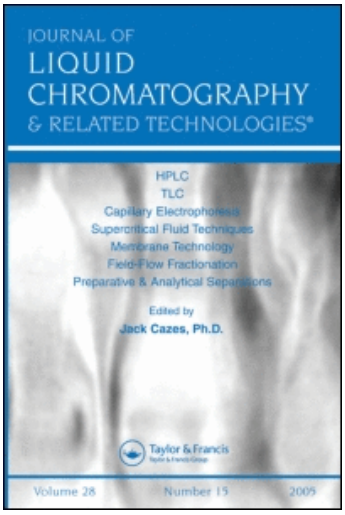

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DETERMINATION OF FLUOXETINE ENANTIOMERS IN HUMAN SERUM AND URINE BY ISOCRATIC HPLC AND PRE-COLUMN FLUORESCENCE DERIVATIZATION WITH (*R*)-(+)-4-NITRO-7-(2-CHLOROFORMYLPYRROLIDIN-1-YL)-2,1,3-BENZOXADIAZOLE

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DETERMINATION OF FLUOXETINE ENANTIOMERS IN HUMAN SERUM AND URINE BY ISOCRATIC HPLC AND PRE-COLUMN FLUORESCENCE DERIVATIZATION WITH (*R*)-(+)-4-NITRO-7-(2-CHLOROFORMYLPYRROLIDIN-1-YL)-2,1,3-BENZOXADIAZOLE

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□ *Fluoxetine (FLX) is a highly selective serotonin reuptake inhibitor used as an antidepressant agent and administered as the racemate. In this study, we developed an isocratic HPLC method with fluorescence detection for the determination of FLX enantiomers ((R)-FLX and (S)-FLX) in human serum and urine, after pre-column derivatization with (R)-(+)-4-nitro-7-(2-chloroformyl-pyrrolidin-1-yl)-2,1,3-benzoxadiazole ((R)-(+)-NBD-Pro-COCl). After basic extraction of the samples into pentane, derivatization with (R)-(+)-NBD-Pro-COCl was conducted in borate buffer (pH 9.0) at 70°C for 5 min. Protriptyline was utilized as an internal standard. The regression equations for (R)-FLX hydrochloride and (S)-FLX hydrochloride in human serum showed good linearity in the range of 0.01–0.5 μg/mL with the detection limit of 0.005 μg/mL and in the range of 0.025–0.5 μg/mL with the detection limit of 0.008 μg/mL, respectively. The corresponding values for human urine were 0.025–0.5 μg/mL with the detection limit of 0.014 μg/mL and 0.025–0.5 μg/mL with the detection limit of 0.010 μg/mL, respectively. The coefficients of variation were less than 11.8%, and recovery was good. Our method using (R)-(+)-NBD-Pro-COCl is useful for simultaneous determination of (R)-FLX and (S)-FLX in human serum and urine, and may be suitable for therapeutic drug monitoring.*

Keywords enantiomer, fluorescence detection, fluoxetine, pre-column derivatization, (*R*)-(+)-4-nitro-7-(2-chloroformylpyrrolidin-1-yl)-2,1,3-benzoxadiazole

INTRODUCTION

Fluoxetine (FLX, (±)-*N*-methyl-Y-[4-(trifluoromethyl)phenoxy]benzene propanamine) is a highly selective serotonin reuptake inhibitor used as an antidepressant agent.[1,2] It has been widely marketed and is prescribed

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as the racemate. However, the isomers do not have the same pharmacological activities,[3,4] and exhibit different disposition kinetics.[5–7] For example, at 3 h (time to reach maximum plasma concentration) after administration of FLX at a dose of 10 mg/kg to rats, the FLX concentration was 268.7 nM, consisting of 46.1 and 53.9% of (*R*)-FLX (123.9 nM) and (*S*)-FLX (144.8 nM), respectively.[5] Subsequently, the FLX concentration gradually decreased, and the (*R*)-FLX and (*S*)-FLX concentrations were 7.4 nM and 16.6 nM at 24 hr.

