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Influence of the stationary phase on the stability of thalidomide and comparison of different methods for the quantification of thalidomide in tablets using high-temperature liquid chromatography

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Abstract

In this paper, three different HPLC methods for the quantification of thalidomide in tablets were developed and compared. The comparison of a conventional method at 30 °C with two high-temperature methods at 180 °C showed equal results. Using high-temperature HPLC (HT-HPLC), faster analysis times could be achieved. We have also focused on analyte stability and could show that the stationary phase has a pronounced effect on the on-column degradation of thalidomide at high temperatures. Virtually no degradation occurs if a polystyrene divinylbenzene column is used, whereas thalidomide is completely degraded at 180 °C when a carbon clad zirconium dioxide column is used.

Keywords: High-temperature liquid chromatography; Active pharmaceutical ingredient; Thalidomide; Superheated water chromatography; Fast chromatography

1. Introduction

In the last few years chromatographic separations have been optimised towards a higher sample throughput. There are different approaches to achieve this aim. One option is the use of ultra high pressures up to 1000 bar with short columns and particle sizes smaller than 2 μm [1–8]. Lee and co-workers [3] even constructed a system which was able to generate 2413 bar, resulting in extremely high theoretical plate numbers per meter. However, conventional analytical pumps are only able to deliver the solvents with a maximum pressure up to 400 bar. Therefore, it is necessary to upgrade the HPLC system if pressures above 400 bar will be applied. In contrast to this, the high-temperature approach makes use of elevated temperatures to which the mobile phase and the stationary phase are heated [9–11]. The useful temperature range extends to 250 °C using ordinary binary solvent mixtures consisting of water and an organic co-solvent [12,13]. A variety of temperature stable columns based on metal oxides [14,15] or a polymeric material [16] are commercially available [17]. High-temperature HPLC (HT-HPLC) can be performed on every HPLC system. The only prerequisite is the integration of a specially designed heating system [18]. As opposed to pressure, temperature can be regarded as a universal parameter which can be used to not only accelerate the separation, but also to optimise the selectivity of the phase system [12,19–26]. Moreover, the efficiency can be increased by changing the eluent temperature [13,21,24,27–29]. A concomitant advantage is that the overall system pressure is reduced by a pronounced decrease of the mobile phase viscosity [30], which offers the potential to use sub-2 μm particle packed columns at moderate flow rates without exceeding the pressure limit of conventional HPLC systems. In this study we focused on the high-temperature approach to highlight these important advantages of this technique, which we think will play an ever-greater role in the future.

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In the University Hospital of Heidelberg, a study was initiated to assess the value of thalidomide in the maintenance therapy of multiple myeloma. Thalidomide was originally introduced as a non-barbiturate hypnotic, but withdrawn from the market due to teratogenic effects. Besides its sedative effect, thalidomide has various mechanisms of action that have not as yet been fully understood [31]. For example anti-angiogenic, immunomodulatory as well as apoptotic effects have been demonstrated on myeloma and plasma cells [32]. Therefore, thalidomide is considered for use as a maintenance therapy in multiple myeloma [33].

Three different methods for the quantification of thalidomide in tablets were developed and compared. The aim was to investigate if temperature has a negative effect on the quantification when the elution is carried out at high eluent temperatures. Moreover, an elution using a water-only mobile phase should be attempted to evaluate if this method can also be transferred to new hyphenation techniques in the future.

2. Experimental

2.1. Chemicals

Acetonitrile (Optigrade) was purchased from LGC Promochem (Wesel, Germany) and Merck (HPLC gradient grade, Darmstadt, Germany). Formic acid was purchased from Sigma–Aldrich (Seelze, Germany). High-purity deionised water was produced in house by an Elix 10–Milli-Q Plus water purification system (Millipore, Eschborn, Germany). Tablets containing thalidomide were provided by Grünenthal (Stolberg, Germany). The regenerated cellulose (RC) syringe filters were purchased from Macherey Nagel (Düren, Germany).

