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A MICRO LIQUID COLUMN CHROMATOGRAPHY PROCEDURE FOR TWELVE ANTICONVULSANTS AND SOME OF THEIR METABOLITES

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SUMMARY

Solvent extracts of 50 μl volumes of serum were sufficient for the determination of twelve anticonvulsant drugs. The liquid chromatography procedure utilized a C-18 reversed-phase column and isocratic elution with 15% acetonitrile in water. No derivatization was required. Eluted anticonvulsants were detected by UV absorption at 195 nm and quantitated by drug—internal standard peak area ratios. The procedure provided linear working curves over the concentration range from 1 to 100 mg/l of drug in the serum. The procedure for serum provided recoveries of the drugs from 92 to 101%. Within-day precision was about 4% and day-to-day precision was about 6.5%. The procedure has been applied to urine samples to facilitate bioavailability studies. Data are also given for several metabolites. There is a discussion of many practical aspects of the procedure to improve the reliability of the results.

INTRODUCTION

It is becoming well-established that knowledge of the blood levels of anticonvulsant drugs can assist in the clinical management of a patient [1]. The determination of these drugs in clinical samples has utilized many analytical techniques. The two techniques most frequently used, as judged by the number of tests carried out, are microchemistry [2] and gas chromatography [3—5].

Interest is increasing in the use of liquid chromatography for the analysis of anticonvulsant drugs [6—12] because sample manipulation is less demanding than for gas chromatography (GC) and because several of the drugs and some of their metabolites [10] can be determined simultaneously.

Several separation modes are available for the chromatography of anticonvulsant drugs [11] but reversed-phase partitioning offers distinct advantages over most of the others. Since the mobile phase is largely water, it is less expensive and generally more transparent in the far UV than the solvents

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used in normal-phase chromatography.

Utilizing chromatography conditions similar to those we have previously described [6, 9, 10], we have modified the extraction procedure to provide recoveries greater than 90% for all the drugs tested. This and other improvements permits us now to determine the anticonvulsant drugs using only 50 μl of serum.

The procedure is adaptable without modification to the quantitation of the major anticonvulsant drugs and several other anticonvulsant drugs less frequently used. The conditions are set so that the major anticonvulsant drugs (ethosuximide, primidone, phenobarbital, phenytoin, and carbamazepine) may be determined simultaneously. It is not possible with the conditions described to analyze all the other drugs simultaneously. However, clinical samples generally do not contain more than several of the drugs. Data are given so that the analyst will be able to determine what combinations of drugs are feasible to determine.

Because of the significance of metabolites in bioavailability studies, e.g. where a metabolite may possess anticonvulsant activity, several metabolites have been studied and pertinent data are given. This procedure has been used to analyze urine samples for the drug metabolites.

MATERIALS AND METHODS

Apparatus

We used a Perkin-Elmer Model 601 liquid chromatograph, with a Rheodyne 7105 injection valve, a Perkin-Elmer Model LC-55 variable-wavelength UV spectrophotometer detector and a reversed-phase C-18 column (ODS-Sil-X-I, 0.26 × 25 cm; particle size, 13 μm). Special glassware included: 5-ml conical centrifuge tubes, 10 × 75-mm disposable culture tubes, 20-μl and 50-μl disposable pipets and 1-ml disposable pipets.

Reagents and standards

Methanol and chloroform, distilled in glass, were obtained from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.). The acetonitrile, distilled in glass, UV grade, was also from Burdick & Jackson Labs.

Phenobarbital and phenytoin drug standards were obtained from Applied Science Labs. (State College, Pa., U.S.A.). Phenylethylmalonamide, *p*-hydroxyphenobarbital, 5-(*p*-hydroxyphenyl)-5-phenylhydantoin and carbamazepine 10, 11-epoxide were a gift from Dr. C.E. Pippenger (Columbia-Presbyterian Medical Center, New York, N.Y., U.S.A.). The internal standard, 5-(*p*-methylphenyl)-5-phenylhydantoin (MPPH) was obtained from Aldrich (Milwaukee, Wisc., U.S.A.). All other drugs were obtained from the Theta Corp. (Media, Pa., U.S.A.).