Further, a patient taking FLX at a dosage of 20 mg/day was found to have a higher plasma level of (*S*)-FLX (183 ng/mL) than (*R*)-FLX (66 ng/mL), though the sampling time was not given.[6] Plasma concentration values in 131 adult patients receiving long-term fluoxetine at 10 to 60 mg/day were 186 nM for (*S*)-FLX and 67 nM for (*R*)-FLX.[7]

There have been relatively few reports on methods for simultaneous determination of (*R*)-FLX and (*S*)-FLX, compared with total FLX.[8–12] Guo et al. reported enantiomer analysis of FLX in rat plasma by column switching HPLC using an amylase-based chiral column after pre-column fluorescence derivatizaion with 4-(*N*-chloroformylmethyl-*N*-methyl)amino-7-nitro-2,1,3-benzoxadiazole (NBD-COCl) and 4-(*N*-chloroformylmethyl-*N*-methyl)amino-7-*N,N*-dimethylaminosulfonyl-2,1,3-benzoxadiazole (DBD-COCl).[5] NBD-COCl was superior to DBD-COCl in terms of the sensitivity. However, switching systems are not popular, the columns are expensive, and derivatization is time-consuming (2 hr). As another approach, (*R*)-1-(1-naphthyl)ethyl isocyanate was used as a labeling agent for chiral separation of FLX, and highly sensitive detection was achieved.[13] However, complicated gradient pump control was needed, the derivatization time was 30 min, and fluvoxamine, used as an internal standard (IS), is expensive. Gas chromatographic methods coupled with mass spectrometry and electron capture detection allowed sensitive detection of FLX after derivatization with a chiral reagent, (*S*)-(-)-*N*-trifluoroacetylprolyl chloride, but require specialized equipment.[14,15]

Although it was reported that about 11% of FLX dosed was excreted unchanged,[13,16] the enantiomer ratio excreted in urine remains unknown. To our knowledge, there has been no report of an analytical method for determination of the enantiomers in human urine.

Therefore, we considered it would be useful to develop a new approach for simultaneous determination of (*R*)-FLX and (*S*)-FLX in biological fluids. In this study, we present a simple method employing isocratic HPLC with fluorescence detection for assay of (*R*)-FLX and (*S*)-FLX in human serum and urine after pre-column derivatization with (*R*)-(+)-4-nitro-7-(2-chloroformylpyrrolidin-1-yl)-2,1,3-benzoxadiazole ((*R*)-(+)-NBD-Pro-COCl). The reaction scheme is presented in Figure 1. Protriptyline was chosen as an inexpensive IS.

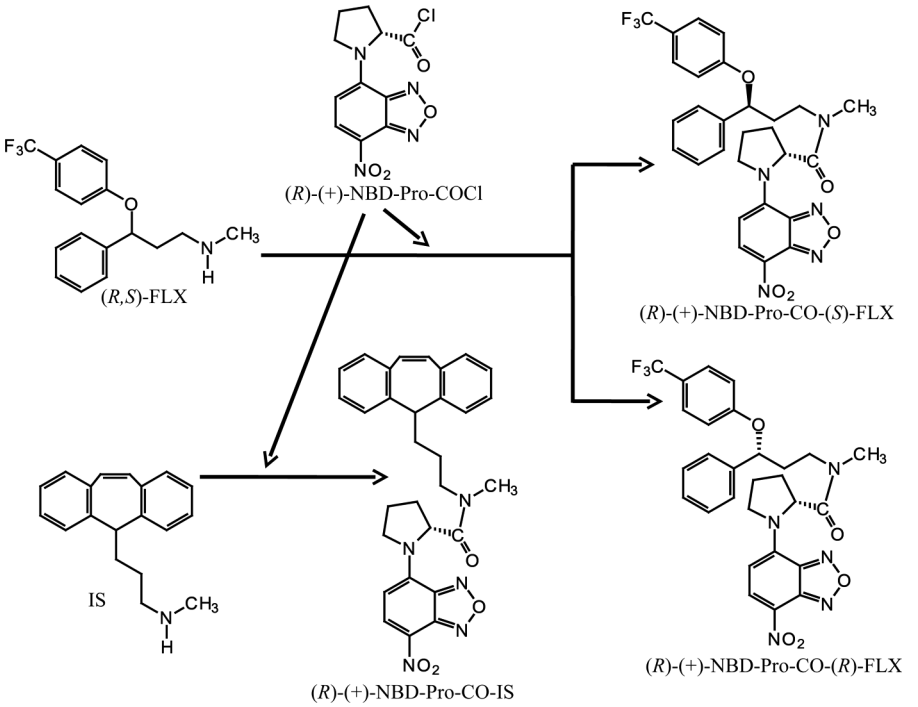

FIGURE 1 Derivatization scheme of (*R,S*)-FLX and IS with (*R*)-(+)-NBD-Pro-COCl.