2.2. Preparation of samples

The thalidomide tablets were weighed on a Mettler Toledo scale MTXS105DU ($d = \pm 0.01$ mg). After milling, 2×25.00 mg of each tablet was dissolved in 25 mL acetonitrile for replicate analysis. These solutions were then treated for 10 min in an ultrasonic bath and afterwards filled up to 100 mL with a solution of deionised water + 0.1% formic acid. The solutions were filtered through a 0.45 μm RC filter. For external calibration a standard solution containing thalidomide was prepared following the same procedure as for the tablets. Calibration solutions were adjusted to 70, 80, 90, 100 and 110 mg/L. Replicate injections were made for all samples.

2.3. HPLC systems

Three different HPLC systems (Beckman Gold, Shimadzu LC 10 and Agilent 1100/1200) were used to collect the chromatographic data. The Beckman System Gold HPLC consists of a System Gold 126 pump, an AS 502e auto sampler, a System Gold 168 diode array detector, an SS420x AD Box for start/stop signal (Beckman, Krefeld, Germany) and a Jetstream 2 column oven (VDS Optilab, Erkerode, Germany). The Shimadzu LC 10 consists of two LC-10AD$_{VP}$ pumps, a DGU-14 A degasser, an SIL 10AD$_{VP}$ auto sampler and an SPD-M10A$_{VP}$ diode array detector (Shimadzu, Duisburg, Germany). The Agilent 1100/1200 HPLC system employed in this study consists of a G1312A binary pump, a G1379A degasser, a G1313A auto sampler and a G1315C diode array detector (Agilent, Waldbronn, Germany). For data acquisition and analysis, the Beckman 32 Karat 7.0, the Shimadzu LabSolutions (Version 1.21, SP1) and the Agilent ChemStation for 3D LC software (Version B01.03) were used. Quantification of thalidomide was always performed at a wavelength of 300 nm.

2.4. HPLC columns

The separation at 30 °C was performed on a silica-based C18 stationary phase (Macherey Nagel, ec150/4 Nucleosil, 100-5 C18 PAH) whereas the high-temperature elution of thalidomide was carried out on a carbon clad zirconium dioxide (ZirChrom Carb, 150 mm × 4.6 mm, 3 μm) and a polystyrene divinylbenzene stationary phase (PLRP-S, 150 mm × 4.6 mm, 3 μm) at 180 °C. For analyte stability measurements both high-temperature columns were used, while for method development for the fast and the aqueous elution of thalidomide only the polystyrene divinylbenzene stationary phase was used.

2.5. Heating system

A homemade heating system was used for controlling the eluent temperature for the high-temperature elution of thalidomide. The heating system was developed for high-temperature liquid chromatography and consists of three modules, which can be controlled independently. The heating range of this system extends from room temperature to 225 °C with maximum heating rates of 40 °C/min. This system can be used for isothermal and temperature programmed operations and is described in detail elsewhere [18]. To keep the mobile phase in the liquid state, even at high temperatures, a 500 psi backpressure regulator (GammaAnalysenTechnik, Bremerhaven, Germany) was connected behind the UVD.

2.6. Analyte stability measurements

A thalidomide standard was eluted isothermally at different temperatures on the ZirChrom Carb and the PLRP-S column. The composition of the mobile phase was adjusted so that the elution of thalidomide was realized between 15 and 20 min at a starting temperature of 60 °C. The flow rate was 1.0 mL/min for the ZirChrom Carb column and 0.2 mL/min for the PLRP-S column. The lower flow rate for the polymeric column was chosen to keep the pressure below the pressure limit recommended by the column manufacturer.

3. Methods for elution of thalidomide

3.1. Standard method

Applying the standard method (Fig. 1a) thalidomide was eluted on a Macherey Nagel Nucleosil C18 PAH column

