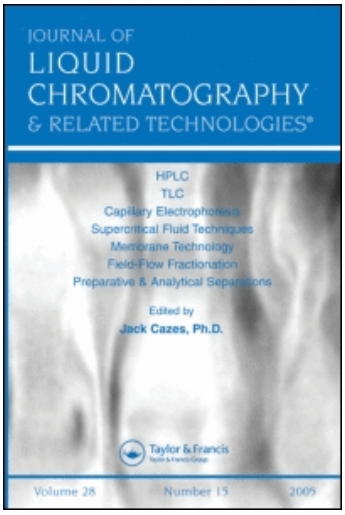

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High Performance Liquid Chromatography of Dithiolthiones

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF DITHIOLTHIONES

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ABSTRACT

Sixteen dithiolthiones including oltipraz (5-(2-Pyrazinyl)-4-methyl-1-1,2 dithiol-3-thione) and anethol dithiolthione (P-methoxyphenyl-1,2-dithiol-3-thione) have been studied using an isocratic high performance liquid chromatographic system. The retention characteristics of these compounds were determined using a conventional C 18 reverse phase column and a μ Bondapack phenyl column. The mobile phases used consisted of several concentrations of methanol and water. All compounds studied were adequately detected in the nanogram range (30-100 ng on column) using

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ultra violet detection (UV) set at 300 nm. Several analogs and isomers were separated. The retention characteristics of the 16 compounds studied are reported for both columns using 4 mobile phases and chromatograms of reference standards are presented.

INTRODUCTION

Oral administration to mice of dithiolthiones results in chemoprotective and radioprotective effects (1). This is associated with significant elevations in tissue glutathione levels and in the activity of a number of enzymes, such as glutathione-S-transferases, catalyzing the inactivation of carcinogens and other toxic products (1). One dithiolthione, oltipraz, (5-(2-pyrazinyl)-4 methyl-1,2 dithiol-3-thione) has antischistosomal activity (2,3,4,) and anethol dithiolthione (ADT) (3-(p-methyoxyphenyl)-1,2-dithiol-3 thione) has been reported to stimulate salivary secretion and to antagonize the dryness of the mouth, produced by certain antidepressant drugs (5).

Because of the numerous biological activities, and because of their presence in several edible plants (Brassica species) (6), this class of compounds is assuming considerable interest.

No methods have been reported in the literature for the separation of dithiolthiones by high performance liquid chromatography (HPLC). In this paper we are describing conditions for the separation and detection of 16 dithiolthiones using two reverse phase columns and four mobile phases. Absorption maxima and the extinction coefficients (α) of these compounds in the UV and visible regions are reported also.

MATERIALS AND METHODS

Instrumentation

An isocratic HPLC system assembled in our laboratory consisted of a Milton Roy

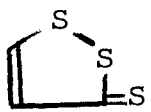
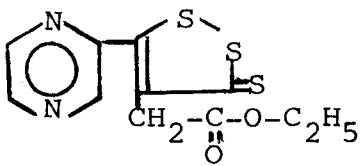
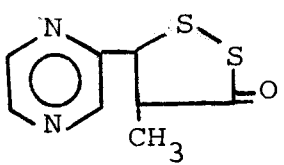
reciprocating minipump, model 396; a stainless steel tube, 1/4 inch (0.64 cm) outer diameter by 1 meter as a pulsation damper; a 5,000 pounds per square inch (34.5 megapascal) pressure gauge; a Rheodyne injector model 7125 and a variable wavelength ultraviolet (UV) detector, model Spectro-Monitor III. All parts were obtained from Laboratory Data Control (Riviera Beach, Florida). A Hewlett Packard computing integrator model 3390A (Avondale, Pennsylvania) was connected to the detector. A Brownlee RP 18, Lichrosorb 3 cm guard column (Santa Clara, California) was connected to the analytical column. Two analytical columns were used in this study; a Whatman, Partisil PXS 10/25, ODS-2 microparticulate, reverse phase column (Clifton, New Jersey) which will be referred to as column A, and a Waters, μ Bondapack phenyl (Milford, Massachusetts), which will be referred to as

