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RAPID AND SIMPLE CLEAN-UP AND DERIVATIZATION PROCEDURE FOR THE GAS CHROMATOGRAPHIC DETERMINATION OF ACIDIC DRUGS IN PLASMA

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SUMMARY

A rapid and simple clean-up and derivatization procedure that can be generally applied to the gas chromatographic (GC) determination of acidic drugs of various chemical and therapeutic classes is described. The drugs are extracted from acidified plasma with chloroform containing 5% of isopropanol, which is then evaporated. The residue is dissolved in toluene, then the drugs are back-extracted into a small volume of a methanolic tetramethylammonium hydroxide solution. The solution obtained is added to N,N-dimethylacetamide and the drugs are treated with *n*-butyl iodide and chromatographed as their *n*-butyl esters. Retention times are given for three different columns; the results show that in all but three instances one derivative is formed. The recoveries in the extraction steps were determined by GC and UV spectrophotometry.

The recovery of the back-extraction and of the extraction from plasma with chloroform–isopropanol is fairly good. For some drugs toluene can be used as the extraction solvent, which has the advantage that no evaporation of the extract is necessary, and it can be extracted directly with the tetramethylammonium hydroxide solution. Chromatograms of blank plasma treated according to the described procedure do not show interfering peaks from endogenous compounds.

The advantages of the proposed method are as follows. It is a standard procedure that can be used for different types of drugs, using one standard column, with no interference from endogenous compounds. Separation is achieved between drugs that differ only in the number or position of the methyl groups and between a drug and its demethylated metabolite(s). No decomposition of the products occurs during the alkylation process and injection of the resulting neutral solution is not detrimental to the column material.

INTRODUCTION

In the gas chromatographic (GC) determination of acidic drugs in plasma, two major problems are encountered. Because of the strong polarity of these compounds, they usually have to be derivatized to make them suitable for GC analysis. Secondly,

it is often impossible to extract them from plasma with a sufficient recovery without extracting interfering compounds. Many methods for the derivatization of acidic drugs have been described[1], but the extraction and clean-up of the sample have attracted less attention[2,3].

One of the recommended procedures for the determination of acidic drugs, especially the anti-epileptics[4], consists in extraction of the compound with chloroform–isopropanol, evaporation of the solvent, dissolution of the residue in toluene and back-extraction of the compounds into methanolic trimethylphenylammonium hydroxide solution. A few microlitres of this solution are injected into the gas chromatograph and the methyl esters of the drugs are formed in the injection port and chromatographed.

This method is very attractive in its simplicity, but has some disadvantages. In many instances decomposition products are formed owing to the high temperature and the strongly alkaline medium during the methylation process, and methylation is inadequate for drugs that give demethylated metabolites and for drugs that differ only in the number or position of the methyl substituents, for instance phenobarbital and methylphenobarbital or theophylline and theobromine. This problem can be overcome by using on-column butylation with tetrabutylammonium hydroxide, but this technique can also give rise to the formation of decomposition products. Finally, the injection of a strongly alkaline solution often results in a very broad solvent peak and multiple injections of these solutions can reduce the column life significantly.

These problems can be overcome by alkylation of the compounds before injection into the gas chromatograph, as described by Greeley[5] and Ijdenberg[6]. The method described by Greeley has the advantage that it is easily combined with the back-extraction of the compounds from toluene into a small volume of methanolic tetramethylammonium hydroxide solution and that derivatization is complete within a few minutes. Recently we described methods for the determination of pentobarbital[7] and nalidixic acid[8] using this technique.

In this paper we describe a rapid and simple procedure that can be used for the determination of acidic drugs using one standard column. Retention times on different columns and recovery data are given for 30 drugs from different chemical and therapeutic classes; blank plasma samples were treated according to the described procedure to see if there is any interference from endogenous compounds.

EXPERIMENTAL

Apparatus

A Packard-Becker Model 419 gas chromatograph equipped with flame ionization detectors was used. The glass columns (150 cm × 2 mm I.D.) were packed with 3% OV-1, 3% OV-17 or 3% SP-1000, all on 100–120-mesh Chromosorb W HP (Chrompack, Middelburg, The Netherlands). Depending on which compounds were studied, the column temperature was set between 100° and 270° and the injection port and detector temperatures were 30° higher than the column temperature. The carrier gas (nitrogen) flow-rate was maintained at 20 ml/min, the hydrogen flow-rate was 25 ml/min and the air flow-rate was 250 ml/min.