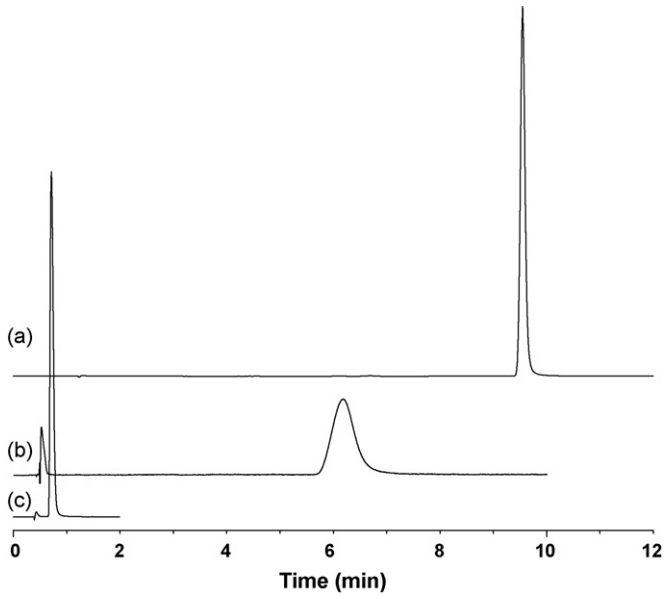

Fig. 1. Elution of thalidomide on an (a) Macherey Nagel Nucleosil C18 PAH column, (b) PLRP-S column with aqueous mobile phase and (c) PLRP-S column for fast separation. Chromatographic conditions are given in Section 3.

(150 mm × 4.0 mm, 5 μm, 100 Å) with a binary solvent gradient. A linear gradient was applied from 5 to 38% B (acetonitrile + 0.1% formic acid) within 10 min. The flow rate was adjusted to 1.0 mL/min and the column was thermally equilibrated at 30 °C in an air bath column oven. The maximum column backpressure was 150 bar. This separation was performed on the Beckman HPLC system and presents the standard HPLC method, which is usually used in house.

3.2. Aqueous high-temperature method

Applying the aqueous high-temperature method (Fig. 1b), thalidomide was eluted isocratically on a temperature stable polystyrene divinylbenzene stationary phase (PLRP-S, 150 mm × 4.6 mm, 3 μm, 300 Å) with a mobile phase of water + 0.1% formic acid. The flow rate was adjusted to 0.7 mL/min and the column temperature was set to 180 °C using the specially designed heating system. The column backpressure did not exceed 275 bar at this flow rate, which is the maximum pressure for the polymeric column. This elution was carried out on the Shimadzu HPLC system.

3.3. Fast high-temperature method

Using the fast high-temperature method (Fig. 1c), thalidomide was eluted on a temperature stable polystyrene divinylbenzene column (PLRP-S, 150 mm × 4.6 mm, 3 μm, 300 Å) with an isocratic mobile phase consisting of 20% acetonitrile and 80% deionised water to each of which 0.1% formic acid was added. The flow rate was adjusted to 1.0 mL/min and the column temperature was set to 180 °C using the specially designed heating system. The column backpressure was 270 bar. This separation was achieved on the Agilent HPLC system.

4. Results and discussion

4.1. Influence of the stationary phase on analyte degradation

The reluctance to use temperature as an active variable in liquid chromatography partially originates from the fear that high eluent temperatures will inevitably lead to a significant decrease of the analyte signal caused by an on-column degradation of the compounds of interest. Most publications dealing with high-temperature LC do not mention the consequences of this possible drawback, although this question is highly relevant for practical reasons. A theoretical concept describing the on-column degradation is represented by the dimensionless Damköhler number, *Da* [34]. The Damköhler number is defined as the ratio of the residence time of an analyte in the column to the relaxation time for the on-column reaction [35]. In analytical chromatography, it is of little consequence whether the reaction has proceeded almost to completion, as is indicated by large *Da* values, or not at all, as suggested by $Da \ll 1$. In the first case, the analyte is fully converted into a single product, whereas in the second, the eluite remains unchanged. For chromatography, intermediate Damköhler numbers of 0.1–50 are always undesirable because under such conditions the interplay of the reaction with the elution process is detrimental to the separation. In contrast to mostly theoretical suggestions the effect of temperature on analyte stability has been verified experimentally in our investigation by a

Table 1
Peak area, peak height, peak asymmetry and peak width at different temperatures on the PLRP-S column

Temperature (°C)	Area (mAU s)	Height (mAU)	Asymmetry[a]	Width (min)[b]
60	12,683,032	322,677	2.01	1.46
80	12,834,336	355,324	1.58	1.24
100	12,898,045	424,675	1.41	0.99
120	12,589,763	473,691	1.33	0.84
140	12,841,541	528,181	1.27	0.72
160	12,597,459	574,049	1.36	0.64
180	12,100,791	604,475	1.38	0.57

Data acquisition was performed using the Shimadzu LC10AD$_{VP}$ HPLC system. Chromatographic conditions—flow rate: 0.2 mL/min, mobile phase: 80% deionised water/20% acetonitrile (+0.1% formic acid each), and detector: DAD at 300 nm.