Prepared serum standards at six concentrations were obtained from Syva Corp. (Palo Alto, Calif., U.S.A.) and were reconstituted according to the manufacturer's instructions. They include ethosuximide, primidone, phenobarbital, phenytoin and carbamazepine. If drugs not present in the Syva material were to be determined, serum pools were prepared to which concentrations of the drug were added that are appropriate for the expected range. Typically,

three serum levels are chosen, at the lower end, the mid-range and the upper end of the expected range.

Individual standard solutions were prepared from the drugs by dissolving them in methanol to yield 1 mg/ml. A chromatography test mixture was prepared by adding 1 ml each of the standard solutions of ethosuximide, primidone, phenobarbital, phenacetin, phenytoin, carbamazepine and MPPH (internal standard) to a PTFE-lined screw-capped tube and evaporating off the solvent with air. The residue was redissolved in 4 ml of methanol to yield a concentration of 250 mg/l of each drug. All drug standards were stored at 4°.

The mobile phase is prepared by adding 75 ml acetonitrile to 425 ml water and mixing. The chloroform extractant contains 2 mg/l of the internal standard. The phosphate buffer is 0.1 *M*, pH 8.0.

Procedure

Add 50 µl of serum and 50 µl of phosphate buffer to a 5-ml conical centrifuge tube. Mix well for 15 sec and add 0.5 ml of the extractant solution. Mix again for 15 sec and centrifuge for 1 min at 710 *g*. Aspirate off the organic phase using a 1-ml Hamilton syringe (No. 1001) and evaporate at room temperature with a gentle current of air. Redissolve the residue in 20 µl of methanol and inject 10 µl of this into the liquid chromatograph.

Chromatography

The mobile phase consisted of 15% acetonitrile in water, delivered at a flow-rate of 1 ml/min. Column temperature was 65°. The UV detector wavelength was 195 nm.

Calibration

Calibration is accomplished by using the procedure on the six Syva serum standards and the prepared pools in the event that the analysis is required for drugs not in the Syva standards. The peak area is measured for the standards. The area ratio is calculated by dividing the peak area of the drug by the peak area of the MPPH internal standard. A working curve is prepared by plotting the peak area ratio of each drug against concentration. The peak area ratios are calculated for drugs in the patient sera. The concentration of each drug is determined from the working curve.

RESULTS

Quality control

Each day, prior to analyzing samples, we establish that the chromatographic separation is acceptable by injecting the test mixture into the chromatograph. A typical chromatogram of this test mixture is illustrated in Fig. 1A. Both the retention time and the relative retention, r_{IS}, for each compound compared to the internal standard are listed in Table I.

We also participated in the very useful quality control program of the Epilepsy Foundation of America (Dr. C.E. Pippenger). This permitted us to compare our results against others, often using different techniques. A chromatogram of a sample from that program is shown in Fig. 1B.

