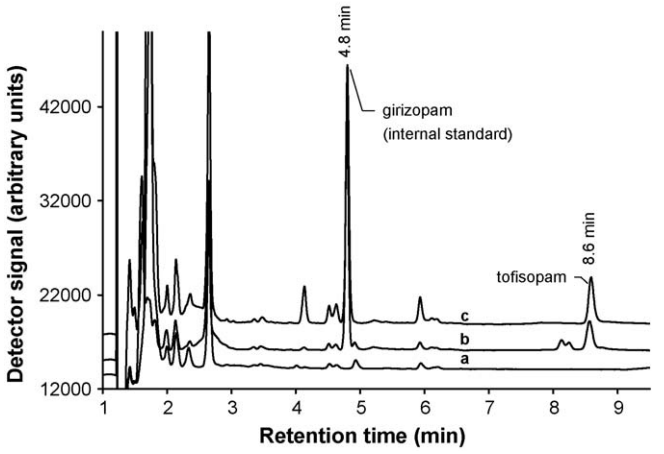

Fig. 2. Typical chromatograms of blank plasma (a), plasma sample obtained from volunteer 05 after 0.5 h of drug administration (b) and plasma spiked with tofisopam (150 ng/ml) (c).

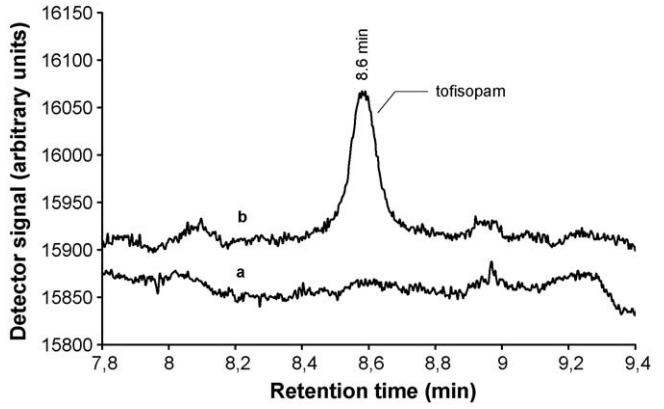

Fig. 3. Chromatograms of blank plasma (a), and plasma spiked at LLOQ (5 ng/ml tofisopam) (b) in the retention time window of tofisopam.

3.2. Pharmacokinetic study

In this pharmacokinetic (PK) study following intake of 100 mg tofisopam, the plasma concentration values of tofisopam were quite variable (highly variable drug). Mean plasma concentrations measured at different time points after the administration of tofisopam are shown in Fig. 4. As in several cases the two-compartment open model could not be applied correctly,

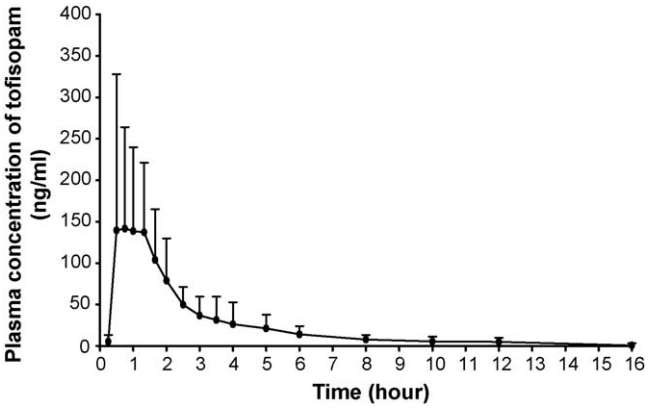

Fig. 4. Pharmacokinetic profile of tofisopam after oral administration of 100 mg ($n = 12$, mean ± S.D.).