Retention times and peak areas were obtained with the Data Analyser System IV B (Spectra-Physics, Santa Clara, Calif., U.S.A.). The UV absorption of solutions

of the compounds was measured with a Perkin-Elmer Model 139 spectrophotometer with matched 1-cm cells.

Reagents and materials

The drugs used (Table I) were purchased from various commercial sources or were obtained as gifts from various companies and were used as such.

Tetramethylammonium hydroxide (20% solution in methanol) (TMAH) was obtained from Aldrich-Europe (Beerse, Belgium) and *n*-butyl iodide from Fluka (Buchs, Switzerland). All other reagents and solvents were of analytical-reagent grade.

Procedures

(A) Determination of retention times of butyl esters on various columns

The compounds under study were dissolved in N,N-dimethylacetamide (DMA) to a contration of 1 mg/ml. To 50 μl of the solution obtained were added 10 μl of TMAH and 10 μl of *n*-butyl iodide. After mixing on a vortex-type mixer, standing at room temperature for 10 min and centrifugation at 2500 *g* for 5 min, 1 μl of the clear supernatant was injected into the gas chromatograph. The column temperature was programmed from 100° to 270° at 10°/min with a 5-min hold at 270°. Once the retention temperature had been determined the compounds were chromatographed isothermally at a suitable temperature to determine exactly their retention times. This procedure was carried out using the 3% OV-1, the 3% OV-17 and the 3% SP-1000 columns.

(B) Gas chromatographic determination of recoveries

(1) Absolute recoveries of benzoic acid, phenobarbital and phenylbutazone. For each of these compounds the same procedure was followed, but with different internal standards and different column temperatures. For benzoic acid, methyl salicylate was used as the internal standard and the column temperature was 140°; for phenobarbital, heptobarbital was used at a column temperature of 210°; and for phenylbutazone, mefenamic acid was used at a column temperature of 230°. In all instances the 3% OV-17 column was used. Each of these compounds, including the internal standards, was dissolved in TMAH to a concentration of 5 mg/ml.

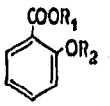
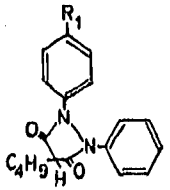
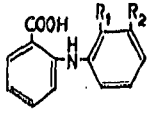
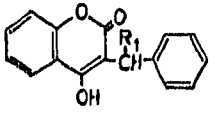
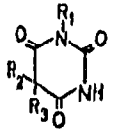
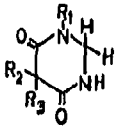
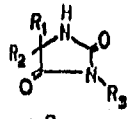
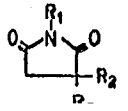
(a) From this solution 5 μl were mixed with 5 μl of the internal standard solution in a glass capillary tube (5 cm × 3 mm I.D.), then 30 μl of DMA and 10 μl of *n*-butyl iodide were added. After mixing, standing for 10 min and centrifugation (2500 *g*) for 5 min, 1 μl of the clear supernatant was injected into the gas chromatograph at the appropriate column temperature and the peak-area ratio was determined.

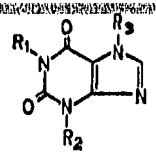
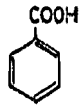
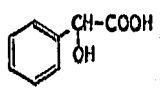
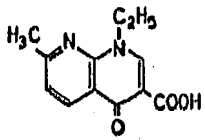
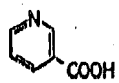
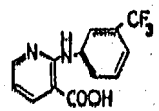
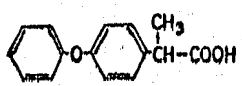
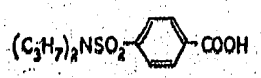
(b) From the solution in TMAH 50 μl were added to 5 ml of toluene and, after shaking for 30 sec and centrifugation (2500 *g*) for 2 min, 5 μl of the methanolic layer were treated as described under (a).