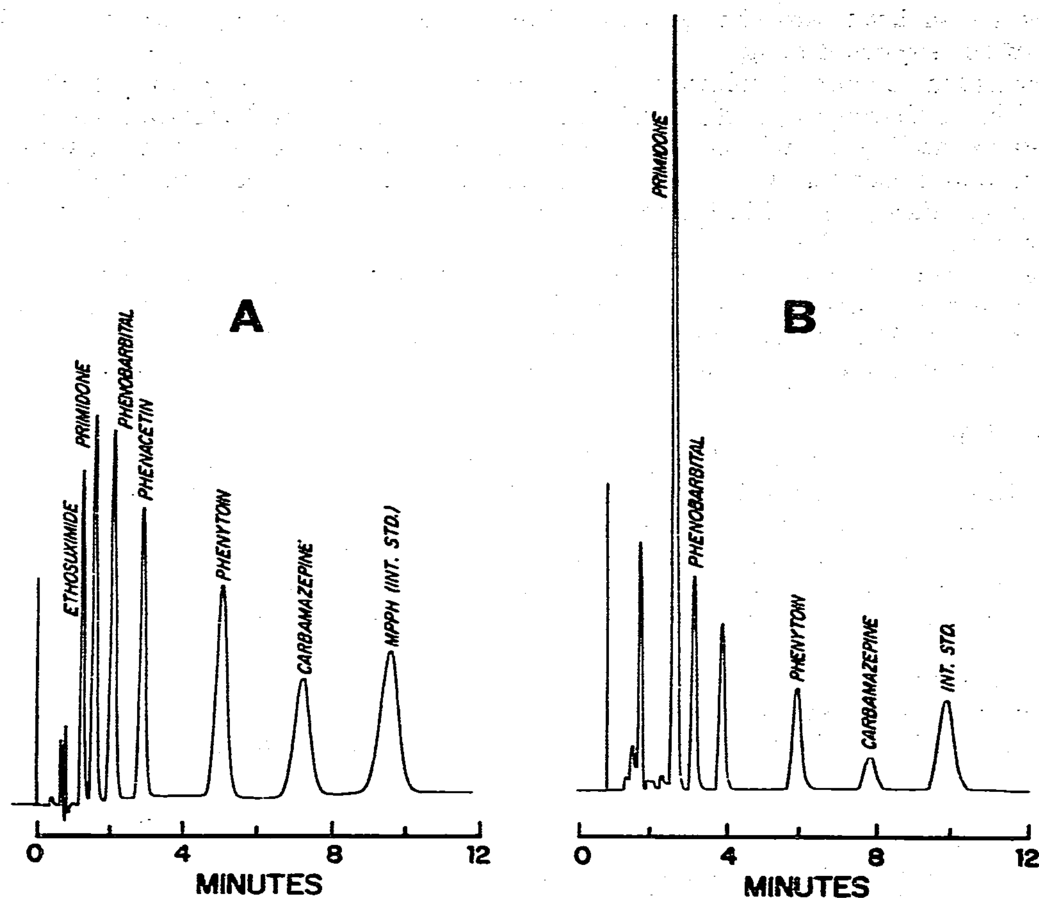

Fig. 1. A, Chromatogram of a mixture of anticonvulsant drug standards, each peak represents 250 ng of each drug. B, Representative chromatogram of a quality control serum; the peak immediately following phenobarbital is phenacetin, which was added to the extractant with the MPPH internal standard.

Recoveries

Recovery studies were made by adding specified quantities of the compounds to pooled sera known to be drug-free. The pool was analyzed in 50-μl aliquots and the peak areas of the extracted drugs were compared with the peak areas obtained by injecting 1 μg of each pure drug standard into the chromatograph. The data are given in Table II for concentrations of 10 and 50 mg/l. The recoveries ranged from 92% to 101%. Table II also includes recovery data for the 10, 11-epoxide metabolite of carbamazepine.

Linearity

We evaluated the linearity of the procedure for each drug by analyzing aliquots of a serum pool to which known quantities of the drugs were added to give a range of concentrations from 1 to 100 mg/l. It was established that for this range a linear relationship existed between the concentration and the

TABLE I

RETENTION TIME AND RELATIVE RETENTION TIME DATA FOR ANTICONVULSANT DRUGS AND METABOLITES

No.	Compound	Retention time (min)	Relative retention*
1	Succinic acid amide	1.86	0.064
2	Acetazolamide	2.07	0.076
3	Ethosuximide	2.66	0.108
4	Trimethadione	2.93	0.123
5	Phenylethylmalonamide	2.94	0.124
6	*p*-Hydroxyphenobarbital	2.96	0.125
7	Primidone	3.28	0.142
8	Paramethadione	3.90	0.177
9	Phenobarbital	4.17	0.192
10	5-(*p*-Hydroxyphenyl)-5-phenylhydantoin	4.48	0.209
11	Ethotoin	4.50	0.210
12	Phensuximide	5.08	0.242
13	Phenacetin	5.70	0.276
14	Mephenytoin	6.72	0.332
15	Mephobarbital	8.18	0.413
16	Metharbital	8.26	0.417
17	Methsuximide	8.28	0.419
18	Carbamazepine 10, 11-epoxide	8.30	0.420
19	Phenytoin	9.80	0.502
20	Carbamazepine	14.41	0.757
21	MPPH	18.81	1.000

*Relative to MPPH, the internal standard, after adjusting the retention time by subtracting 0.70 min mobile phase hold-up time.

peak area ratio of the drug to the internal standard. Of the metabolites, only the 10, 11-epoxide of carbamazepine was studied. A linear relationship was confirmed for this metabolite.