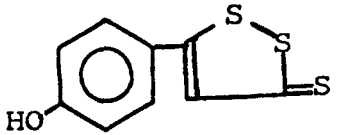
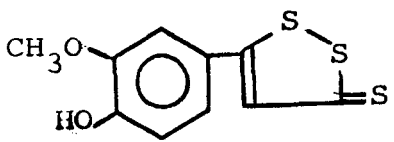
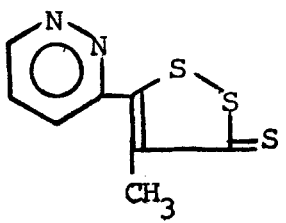
Column B. Columns were kept inside a Bioanalytical Systems heater model LC-23A (West Lafayette, Indiana) and column temperature was maintained at 35°C throughout this study.

Materials:

The compounds investigated were obtained courtesy of Dr. Baronnet of Laboratoires Therapeutique Moderne, Suresnes, France (Latema), Dr. Benazet of Rhone Poulenc, (RP), Research Division Vitry Sur-Seine, France and Dr. R. Gyurick of Smith Kleine and French, USA. They were used without further purification. The sources of these compounds, their structures, absorbance maxima, excitation coefficients and their names or their company identification numbers are listed in Table 1. These compounds were arranged in order of their elution on column A with 70% methanol as a mobile phase

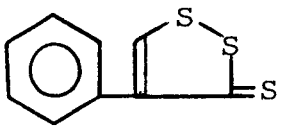
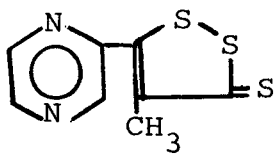
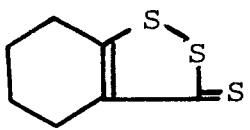
TABLE 1
Compounds Studied, Their Structures, Sources, Names or Code Numbers, Absorbance Maxima and Extinction Coefficients.

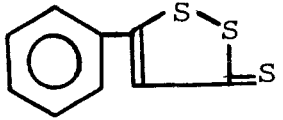
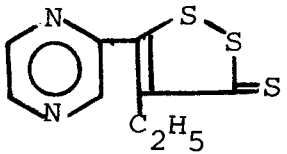
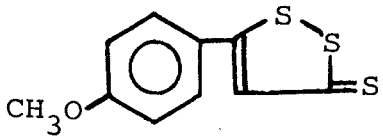
Roman Numeral of Comp.[1]	Structure	Name or Code Number[2]	Source[3]	Abs. Max. (nm)	Extinc. Coef. (ϵ)
I		Dithiocylopen-tenethione	Latema	225	46.0
				245	29.0
				320	25.0
				405	60.0
II		38656	RP	230	52
				270	45.2
				420	39.5
III		36642	RP	230	26.5
				288	64.5
				350	20.5

IV		Des-methyl ADT	Latema	230	31.0
				350	50.0
				425	35.0
V		120L	Latema	230	68.0
				255	65.5
				370	92.0
				430	79.0
VI		36733	RP	220	43.0
				265	40.0
				435	29.0

(continued)

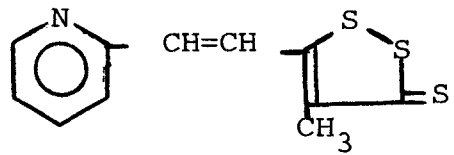
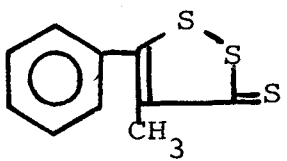
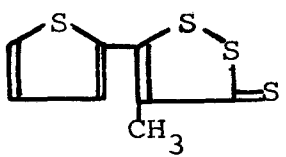
Table 1 Cont.

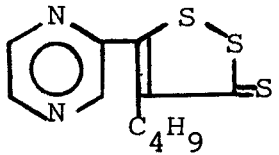
VII		1129L	Latema	210	65.3
				230	45.9
				320	18.8
				420	33.5
VIII		Oltipraz (OLT)	RP	220	50.5
				295	63.5
				430	39.0
IX		82013	SKF	220	58.0
				275	40.5
				315	32.5
				404	66.5

X		116L	Latema	220	57.0
				265	49.0
				310	94.5
				425	52.0
XI		36334 (Ethyl OLT)	RP	225	45.5
				290	56.0
				435	36.5
XII		Anethol-dithiolthione (ADT)	Latema	225	61.5
				265	35.5
				342	96.0
				425	57.0

(continued)

Table 1 Cont.