(c) From the solution in TMAH, 50 μl were added to 3 ml of a phosphate buffer (pH 3), to which were added 6 ml of toluene. After shaking for 30 sec and centrifugation (2500 *g*) for 2 min, 5 ml of the toluene layer were transferred into another tube and evaporated to dryness under nitrogen at 70° in a metal heating block. The residue was re-dissolved in 50 μl of the internal standard solution, 10 μl of this

TABLE I

STRUCTURE AND THERAPEUTIC CATEGORY[9] OF THE COMPOUNDS STUDIED

Chemical class	*Structure*	R_1	R_2	R_3	*Name*	*Therapeutic category*
Salicylates		H	H		Salicylic acid	Topical keratolytic, food preservative
		CH_3	H		Methyl salicylate	Counter-irritant
Pyrazolinone derivatives		H			Phenylbutazone	Anti-inflammatory
		OH			Oxyphenbutazone	Anti-inflammatory
Anthranilic acid derivatives		H	CF_3		Flufenamic acid	Anti-inflammatory, analgesic
		CH_3	CH_3		Mefenamic acid	Anti-inflammatory, analgesic
Coumarin derivatives		CH_2–CO–CH_3			Warfarin	Anticoagulant
		CH_2–CH_3			Phenprocoumon	Anticoagulant
Barbiturates		H	C_2H_5	C_6H_5	Phenobarbital	Anti-convulsant, hypnotic, sedative
		H	CH_3	C_6H_5	Heptobarbital	Anti-convulsant, hypnotic, sedative
		CH_3	C_2H_5	C_6H_5	Methylpheno-barbital	Anti-convulsant, hypnotic, sedative
Dioxopyrimidine derivatives		H	C_2H_5	C_6H_5	Primidone	Anti-convulsant
Hydantoins		C_6H_5	C_6H_5	H	Phenytoin	Anti-convulsant, anti-epileptic
		C_6H_5	C_2H_5	CH_3	Mephenytoin	Anti-convulsant, anti-epileptic
Succinimides		H	C_2H_5	CH_3	Ethosuximide	Anti-convulsant

Xanthines		CH_3	CH_3	H	Theophylline	Smooth muscle relaxant, cardiac stimulant
		H	CH_3	CH_3	Theobromine	Smooth muscle relaxant, cardiac stimulant
		CH_3	CH_3	CH_2-COOH	Acefylline	Smooth muscle relaxant, cardiac stimulant
		CH_3	H	CH_3	1,7-Dimethyl-xanthine	Metabolite of caffeine and/or theophylline
		CH_3	H	H	1-Methylxanthine	Metabolite of caffeine and/or theophylline
		H	CH_3	H	3-Methylxanthine	Metabolite of caffeine and/or theophylline
		H	H	CH_3	7-Methylxanthine	Metabolite of caffeine and/or theophylline
Miscellaneous carboxylic acids					Benzoic acid	Pharmaceutical aid, antifungal
					Mandelic acid	Urinary antiseptic
					Nalidixic acid	Antibacterial
					Nicotinic acid	Vitamin
					Niflumic acid	Anti-inflammatory
					Phenoprofen	Anti-inflammatory
					Probenecid	Uricosuric
					Valproic acid	Anti-convulsant, anti-epileptic

solution were placed in a glass capillary tube and 30 μl of DMA and 10 μl of *n*-butyl iodide were added. After standing for 10 min and centrifugation at 2500 *g* for 5 min, 1 μl of the clear supernatant was injected.

(d) The same procedure as described under (c) was followed, but instead of toluene, chloroform–isopropanol (95:5) was used as the extraction solvent.

(2) Revoveries of the other compounds, determined with benzoic acid, phenobarbital and phenylbutazone as the internal standards. Solutions containing three or four of the compounds that were well separated by GC were prepared in TMAH to a concentration of 4 mg/ml for each compound, together with the appropriate internal standard.

(a) From this solution, 10 μl were transferred into a glass capillary tube, then 30 μl of DMA and 10 μl of *n*-butyl iodide were added. After standing for 10 min and centrifugation at 2500 *g* for 5 min, 1 μl of the clear supernatant was injected at the appropriate column temperature and the peak-area ratios were determined.

(b) From the solution in TMAH, 20 μl were added to 2 ml of toluene. After shaking for 30 sec and centrifugation at 2500 *g* for 2 min, 10 μl of the methanolic layer were treated as described under (a).