Sensitivity

The quantity of sample that is injected into the chromatograph is equivalent to 25 µl of serum. In this sample volume our procedure readily detected 5 ng (0.2 mg/l) of each of the compounds.

Precision

We studied within-day precision by analyzing 15 aliquots each of two sets of serum pools to which were added 5 and 50 mg/l of each drug, respectively. One serum pool contained ethosuximide, phenylethylmalonamide, primidone, phenobarbital, phensuximide, mephenytoin, methsuximide, phenytoin and carbamazepine. The other serum pool we used contained acetazolamide, trimethadione, paramethadione, ethotoin and metharbital. The other drugs not formulated into one of our serum pools were studied individually. For most compounds, either the 5-mg/l or 50-mg/l concentration is in the therapeutic range. The results are summarized in Table III and indicate that a within-day precision between 3 and 4% is obtainable for concentrations of 5 mg/l and

TABLE II

RECOVERY DATA FOR ANTICONVULSANT DRUGS FROM SERUM

— = Not determined

No.	Compound	Concentration	
		10 mg/l	50 mg/l
1	Succinic acid amide	91	89
2	Acetazolamide	93	96
3	Ethosumixide	96	98
4	Trimethadione	93	93
5	Phenylethylmalonamide	—	—
6	*p*-Hydroxyphenobarbital	—	—
7	Primidone	100	99
8	Paramethadione	96	95
9	Phenobarbital	100	98
10	5-(*p*-Hydroxyphenyl)-5-phenylhydantoin	—	—
11	Ethotoin	94	97
12	Phensuximide	94	96
13	Phenacetin	95	96
14	Mephenytoin	98	101
15	Mephobarbital	96	96
16	Metharbital	93	94
17	Methsuximide	95	95
18	Carbamazepine 10, 11-epoxide	97	98
19	Phenytoin	101	98
20	Carbamazepine	100	98

TABLE III

WITHIN-DAY PRECISION DATA FOR ANTICONVULSANT DRUGS FROM SERUM

$\bar{x}$ = Mean; SD = standard deviation; CV = coefficient of variation.

Compound	Concentration					
	5 mg/l			50 mg/l		
	$\bar{x}$	SD	CV (%)	$\bar{x}$	SD	CV (%)
Ethosuximide	4.6	0.19	4.2	51.3	1.95	3.8
Trimethadione	4.8	0.18	3.8	50.8	1.63	3.2
Primidone	5.1	0.16	3.1	48.9	1.66	3.4
Paramethadione	4.7	0.18	3.9	49.0	1.86	3.8
Phenobarbital	4.7	0.15	3.2	46.8	1.40	3.0
Ethotoin	5.2	0.20	3.8	50.1	1.55	3.1
Phensuximide	4.9	0.19	3.9	48.3	1.64	3.4
Mephenytoin	5.0	0.19	3.8	52.1	1.88	3.6
Mephobarbital	5.0	0.18	3.5	48.5	1.75	3.6
Metharbital	4.7	0.17	3.6	48.9	1.57	3.2
Methsuximide	4.9	0.17	3.4	48.7	1.51	3.1
Carbamazepine 10, 11-epoxide	5.1	0.19	3.7	50.5	1.57	3.1
Phenytoin	5.0	0.20	4.0	48.9	1.66	3.4
Carbamazepine	5.0	0.16	3.1	51.0	1.48	2.9

TABLE IV

DAY-TO-DAY PRECISION DATA FOR ANTICONVULSANT DRUGS FROM SERUM

Compound	Concentration					
	5 mg/l			50 mg/l		
	$\bar{x}$	SD	CV (%)	$\bar{x}$	SD	CV (%)
Ethosuximide	4.8	0.31	6.4	51.2	3.17	6.2
Trimethadione	4.7	0.28	5.9	50.4	2.47	4.9
Primidone	4.9	0.28	5.7	51.0	2.35	4.6
Paramethadione	5.2	0.27	5.2	48.0	2.26	4.7
Phenobarbital	5.1	0.30	5.9	52.2	2.71	5.2
Ethotoin	4.6	0.29	6.2	50.6	2.88	5.7
Phensuximide	4.8	0.29	6.1	50.4	2.42	4.8
Mephenytoin	4.5	0.28	6.3	47.0	2.30	4.9
Mephobarbital	4.9	0.27	5.6	48.9	2.35	4.8
Metharbital	5.2	0.26	4.9	47.3	2.46	5.2
Methsuximide	4.8	0.27	5.6	48.7	2.97	6.1
Carbamazepine 10, 11-epoxide	4.7	0.24	5.1	49.3	3.16	6.4
Phenytoin	5.1	0.30	5.8	48.7	2.78	5.7
Carbamazepine	4.9	0.24	4.8	49.6	2.18	4.4