[a] Calculations referring to USP.

[b] Peak width at 5% height.

Table 2
Peak area, peak height, peak asymmetry and peak width at different temperatures on the ZirChrom Carb column

Temperature (°C)	Area (mAU s)	Height (mAU)	Asymmetry[a]	Width (min)[b]
60	5202	78	2.57	2.43
80	4990	112	2.48	1.60
100	4455	114	2.50	1.08
120	3077	134	2.54	0.78
140	996	57	3.02	0.75
160	63	4	1.60	0.68
180	n/A[c]	n/A[c]	n/A[c]	n/A[c]

Data acquisition was performed using the Agilent 1200/1100 HPLC system. Chromatographic conditions—flow rate: 1.0 mL/min, mobile phase: 75% deionised water/25% acetonitrile (+0.1% formic acid each), and detector: DAD at 300 nm.
[a] Calculations referring to USP.
[b] Peak width at 5% height.
[c] Not analysable.

UV-detector. In UV detection, the injected amount of the analyte is proportional to the peak area, provided that the flow rate is kept constant and the detector response is linear. Therefore, if a constant amount is injected at different temperatures, a degradation of the analyte results in a decrease of the peak area, whereas a constant peak area indicates that the analyte is not degraded on-column. The formation of split peaks or peak shoulders then also indicates that there is an interconversion of two species.

For this reason, isothermal and isocratic measurements for the determination of the analyte stability as described in Section 2.6 were performed. Data for peak area, peak height, asymmetry and peak width at different column temperatures for both columns are given in Tables 1 and 2. Figs. 2 and 3 show the elution behaviour of thalidomide on a carbon clad zirconium dioxide stationary phase and on a polystyrene divinylbenzene stationary phase, respectively. Usually, the theoretical dependence of the retention factor on temperature is described by the van't Hoff equation [41–43]. The plot of the natural logarithm of the retention factor against the inverse of the absolute temperature normally shows a linear behaviour, which is also the case in our investigation (data not shown here).

Chromatographic theory predicts that high eluent temperatures positively affect the overall column performance. Nevertheless, due to various temperature dependent equilibria this will not necessarily be observed if a low temperature and a high-temperature elution of an analyte on a stationary phase are compared. The reason is that temperature can influence the pH of the mobile phase [43,44]. Moreover, an increase in efficiency

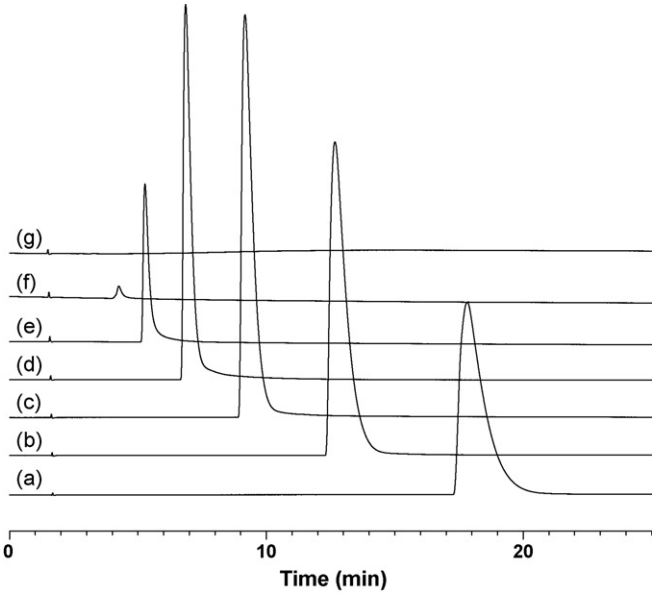

Fig. 2. Monitoring of the on-column degradation of thalidomide on a ZirChrom Carb column (150 mm × 4.6 mm, 3 μm) from (a) 60 °C to (g) 180 °C in 20 °C increments. Chromatographic conditions—flow rate: 1 mL/min, mobile phase: 75% deionised water/25% acetonitrile (+0.1% formic acid each), and detector: DAD at 300 nm.