(c) From the solution in TMAH, 20 μl were added to 1 ml of buffer (pH 3), to which were added 2 ml of toluene. After shaking for 30 sec and centrifugation at 2500 *g* for 2 min, the toluene layer was transferred to another tube to which were added 20 μl of TMAH. After shaking for 30 sec and centrifugation at 2500 *g* for 2 min, 10 μl of the methanolic layer were treated as described under (a).

(d) From the solution in TMAH, 20 μl were added to 1 ml of buffer (pH 3), to which were added 2 ml of chloroform–isopropanol (95:5). After shaking, the organic layer was transferred into another tube by filtration through phase separating paper (Whatman 1 PS) and evaporated to dryness under nitrogen in a metal heating block at 50°. After shaking for 30 sec and centrifugation at 2500 *g* for 2 min, 10 μl of the methanolic layer were treated as described under (a).

(C) Spectrophotometric determination of recoveries

The compounds were dissolved in buffer (pH 3) to a concentration that resulted in an absorbance between 0.7 and 1.0 at the wavelength of maximum absorption.

Of these solutions, 5 ml were extracted with 10 ml of toluene or 10 ml of chloroform–isopropanol (95:5) and the absorbance of the aqueous layer was measured before and after extraction in 1-cm cells against blanks treated in the same way.

For phenobarbital and methylphenobarbital, the procedure described by Hulshoff *et al.*[7] was used.

RESULTS AND DISCUSSION

Table II gives the retention times of the compounds obtained with the three different columns. It can be seen that almost all of the compounds give a single peak on all three columns, so it can be assumed that only one product is formed. Only with phenytoin, mephenytoin and primidone is more than one product formed, although with primidone the major peak is at least 95% of the total peak area. When these three drugs were methylated according to the procedure described here, using methyl iodide instead of *n*-butyl iodide, they all gave one peak on the three different

TABLE II

RETENTION TIMES OF BUTYL ESTERS OF COMPOUNDS STUDIED ON 1.5-m GLASS COLUMNS, PACKED WITH VARIOUS STATIONARY PHASES

Compound	*3% OV-1*		*3% OV-17*		*3% SP-1000*	
	Temp. (°C)	*Retention time (sec)*	*Temp. (°C)*	*Retention time (sec)*	*Temp. (°C)*	*Retention time (sec)*
Valproic acid	100	341	12·	144	120	—*
Benzoic acid	120	229	140	167	150	232
Ethosuximide	120	250	140	213	150	260
Nicotinic acid	120	275	140	218	150	419
Salicylic acid	120	344	140	9243	150	257
Mandelic acid	120	457	140	402	170	440
Methyl salicylate	120	532	140	488	170	256
Theophylline	200	159	210	336	230	347
Theobromine	200	165	210	376	230	414
1,7-Dimethylxanthine	200	160	210	378	230	412
1-Methylxanthine	200	210	210	571	230	430
3-Methylxanthine	200	191	210	552	230	421
7-Methylxanthine	200	205	210	653	230	516
Mephenytoin	200	126; 150	210	184; 256	230	175; 356
Methylphenobarbital	200	173	210	258	230	157
Phenobarbital	200	309	210	417	230	192
Heptobarbital	200	263	210	386	230	188
Primidone	200	306; 431	210	554; 863	230	230; 516
Flufenamic acid	200	234	210	271	230	218
Niflumic acid	200	306	210	369	230	315
Phenoprofen	200	222	210	347	230	253
Mefenamic acid	210	319	230	349	260	173
Acefylline	210	293	230	497	260	570
Probenecid	230	204	230	455	230	926
Phenytoin	200	380; 565	230	832; 905	230	712; 789
Phenprocoumon	230	315	250	382	270	—**
Nalidixic acid	230	254	250	445	270	—**
Phenylbutazone	230	220	250	280	270	202
Warfarin	250	232	250	751	270	—**
Oxyphenbutazone	250	397	250	1140	270	723

* Not detectable, due to the broad dimethylacetamide pesk.
** Not eluted.

columns, so in that instance only one product is formed. The identity of the products obtained after butylation is at present being studied using GC combined with mass spectrometry.