the characterisations of the main pharmacokinetic parameters of tofisopam are based on the non-compartment analysis presented in the Table 2. Nevertheless, the results from compartment analysis correlated extremely well with data from non-compartment calculation; therefore, they are used as supportive data. The maximum measured plasma concentration ranged from 40.9 ng/ml to 655.6 ng/ml. The average value of C_{max} was somewhat higher than that observed in most of the other studies [18]. The relatively small t_{max} values (ranging from 0.5 h to 3.5 h), which with the short absorption half-life ($t^{k_a}_{1/2}$) and absorption rate constant (k_a) values indicated a rapid absorption process. Only one volunteer showed a delayed absorption and C_{max} was reached 3.5 h after oral administration. In the clinical and laboratory data there were no any explanation of this atypical absorption profile, but this phenomenon also occurred in a previous study [18]. $AUC_{0-\infty}$ values were somewhat less variable it ranging from 193.7 ng h/ml to 780.0 ng h/ml. The AUC value was higher than that observed in most of the other studies [18,19]. The difference may be consequence partly of the higher sensitivity of the current technique. The AUC_{Rest} values for the parent compound were lower than the 20% limit in all but one case. The apparent volume of distribution (V_d) ranged from 523.8 l^{-1} to 5154.1 l^{-1} indicating that tofisopam has higher concentration in extravascular tissue than in the vascular compartment. However, in the calculation of V_d, 100% bioavailability was postulated as no information about the human bioavailability of the compound is known. The distribution phase of tofisopam was found to be relatively rapid according to the alpha and $t^{\alpha}_{1/2}$ values resulting from compartment analysis. Among the typical parameters of elimination phase, the oral clearance (Cl_{oral}), and elimination half life ($t^{\beta}_{1/2}$) were found to be ranging from 178.8 l/h to 516.3 l/h and from 2.02 h to 8.09 h, respectively. The elimination hybrid rate constant (β) of tofisopam was relatively small (0.198 ± 0.081 1/h).

Table 2
Pharmacokinetic constants and parameters of tofisopam calculated by non-compartment model ($n = 12$) and two-compartment model ($n = 6$)

Pharmacokinetic parameters	Calculated by non-compartment model ($n = 12$) Mean ± S.D.	Calculated by two-compartment model ($n = 6$) Mean ± S.D.
C_{max} (ng/ml)	230.5 ± 162.6	328.8 ± 280.0
t_{max} (h)	1.15 ± 0.81	0.60 ± 0.34
AUC_{0-t} (ng h/ml)	382.0 ± 171.9	432.6 ± 224.9
$AUC_{0-\infty}$ (ng h/ml)	422.1 ± 172.5	–
AUC_{Rest} (%)	10.9 ± 6.6	–
$AUMC_{0-\infty}$ (ng h h/ml)	1774.2 ± 983.9	1247.9 ± 584.8
k_a (1/h)	–	31.06 ± 33.33
α (1/h)	–	1.627 ± 0.488
β (1/h)	0.19 ± 0.081	0.21 ± 0.085
$t^{k_a}_{1/2}$ (h)	–	0.10 ± 0.12
$t^{\alpha}_{1/2}$ (h)	–	0.46 ± 0.13
$t^{\beta}_{1/2}$ (h)	4.12 ± 1.82	4.11 ± 2.55
t_{lag} (h)	–	0.37 ± 0.18
MRT (h)	4.34 ± 2.39	2.8 ± 2.0
Cl_{oral} (l/h)	279.2 ± 121.4	280.4 ± 120.3
V_d (l)	1679.3 ± 1235.0	1842.7 ± 1818.6

All data from each 12 volunteers were included in the safety analyses. Extensive evaluation of vital signs, ECG, and physical findings did not indicate toxicity associated with tofisopam administration. The results of the post-study laboratory tests confirmed absence of significant changes in the volunteers' state of health and the drug is safe administered in 100 mg as a single dose.

In conclusion, a new, simple GC procedure with a nitrogen phosphorus detector (NPD) was developed and validated for the determination of tofisopam level in the plasma. To our knowledge no other GC method for the determination of tofisopam in human plasma has been reported which was validated in accordance with GLP regulations and for purpose of pharmacokinetic study. The simple GC-NPD assay presented in this paper involves simple and cost effective liquid–liquid extraction and meets the requirements of pharmacokinetic applications. The method has shown advantages such as sensitivity, reproducibility and speed. The applicability of the new bioanalytical method was demonstrated in a study for the evaluation of tofisopam single dose pharmacokinetics. According to the results tofisopam can be characterised with rapid absorption and distribution, relatively short biological half-life and considerable inter-individual variation in the plasma concentration levels.

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