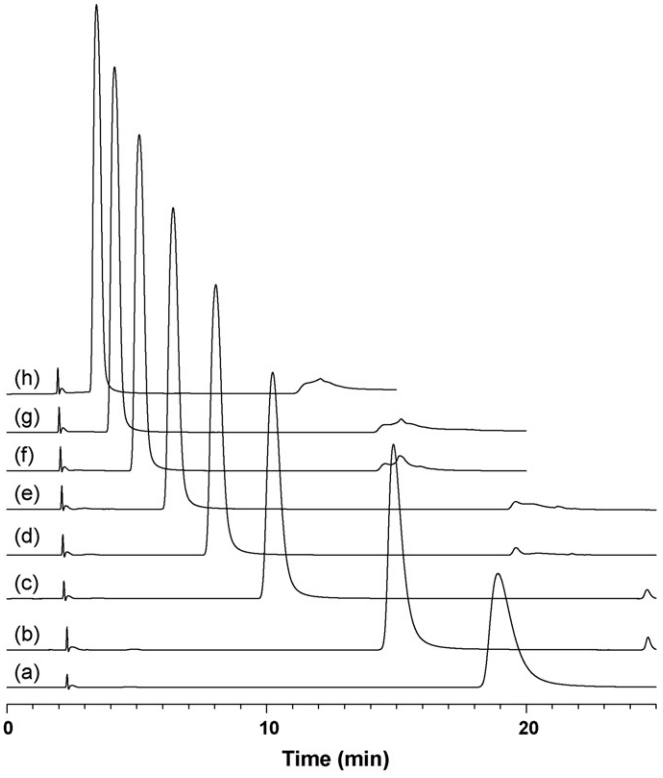

Fig. 3. Monitoring of the on-column degradation of thalidomide on a PLRP-S column (150 mm × 4.6 mm, 3 μm) from (a) 40 °C to (h) 180 °C in 20 °C increments. Chromatographic conditions—flow rate: 0.2 mL/min, mobile phase: 80% deionised water/20% acetonitrile (+0.1% formic acid each), and detector: DAD at 300 nm.

can be compromised due to experimental reasons. If there is a thermal mismatch between the stationary phase and the incoming mobile phase, excessive peak broadening will occur [17,45]. Our results given in Tables 1 and 2 reveal that for the elution of thalidomide on the polymeric column, peak height increases while peak area remains constant. Furthermore, peak asymmetry is significantly reduced. Thalidomide is eluted with an asymmetry factor smaller than 1.41 for temperatures above 100 °C. In contrast to this, an elution at 60 and 80 °C yields an asymmetry factor of 2.01 and 1.58, respectively. Therefore, the quantification is significantly improved at higher temperatures since more accurate peak detection is achieved. In contrast to this the elution of thalidomide on the zirconium dioxide column is not improved since asymmetry factors are even worse at high eluent temperatures. This means that the polystyrene divinylbenzene column is superior in terms of peak detection and quantification at high-temperature conditions.

The most interesting phenomenon we observed was that on the carbon clad zirconium dioxide stationary phase the peak areas decrease with increasing temperature. This can be explained with an on-column degradation of thalidomide. It is obvious that this degradation occurs only on the carbon clad zirconium dioxide stationary phase. Degradation peaks have not been observed and a shorter residence time on the heated column due to higher flow rates showed no influence on the degradation. A reason for this could be that the carbon cladded surface of the zirconia acts as a catalyst [36–40], but further investigations have to be carried out to determine the on-column degradation process. In contrast to this, the peak area on the polystyrene divinylbenzene column remains constant with an increase of the column temperature, which means that degradation is not evident. This shows that the phase system has a pivotal influence on the analyte stability. Therefore, analyte stability should not be measured on a reference column and the method then transferred to another column with different surface properties. The result might be that the analyte is temperature stable on the first column but will degrade rapidly on the second column by on-column reactions. As a result of these investigations, the polystyrene divinylbenzene column was chosen for all further investigations in this study.