From Table II it is clear that the drugs can be chromatographed on the OV-17 column, using only a few fixed column temperatures, *e.g.*, 140°, 210° and 250°. A good separation is obtained between theophylline and the other xanthines and also between phenobarbital and methylphenobarbital, indicating that after butylation a sufficiently good separation between a drug and its demethylated metabolite(s) is obtained.

Table III gives the results of the GC recovery studies for the various compounds (Experimental, procedure B). In the absolute recovery studies a correction was made

TABLE III

RECOVERIES OF THE COMPOUNDS IN THE VARIOUS EXTRACTION STEPS
Each result is the mean of three determinations.

Compound	*Recovery with back-extraction with TMAH (%)*	*Recovery with toluene (%)*	*Recovery with chloroform–isopropanol (%)*
Valproic acid	72	87	79
Benzoic acid	100	73	98
Ethosuximide	83	41	101
Nicotinic acid	1·0	<1	16
Salicylic acid	95	57	96
Mandelic acid	93	21	55
Methyl salicylate	91	100	98
Theophylline	104	<1	73
Theobromine	103	<1	63
1,7-Dimethylxanthine	86	<1	61
1-Methylxanthine	97	<1	4
3-Methylxanthine	86	<1	3
7-Methylxanthine	86	<1	4
Mephenytoin	103	97	92
Methylphenobarbital	80	89	102
Phenobarbital	95	47	95
Heptobarbital	98	31	91
Primidone	102	7	62
Flufenamic acid	62	95	69
Niflumic acid	61	94	101
Phenoprofen	80	101	88
Mefenamic acid	80	72	88
Acefylline	95	9	19
Probenecid	89	66	82
Phenytoin	102	69	86
Phenprocoumon	73	67	95
Nalidixic acid	77	102	95
Phenylbutazone	99	73	77
Warfarin	47	101	102
Oxyphenbutazone	98	95	98

for the 25% reduction in the volume of the TMAH layer when extracting toluene with this solution. For the studies with an internal standard (Experimental, procedure B2), this correction is not necessary. The recovery of the extraction of the drugs from toluene with TMAH is in general very good; only the anthranilic acid derivatives and warfarin show a low recovery. The recovery of the extraction with toluene from the buffer solution is very poor for many of the drugs, especially the xanthine derivatives, nicotinic acid, mandelic acid and primidone.

With chloroform–isopropanol the recovery is much better for these compounds and in general is acceptable, except perhaps for the monomethylxanthines, acefylline and nicotinic acid. In most instances it is therefore advisable to use chloroform–isopropanol as the extraction solvent, although this approach has the disadvantage that sometimes emulsions are formed upon mixing with vortex-type mixers and that the extract has to be evaporated. An advantage of this solvent is that the organic layer can be very easily transferred into another tube by filtration through phase separating

paper. When the tubes containing acidified plasma and chloroform–isopropanol are shaken by hand instead of on a vortex mixer, no emulsions are formed. Separation of the two phases by filtration without centrifugation of the tubes is then possible, although this frequently results in considerable losses of the extract.

In Table IV the results of the gas chromatographic and spectrophotometric recovery studies are compared, showing very good agreement between the results obtained by the two methods. On the basis of the data obtained the following procedure is suggested for the determination of acidic drugs in plasma.

TABLE IV

COMPARISON OF RECOVERIES OBTAINED BY GC AND UV SPECTROPHOTOMETRY

Each result is the mean of three determinations.

Compound	*Recovery with toluene (%)*		*Recovery with chloroform–isopropanol (%)*	
	GC	*UV*	*GC*	*UV*
Salicylic acid	57	62	96	94
Methyl salicylate	100	96	98	100
Theophylline	<1	<1	73	72
3-Methylxanthine	<1	<1	3	5
Methylphenobarbital	89	93	102	96
Phenobarbital	47	49	95	98
Niflumic acid	94	95	101	99
Phenoprofen	101	93	88	98
Nalidixic acid	102	100	95	100
Warfarin	101	100	102	100

To 1 ml of plasma in a centrifuge tube add 50 μl of 4 *M* hydrochloric acid and 2 ml of chloroform–isopropanol (95:5), containing a suitable internal standard. Shake by hand for 30 sec and separate the two phases by filtration through phase separating paper, if necessary after centrifugation. Evaporate the organic phase to dryness under nitrogen at 50° and dissolve the residue in 2 ml of toluene and 20 μl of a solution of 20% TMAH in methanol. After mixing on a vortex-type mixer (30 sec) and centrifugation (2500 *g*, 2 min), transfer 10 μl of the TMAH layer into a glass capillary tube (5 cm × 3 mm I.D.) containing 30 μl of DMA. Add 10 μl of *n*-butyl iodide, mix well and, after 10 min, centrifuge (2500 *g*, 2 min). Inject 1 or 2 μl of the clear supernatant into the gas chromatograph, equipped with a 3% OV-17 column, maintained at 140°, 210° or 250°, depending on the compound(s) to be determined.