EXPERIMENTAL

Reagents

FLX hydrochloride, (*R*)-FLX hydrochloride, (*S*)-FLX hydrochloride, norfluoxetine (NFLX) hydrochloride, protriptyline hydrochloride, maprotiline hydrochloride, and nortriptyline hydrochloride, human serum, and acetonitrile were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Human urine as a freeze-dried powder (Normal, quantitative urine control) and (*R*)-(+)-NBD-Pro-COCl were obtained from Bio-Rad Laboratories (Irvine, CA, U.S.A.) and Tokyo Chemical Industry Co. (Tokyo, Japan), respectively. Fluvoxamine maleate, desipramine hydrochloride, paroxetine hydrochloride, and general reagents were obtained from Wako Pure Chemical Industries (Osaka, Japan).

Equipment

The HPLC system comprised a model L-6200 pump (Hitachi, Tokyo), a Rheodyne injection valve (Cotati, CA, USA) with a 50-μL loop and a model RF-10A fluorometer (Shimadzu, Kyoto, Japan) operating at an excitation

wavelength of 470 nm and an emission wavelength of 540 nm. A C_{18}-MS-II column (Nacalai Tesque, Kyoto) of 150 × 4.6 mm i.d. with 5 μm particles was used. Quantification of the peaks was performed with a Chromatopac Model CR-8A integrator (Shimadzu). The mobile phase was prepared by the addition of acetonitrile (550 mL) to a solution of trifluoroacetic acid (0.1 v/v%) in 450 mL of water. The samples were eluted from the column at a flow rate of 1.0 mL/min, at room temperature.

Extraction from Human Serum and Urine

An aliquot of 100 μL of sample was rendered alkaline by the addition of NaOH (1 M, 100 μL). Protriptyline hydrochloride solution in water (0.1 μg/mL, 100 μL) was added as an IS to prepare the standard curves for (*R*)-FLX and (*S*)-FLX. Then, the mixture was vortexed for 1 min and extracted with pentane (3 mL, twice). The pentane phases were combined and evaporated, and derivatization was performed as follows.

Derivatization

Borate buffer (0.1 M) was adjusted to pH 9.0 by the addition of NaOH (1 M). Borate buffer (300 μL) was added to the extract. (*R*)-(+)-NBD-Pro-COCl solution in acetonitrile (1 mg/mL, 100 μL) was added and the mixture was vortexed, then allowed to stand for 5 min at 70°C. Next, it was set on ice for 1 min, and an aliquot (50 μL) was injected into the HPLC system.

Separation Parameters of Diastereomers

The separation factor (α) and resolution value (*R*s) were calculated from the following equations:

$$\alpha = (t_{R2} - t_0)/(t_{R1} + t_0)$$

$$R\text{s} = 1.18 \times (t_{R2} - t_{R1})/(W_1 + W_2)$$

where t_0 is the retention time on the void volume of the column; t_{R1}, and t_{R2} are the retention times of the peaks; W_1 and W_2 are the widths at half height from the bases formed by triangulation of the peaks.

Calibration Curve

A solution of FLX hydrochloride (1 mg/mL) in water was added to human biological fluids. The concentrations of FLX hydrochloride were

0, 0.02, 0.05, 0.1, 0.2, 0.5, and 1 μg/mL (0, 0.01, 0.025, 0.05, 0.1, 0.25, and 0.5 μg/mL, respectively, as (*R*)-FLX hydrochloride and (*S*)-FLX hydrochloride concentrations). All samples were extracted and analyzed using the procedures described above. Calibration curves based on the peak area ratios of (*R*)-FLX hydrochloride and (*S*)-FLX hydrochloride to the IS were constructed.

RESULTS AND DISCUSSION

Reaction Time Course and pH Dependency

For the time course study, the reaction time was set at 3, 5, 10, 20, and 30 min. A sample (100 μL) of (*R*)-FLX hydrochloride and (*S*)-FLX hydrochloride (each 0.25 μg/mL) mixed solution in water was added to borate buffer (0.1 M, 200 μL, pH 9.0), and derivatized with (*R*)-(+)-NBD-Pro-COCl (1 mg/mL, 100 μL). Both derivatives reached a plateau at 5 min, subsequently their peak areas tended to decrease (data not shown). The IS derivative reached a plateau at 3 min.

Next, pH dependency (pH 7.0 to 9.5) was examined at the derivatization time of 5 min. Peak areas of both derivatives at pH 7.0 and 7.5 were about 70 to 90% of the maximum at pH 9.0, and the areas at pH 8.5 and 9.5 were more than 95% of the maximum. The behavior of the IS derivative was similar. Thus, the derivatization time of 5 min at pH 9.0 was selected.