XIII		40863	RP	238	29.0
				350	49.5
				460	19.5
XIV		37528	RP	215	63.5
				238	46.5
				295	56.0
				415	51.0
XV		36731	RP	225	56.5
				265	37.5
				335	65.5
				430	51.0

XVI		35919 (Butyl OLT)	RP	222	53.0
				290	62.0
				440	41.5

1. These compounds eluted in that order on a Whatman partisil, PXS, 10/25, ODS-2, Column and when mobile phases containing 70% or 75% methanol were used.
2. These numbers are the numbers assigned to these compounds by the manufacturers.
3. Latema - Laboratoires de Therapeutique Moderne; RP - Rhone Poulenc; SKF- Smith Klein and French.

and assigned a Roman numeral based on that order for the purpose of referring to these compounds throughout this paper.

Methanol, (Glass-distilled) was purchased from Burdick and Jackson laboratories (Muskegan, Michigan). Water was distilled, deionized and demineralized.

Individual reference standards were dissolved in methanol and further dilutions and mixtures were also prepared in methanol. Solutions were stored in vials provided with teflon-lined caps and the vials were wrapped with aluminum foil to protect the samples from light. Samples were gassed with nitrogen and kept refrigerated when not in use. No decomposition was observed under these conditions for at least two months.

Mobile Phases

Four mobile phases containing 75, 70, 65 and 60% methanol in water were used. These

mobile phases will be referred to throughout this paper as 75%, 70%, 65% and 60% methanol respectively. The mobile phases were degassed under vacuum immediately prior to use and kept at approximately 40°C during chromatography to prevent the introduction of air bubbles into the system. The flow rate was kept at 1.1 ml/min throughout this study.

Detector and Integrator Settings:

The absorption characteristics of the compounds were studied using a Varian scanning spectrophotometer model Cary 219 in the UV and visible range (Table 1). All the compounds were found to possess a maximum at a wavelength close to 300 nm. Thus when the UV detector was set at that wave length, all the compounds were adequately detected when nanogram amounts (30-100 ng on column) were chromatographed.

The UV detector provided a constant signal to the computing integrator of 1 absorbance unit (AU)/volt. Thus height counts provided by the integrator were constant for a given peak, regardless of the attenuation setting of the integrator. The actual size of the peaks as they appear on the chromatograms however are determined by the attenuation of the integrator. Throughout this study, the attenuation was set at 3 which equals to 8 mV full scale. Since the detector provides 1 AU/V, the sensitivity of the system was 0.008 absorbance units full scale, (AUFS).

RESULTS AND DISCUSSION

Working standards of individual compounds were chromatographed to determine their retention time (t_R) and their detector response. The amount introduced onto the column was in the range of 3 to 9 µl

of solutions containing 10 μg/ml. The capacity factor (k') for each compound was determined using the 4 mobile phases on each column according to the equation $K' = t_R - t_0/t_0$ where t_0 represents the solvent front which was 2.70 min. on column A and 3.35 min. on column B. Table 2 lists the K' values of these compounds. Figures 1 and 2 provide a graphic representation of these data.

As expected the compounds were generally retained more on column A than on column B. Also their retention was increased as the percentage of methanol was decreased in the mobile phase. The order of elution was consistent for most of the compounds with all mobile phases used. Only few compounds reversed order as the amount of methanol was decreased and the degree of reversal was not very significant.

TABLE 2
K' Values and Detection Responses of the Compounds Studied

	COLUMN A				COLUMN B				
	PERCENT METHANOL IN MOBILE PHASES								
Compound	75	70	65	60	75	70	65	60	mAU[1] Per µg on column
I	0.66	0.74	1.00	1.39	0.38	0.32	0.46	0.61	77.17
II	1.21	1.45	2.15	3.37	0.80	0.90	1.44	2.03	63.33
III	1.47	1.62	2.21	3.17	0.70	0.71	1.04	1.45	156.67
IV	1.51	1.98	3.02	4.94	0.76	0.91	1.54	2.33	34.67
V	1.67	2.21	3.46	5.52	0.83	0.98	1.67	2.56	47.11
VI	1.72	2.25	3.18	4.70	0.74	0.80	1.23	1.70	71.56