slightly better precision is found for concentrations of 50 mg/l. Similarly, aliquots of the same pools and individual sera used to determine within-day precisions were analyzed daily for 15 days and the day-to-day precision estimated. Table IV shows that about 5% precision is found at both levels.

Accuracy

Because there are no accepted reference or routine methods for the determination of the less frequently used anticonvulsant drugs, we were unable to compare results obtained by different procedures. In our earlier study [6] comparing a GC procedure and a procedure very similar to the present one for fifteen samples, we found excellent agreement for primidone, phenytoin, phenobarbital and ethosuximide. We were unable to obtain good correlation with a GC procedure for carbamazepine [13]. We believe this was because of the poor precision of the GC procedure, in our hands. We found within-day precision of 21% for the carbamazepine gas chromatography procedure.

Patient sera

Over a period longer than 6 months this procedure has been applied to numerous sera from patients on anticonvulsant therapy. An example is shown in Fig. 2A from a subject on primidone, phenobarbital and phenytoin therapy. The sample was calculated to contain 8, 15 and 6 mg/l each of the compounds, respectively.

Fig. 2B was obtained by the analysis of a clinical serum from a patient on carbamazepine therapy. Carbamazepine was quantitated together with its metabolite, the 10, 11-epoxide of carbamazepine. The quantities calculated

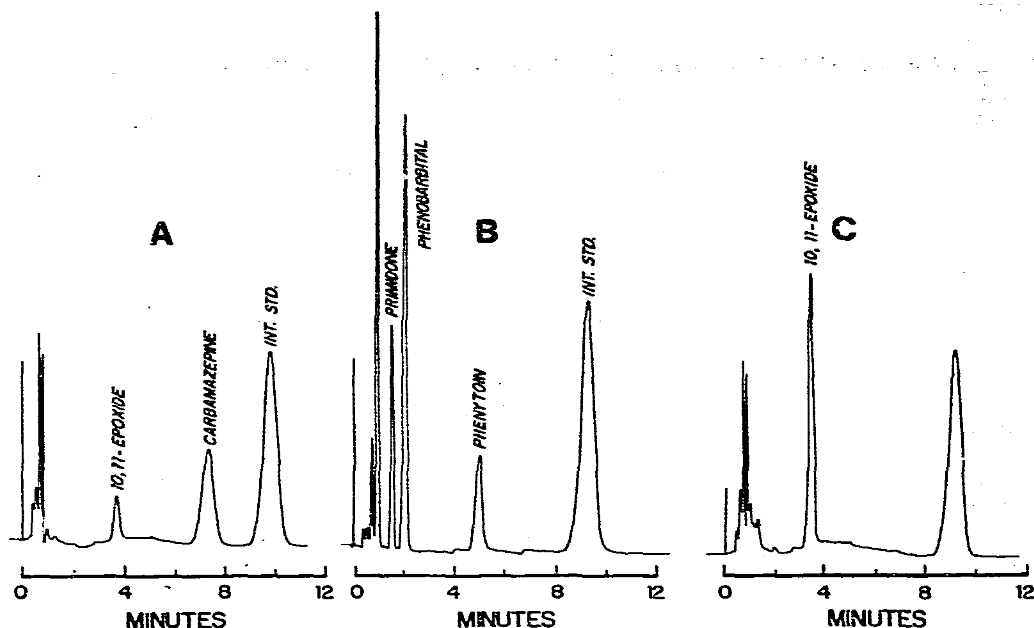

Fig. 2. Several clinical samples. A, Chromatogram of a patient serum containing primidone, phenobarbital and phenytoin at concentrations of 8, 15 and 6 mg/l, respectively. B, Chromatogram of a serum from a patient on carbamazepine; This drug and the carbamazepine 10, 11-epoxide were calculated to be 12 and 2 mg/l, respectively. C, Chromatogram of a urine sample from a patient receiving carbamazepine showing a carbamazepine 10, 11-epoxide concentration of 11 mg/l.