Chromatogram

Figure 2 shows typical chromatograms obtained from (A-1) blank serum, (A-2) serum spiked with FLX hydrochloride (1 μg/mL), NFLX hydrochloride (1 μg/mL), and IS (0.1 μg/mL), (B-1) blank urine and (B-2) urine spiked with FLX hydrochloride (1 μg/mL), NFLX hydrochloride (1 μg/mL), and IS (0.1 μg/mL). The retention times of the IS, (*R*)-FLX, and (*S*)-FLX derivatives were 23.8, 26.4, and 28.5 min, respectively. The running time was 31 min.

It is well known that FLX is mainly converted to NFLX (the *N*-demethylated metabolite of FLX), which has similar pharmacological activity to FLX.[1,17,18] Therefore, we examined the simultaneous detection of derivatives of FLX and NFLX enantiomers in this assay. Two peaks of NFLX derivative were detected at 17.6 and 19.8 min. However, identification could not be performed since (*R*)-NFLX and (*S*)-NFLX are not commercially available.

On the other hand, the values of α and *R*s in human serum and urine at 0.5 μg/mL in Figure 2 were 1.08 and 1.38, respectively, suggesting that our

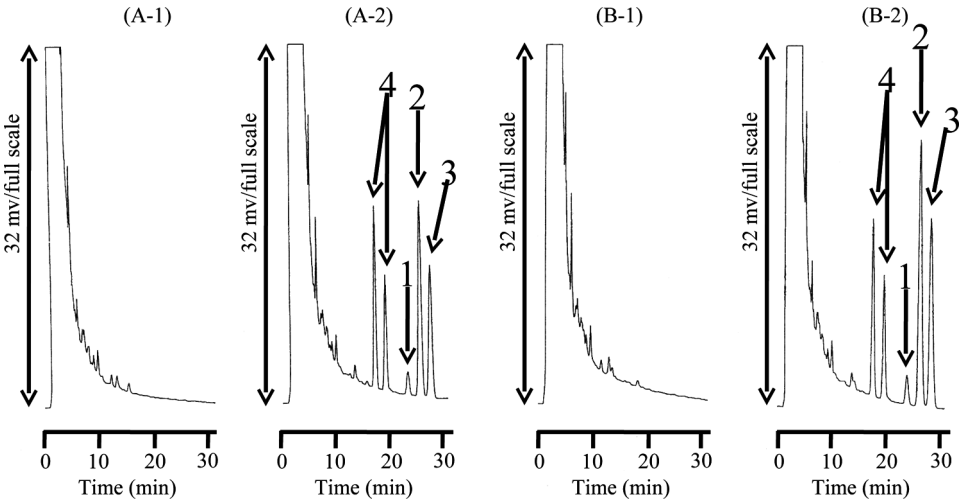

FIGURE 2 Typical chromatograms of (*R*)-FLX, (*S*)-FLX, NFLX, and IS derivatives with (*R*)-(+)-NBD-Pro-COCl in human serum (A) and urine (B). (A-1) and (B-1), Blank; (A-2) and (B-2), Sample spiked with FLX hydrochloride (racemate, 1 mg/mL), NFLX hydrochloride (racemate, 1 mg/mL), and IS (0.1 mg/mL). Peaks: 1 = IS derivative, 2 = (*R*)-FLX derivative, 3 = (*S*)-FLX derivative, 4 = NFLX derivatives, not identified as enantiomer of NFLX.

method using (*R*)-(+)-NBD-Pro-COCl will be useful for determining FLX enantiomers in human biological fluids.

Standard Curves of (*R*)-FLX and (*S*)-FLX and Detection Limits

Standard curves of (*R*)-FLX and (*S*)-FLX were constructed by plotting integrated peak area ratios of (*R*)-FLX hydrochloride and (*S*)-FLX hydrochloride to IS *vs.* (*R*)-FLX hydrochloride and (*S*)-FLX hydrochloride concentration (Table 1). In human serum samples, linear relationships were obtained for (*R*)-FLX hydrochloride ($y = 16.2x + 0.0270$, concentrations ranging from 0.01 to 0.5 μg/mL, $r = 0.9994$) and for (*S*)-FLX hydrochloride ($y = 12.2x + 0.0325$, concentrations ranging from 0.025 to 0.5 μg/mL, $r = 0.9995$). The lower limits of detection for (*R*)-FLX hydrochloride and