VII	2.08	2.57	3.94	6.31	1.02	1.24	2.13	2.96	44.00
VIII	2.92	3.64	5.08	7.70	1.18	1.18	2.13	2.95	85.67
IX	3.11	3.81	5.87	8.39	1.25	1.55	2.39	3.37	50.17
X	3.66	4.83	7.63	12.68	1.49	1.93	3.28	4.91	111.33
XI	3.72	4.99	7.60	12.24	1.42	1.78	2.90	4.19	76.67
XII	4.30	5.91	9.61	16.52	1.67	2.24	3.93	6.06	33.50
XIII	4.80	7.00	10.23	17.02	1.38	1.95	3.23	4.77	10.77
XIV	4.92	7.32	11.84	20.58	2.03	2.84	5.07	7.80	30.17
XV	5.66	8.05	13.13	22.50	2.07	2.84	5.007	7.64	31.77
XVI	7.37	10.67	18.09	32.31	2.35	3.35	6.06	9.63	52.44

1. These responses were calculated for individual compounds when chromatographed on column A and eluted with 75% methanol in water.

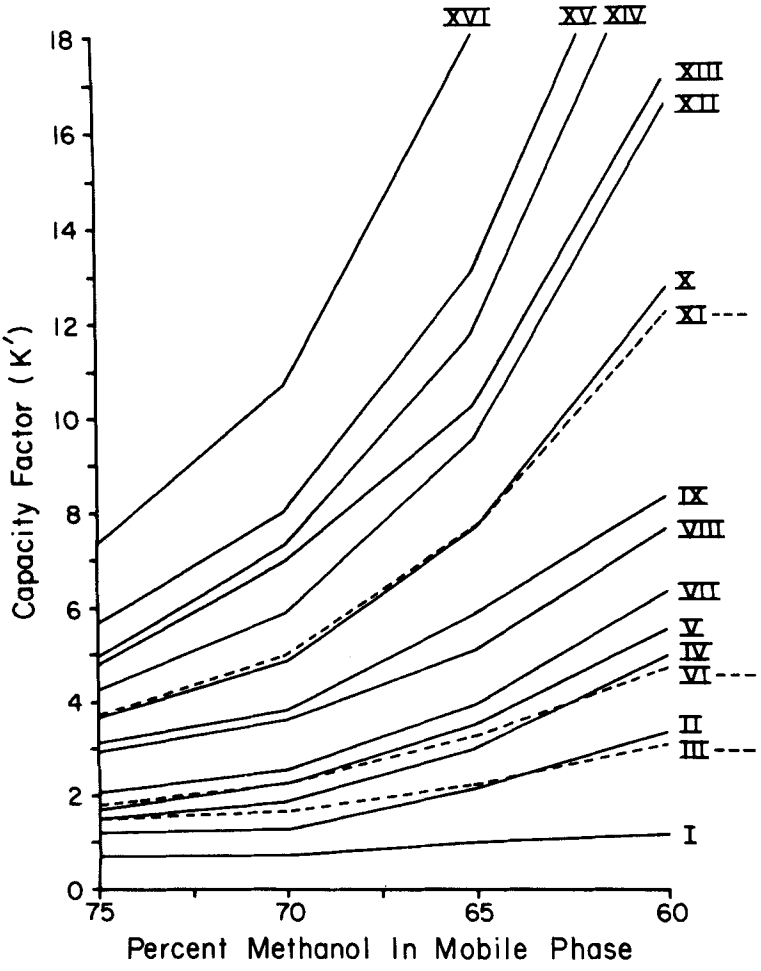


FIGURE 1

Capacity factors (k') of the compounds studied when chromatographed on column A.