4.2. Statistical comparison of different separation methods

The elution of thalidomide as depicted in Fig. 1 was performed as described in Section 3. The first method, described as standard method, is a solvent gradient method. The respective chromatogram is shown in Fig. 1a. Our aim was to transform the solvent gradient method to an isocratic method because no other impurities could be detected in the solvent gradient run. The time between consecutive injections could therefore be significantly reduced, since isocratic elution does not require a re-equilibration step of the stationary phase. Applying an isocratic elution at a high temperature then allows for a fast elution and monitoring of the concentration of the main component of about 1 min. This was achieved by using a mobile phase composed of water and acetonitrile 80/20 (v/v) at 180 °C and is shown in Fig. 1c.

Table 3
Standard deviations of the mean absolute concentration of 18 thalidomide tablets with standard method, aqueous HT-HPLC method and fast HT-HPLC method

	Standard method	Aqueous method	Fast method
Mean (mg)	97.32	94.78	97.43
Standard deviation (mg)	±2.43	±10.57	±4.38
Relative standard deviation (%)	2.50	11.15	4.50

Another strategy was to perform the elution of thalidomide without the addition of the organic modifier. The temperature was also set to 180 °C. The absence of the organic co-solvent resulted in a later elution of thalidomide as was expected (Fig. 1b). Therefore, both objectives using HT-HPLC for fast and aqueous separation have been achieved.

Afterwards, a series of 18 tablets containing thalidomide has been analysed. The content of thalidomide was determined by analysing all samples with all three methods described in the experimental part. The samples were aliquoted after sample preparation in order to guarantee that there was no bias towards the sample preparation. An individual calibration was performed for each method to compare all methods in terms of their analytical response. The concentration range was from 70 to 110 mg/L. A linear regression model was assumed for all three methods. The suitability of this model was performed according to the German standard DIN 32645 (evaluation of calibration). The correlation coefficient for the standard and the fast method was >99% while the coefficient for the aqueous method was only 94%. Nevertheless, the coefficient of correlation is often not an appropriate measure for a proof of linearity. Therefore, this parameter is not suitable as a general acceptance criterion for the performance of the analytical procedure. A generally superior method for determining the linearity of a calibration is the response or sensitivity plot [46]. This approach involves determining the response factor (RF) at each measured concentration and plotting this RF versus analyte concentration. This RF is calculated as $RF = DR/C$, where DR is the detector response (peak area) and C is the concentration of the analyte. Ideally, the response factor should be independent of concentration if the method is truly "linear" over an extended range of concentrations. We obtained a nearly horizontal line, although the scatter of the data points is more pronounced for the aqueous method. The relative standard deviation (% R.S.D.) for the response factors should be <2% for a linear relationship. While the standard method (0.91% R.S.D.) and the fast high-temperature method (1.31% R.S.D.) comply with these criteria, the aqueous method (4.63% R.S.D.) failed to meet this specification.

The data in Table 3 show that the mean value for the aqueous method is smaller than for the standard or fast method. Moreover, the R.S.D. is highest for the aqueous method. Therefore, it should be investigated if there is a statistically significant difference between the mean of the standard method and the mean of the other methods. For this reason, an F-test was made to prove the homogeneity of the variances. With this test it could be shown that the S.D. of the three different methods did not differ statistically. To scrutinise if the three methods showed any

systematic difference, a *t*-test (test of variations) was performed. The results display that the mean values of the three methods do not differ statistically. With these test procedures it could be shown that the three different methods give the same results concerning the content of the active pharmaceutical ingredient of the analysed tablets. During the whole investigation, a degradation of the columns was not observed.

5. Conclusions

We demonstrated that the column can have a pronounced effect on analyte stability if high eluent temperatures are applied. This means that for method development it must be ensured that analyte stability is investigated on the same column on which the method will be run. It should be avoided to use a reference column on which analyte stability is measured and then use a different column on which the method is run. We have compared three methods for the elution and quantification of thalidomide in tablets. The standard method was performed at room temperature using a silica-based stationary phase, while the other methods applied 180 °C using a polystyrene divinylbenzene column. Working at a high temperature allowed either a fast elution using an organic co-solvent in the mobile phase or the use of a water-only mobile phase. There were no statistically significant differences between the means of all three methods when a series of 18 tablets was analysed. Therefore, all methods can be used equivalently for the quantification of thalidomide in tablets. A degradation of the high-temperature stable columns was not observed during 12 h at 180 °C and 120 injections.

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