If the compound to be determined is extracted in sufficient yield with toluene, the procedure can be simplified by using toluene instead of chloroform–isopropanol for the extraction. After centrifugation the toluene layer is then transferred into another tube by means of a pipette and extracted with 20 μl of TMAH, so no evaporation of the solvent is needed.

In order to evaluate possible interferences from endogenous compounds, blank plasma samples were treated according to the procedure described above. The results are shown in Fig. 1, from which it is obvious that without the back-extraction step a number of interfering peaks show up, whereas the chromatograms obtained after back-extraction with TMAH are almost completely clean. When toluene is used as the extraction solvent the differences are less pronounced, but back-extraction is still

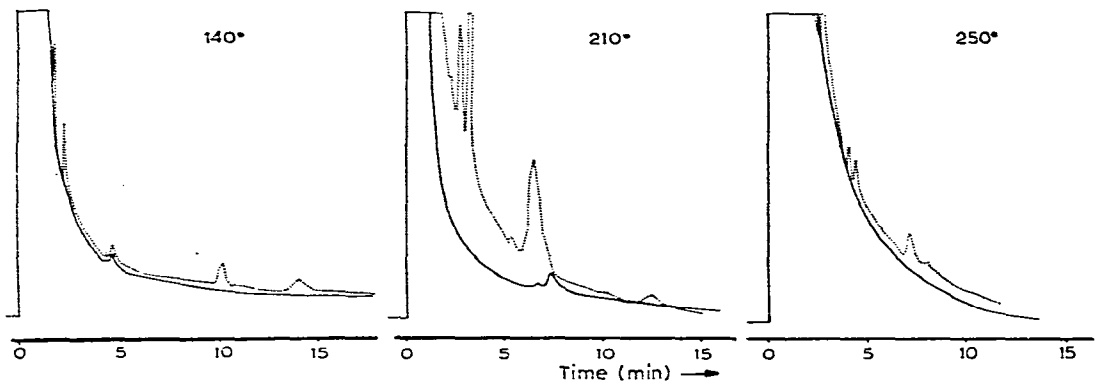


Fig. 1. Chromatograms at the indicated column temperatures of blank plasma samples after extraction with chloroform–isopropanol and evaporation of the solvent. Broken line, residue dissolved in DMA and TMAH and treated with *n*-butyl iodide; solid line, residue dissolved in toluene, back-extracted with TMAH and treated as described.

worthwhile, if only as a convenient concentration step. Fatty acids are likely to interfere in the assay when an extract, obtained after a single extraction step, is injected because these compounds can be derivatized and chromatographed in the same way. However, a recovery study with palmitic acid showed that this compound is extracted from toluene with TMAH to the extent of about 0.1%, which explains the virtual absence of peaks contributable to the higher fatty acids.

The procedure described above was used to construct calibration graphs for theophylline in plasma and saliva to be used in bioavailability studies. In this instance 200 μl of plasma or saliva were extracted with chloroform–isopropanol, containing phenobarbital as the internal standard. The calibration graphs were straight lines passing through the origin, the coefficient of variation at a concentration of 9 μg/ml was 3% and the minimum detectable concentration in 200 μl of plasma was 1 μg/ml.

The determination of acidic drugs in plasma using this procedure is comparable to other methods with respect to speed and sensitivity. Its advantages are as follows. It is a standard procedure, using one standard column, and can be applied to many drugs from various chemical and therapeutic classes, without interference from endogenous compounds; no decomposition of the products occurs in the alkylation process and the injection of solutions from which the excess of quaternary ammonium base has been removed is less damaging to the column material than when using an on-column alkylation technique.

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