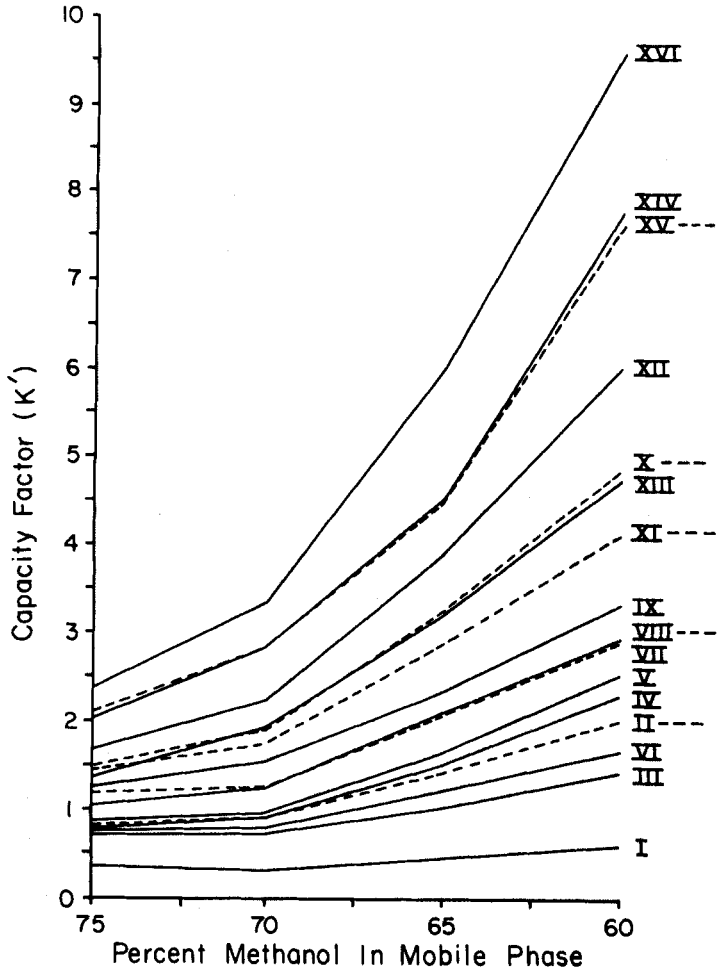


FIGURE 2

Capacity factors (K') of the compounds studied when chromatographed on column B.

For column A, compound III was eluted slightly earlier than compound II when 60% methanol was used. Compound VI was eluted earlier than V with 65% methanol and earlier than IV with 60% methanol. Compounds X and XI also reversed order in a similar fashion when the concentration of methanol in the mobile phase was reduced to 60%. It is difficult to interpret with certainty this difference in response to a lower methanol concentration by compounds III, VI and XI. However, the only structural characteristic common to the three compounds is the presence of two nitrogens in the ring of the side chain of the dithiocyclopetene thione. This effect could not be observed in compounds II and XV possibly due to the presence of a reasonably large side chain which could have overcome that effect. The order of elution from column A could be used as an index of the lipophilicity of these compounds.

The order of elution from column B was significantly different from that of column A. Retention on column B was no longer dependent on the lipophilicity of the compounds and the affinity of the compounds to the phenyl function of the stationary phase resulted in a different pattern of separation. As with column A, few compounds reversed the order of elution as the concentration of methanol in the mobile phase was reduced, however this phenomenon was less pronounced than with column A.

The choice of the column and of the mobile phase to be used for the separation of a given mixture is clearly dependent on the components of the mixture and the particular compound(s) of interest. Figures 3 and 4 illustrate the separation of the same mixture containing the 16 compounds studied using two different chromatographic systems.

Figure 3 reproduces the separation obtained on column A using a mobile phase

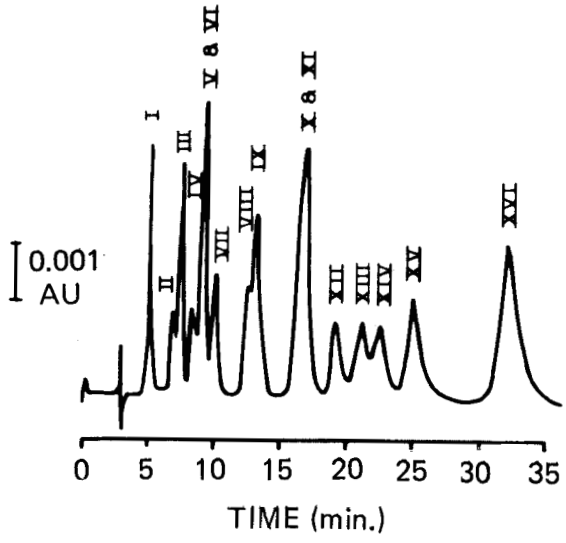


FIGURE 3

A chromatogram representing the separation of the compounds studied on a reverse phase column. The chromatographic conditions are as follows: column, Whatman PXS, 10/25 ODS-2 (column A); mobile phase, 70% methanol in water; detection, UV, 300 nm at 0.008 AUFS; flow rate, 1.1 ml/min; chart speed 0.2 cm/min.