were 12 mg/l and 2 mg/l for the carbamazepine and the 10, 11-epoxide, respectively. The chromatogram in Fig. 2C was obtained from a urine sample from a subject on carbamazepine therapy. A trace of the parent compound was detected, but the 10, 11-epoxide metabolite is strongly evident. The concentration of the 10, 11-epoxide was calculated to be 11 mg/l.

Interferences

The procedure as described is not adequate to separate all the compounds simultaneously. It is adequate to separate all major anticonvulsants and several minor ones simultaneously without interference. Although it is feasible to effect a complete separation of all the drugs simultaneously, this would be at the expense of analysis time. We have chosen to optimize the analysis for the five major anticonvulsants, and the analysis time is about 19 min. Sufficient analysis time is allowed so that metabolites can also be monitored. Significant shortening of the analysis time would most probably reduce resolution to the point where the primidone metabolite, phenylethylmalomide, would overlap the parent compound. It is important to avoid this interference.

To assess the usefulness of the procedure against interfering drug peaks, we injected into the chromatograph the following compounds: amobarbital, pentobarbital, secobarbital, glutethimide, propoxyphene and salicylate. Amobarbital and pentobarbital, if present in the sample, will interfere with the

determination of methsuximide. Propoxyphene, secobarbital and glutethimide have retention times similar to phenytoin and will interfere with the determination of phenytoin, if they are present. No study was undertaken to determine these drugs in physiological samples.

The procedure, equipment and columns that we have used readily permitted identification of compounds that differed in relative retention by more than about 5%. However, if two compounds of similar relative retention were present in the same sample, reliable quantitation required that their relative retention differ by at least 10%.

DISCUSSION

This procedure is a useful approach to the determination of most anticonvulsant compounds, including some metabolites. With the procedure, the analyst will be able to analyze samples containing not only the major anticonvulsant drugs, but also those which are used less frequently. The procedure offers sufficient sensitivity to determine each drug over its entire therapeutic range, all compounds being chromatographed without derivatization.

The growing importance of bioavailability studies, especially where drug metabolites are to be determined, often requires the analysis of urine samples. Urine samples may be analyzed as readily as serum samples.

There are some practical considerations that will improve the reliability of this liquid chromatographic procedure. It is important to monitor on a routine basis the performance of the chromatography system. Periodically injecting a test mixture of drugs into the chromatograph will allow the analyst to confirm the separation capability of the column and to monitor degradation of column efficiency due to usage. A system check of this type should be performed at the beginning of each working day and after every ten analyses.

Clogging of the porous packing retainer located at the top of the column by matrix material extracted from clinical samples will eventually increase the back-pressure of the column to a point where the system becomes unusable. A daily check of system back-pressure will often identify this problem if it arises. Usually, replacement of the packing retainer will reduce the back pressure.

Increasing the acetonitrile percentage in the mobile phase to 100% for 1 h will strip from the column any compounds which have been completely retained on the packing material while operating with the suggested mobile phase for the procedure. This will result in higher column efficiency and reduced back-pressures.

Settling of the column packing will result in loss of column efficiency due to band spreading of the compounds being analyzed. The addition of packing to the top of the column will help correct this.

Carry-over in the injection valve can provide a potential source of error if a previous sample contains large amounts of drug. To a large extent, this problem can be alleviated by routine replacement of the PTFE valve seal every six months. The microliter syringe used for injection should also be inspected routinely for defects. Any analysis immediately following a very high sample should be repeated.

We have listed both the retention time of each anticonvulsant drug and its relative retention as compared to the MPPH internal standard. It should be noted that although the retention time can be used to identify the compound for a particular column under a given set of chromatography conditions, the relative retention is more readily transferred between columns or when chromatographic performance changes.

It is important to evaporate the chloroform extracts at room temperature with an air current not exceeding 50 ml/min. The use of heat during evaporation or continuing to evaporate after the extract is thoroughly dry will result in loss of ethosuximide due to its high volatility.

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