The compounds chromatographed and the amounts on column are as follows: I = 60 ng; II = 30 ng; III = 30 ng; IV = 60 ng; V = 60 ng; VI = 60 ng; VII = 60 ng; VIII - 30 ng; IX = 60 ng; X = 30 ng; XI = 60 ng; XII = 60 ng; XIII = 120 ng; XIV = 60 ng; XV = 90 ng; XVI = 90 ng.

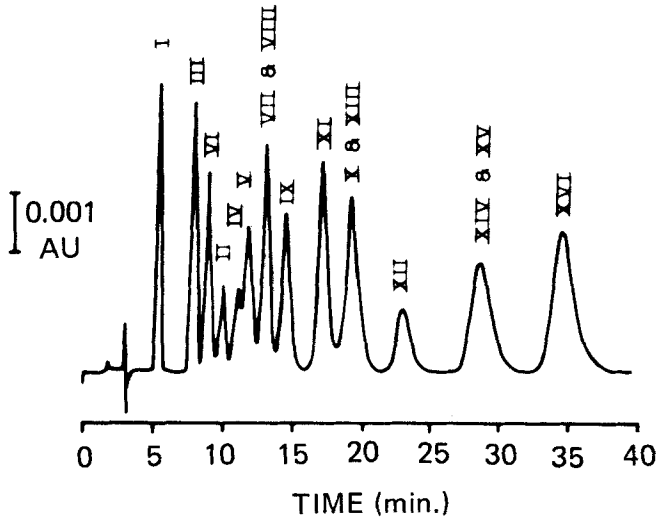


FIGURE 4

A Chromatogram representing the separation of the compounds studied on a phenyl column. The chromatographic conditions are as in Figure 3 except the column used was Waters μ Bondapack Phenyl and the mobile phase containing 60% methanol in water. The components and amounts were the same as in Figure 3.

containing 70% methanol. Under these conditions compounds V and VI; VIII and IX and X and XI co-eluted. Also, most of early eluting compounds, II through VII, did not produce adequate separations.

Figure 4 represents the separation of the same mixture obtained on column B using a mobile phase containing 60% methanol. Under these conditions, compound V (which co-eluted with VI under the previous conditions) was clearly separated from VI but not from IV. Compound VIII, similarly was adequately separated from IX but co-eluted with VII. Finally compound X was adequately resolved from XI but co-eluted with XIII.

In summary, the use of the combination of two columns and four mobile phases allowed most of the compounds studied to be separated adequately.

Detection Responses

Peak height counts computed for the compounds chromatographed on column A using 75% methanol as a mobile phase by the computing integrator, were converted to MV's using the conversion factor provided by the

Hewlett Packard Instruction Manual for the instrument. These values were used to calculate the milli-absorbance units (mAU) per μg on the column which is reproduced in column 10 of Table 2. This value is a useful indication of the signal produced by each compound and can provide an approximation of the detection limits of the method for each compound. Assuming that a sensitivity setting of 0.01 absorbance units full scale (AUFS) is a reasonable setting for a routine operation of most instruments, thus a signal of 1 mAU would represent 10% of the full scale which would certainly be considered a detectable signal (the chromatograms reproduced in Figures 3 & 4 a sensitivity setting of 0.008 AUFS was used). Based on these data and those recorded in column 10 of Table 2, the lowest detectable amounts on column can be calculated by dividing 1000 by the mAU produced by 1 μg on column to result in the

amount in nanograms necessary to produce a signal of 1 mAU. The range of sensitivity of the compounds studied will then be approximately 100 ng on column for compound XIII to 5 ng on column for compound III.

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