TABLE 1 Linear Correlation Parameters of (*R*)-FLX Hydrochloride and (*S*)-FLX Hydrochloride

	Slope	Intercept	Concentration Range	*r*
Serum				
(*R*)-FLX hydrochloride	16.2	0.0270	0.01 to 0.5 μg/mL	0.9994
(*S*)-FLX hydrochloride	12.2	0.0325	0.025 to 0.5 μg/mL	0.9995
Urine				
(*R*)-FLX hydrochloride	14.2	0.0663	0.025 to 0.5 μg/mL	0.9994
(*S*)-FLX hydrochloride	10.1	0.0337	0.025 to 0.5 μg/mL	0.9995

(*S*)-FLX hydrochloride were 0.005 μg/mL (14 nM, 0.0045 μg/mL as (*R*)-FLX) and 0.008 μg/mL (23 nM, 0.0072 μg/mL as (*S*)-FLX), respectively (signal-to-noise ratio of 3:1). In human urine samples, linear relationships were obtained for (*R*)-FLX hydrochloride ($y = 14.2x + 0.0663$, concentrations ranging from 0.025 to 0.5 μg/mL, $r = 0.9994$) and for (*S*)-FLX hydrochloride ($y = 10.1x + 0.0337$, concentrations ranging from 0.025 to 0.5 μg/mL, $r = 0.9995$). The lower limits of detection for (*R*)-FLX hydrochloride and (*S*)-FLX hydrochloride were 0.014 μg/mL (40 nM, 0.013 μg/mL as (*R*)-FLX) and 0.010 μg/mL (29 nM, 0.0089 μg/mL as (*S*)-FLX), respectively (signal-to-noise ratio of 3:1).

Previous reports on determination of FLX enantiomers gave the limits of quantification of 10 nM for FLX[5] and 0.2 ng/mL (signal-to-noise ratio of 2:1) for each enantiomer.[13] These methods are superior to ours in terms of sensitivity, and were applied for pharmacokinetic studies in healthy volunteers (dose of 40 mg of FLX hydrochloride) and in rats (dose of 10 mg/kg of FLX hydrochloride). However, reported levels of (*R*)-FLX and (*S*)-FLX in various biological fluids[5–7] are above the limit of quantification of our method. Therefore, our method will be suitable for (*R*)-FLX and (*S*)-FLX determination in serum samples. In addition, our method is applicable to determine (*R*)-FLX and (*S*)-FLX in human urine.

Precision and Accuracy

Precision and accuracy for intra-day and inter-day assays of (*R*)-FLX and (*S*)-FLX derivatives are shown in Tables 2 and 3. In the intra- and inter-day

TABLE 2 Intra- and Inter-Day Assay Reproducibility for Determination of (*R*)-FLX Hydrochloride and (*S*)-FLX Hydrochloride in Human Serum

Concentration (μg/mL)	Measured (μg/mL) (Mean ± S.D., $n = 5$)	C.V. (%)	Recovery (%)
Intra-day assay			
(*R*)-FLX hydrochloride			
0.01	0.0108 ± 0.0011	10.2	108.0
0.025	0.0236 ± 0.0020	8.5	94.4
0.5	0.536 ± 0.022	4.1	107.2
(*S*)-FLX hydrochloride			
0.025	0.0242 ± 0.0022	9.1	96.8
0.5	0.540 ± 0.030	5.6	108.0
Inter-day assay			
(*R*)-FLX hydrochloride			
0.01	0.0110 ± 0.0013	11.8	110.0
0.025	0.0240 ± 0.0021	8.8	96.0
0.5	0.548 ± 0.046	8.4	109.6
(*S*)-FLX hydrochloride			
0.025	0.0234 ± 0.0017	7.3	93.6
0.5	0.522 ± 0.040	7.7	104.4

TABLE 3 Intra- and Inter-Day Assay Reproducibility for Determination of (*R*)-FLX Hydrochloride and (*S*)-FLX Hydrochloride in Human Urine

Concentration (μg/mL)	Measured (μg/mL) (Mean ± S.D., $n = 5$)	C.V. (%)	Recovery (%)
Intra-day assay			
(*R*)-FLX hydrochloride			
0.025	0.0259 ± 0.0017	6.6	103.6
0.5	0.532 ± 0.046	8.6	106.4
(*S*)-FLX hydrochloride			
0.025	0.0244 ± 0.0023	9.4	97.6
0.5	0.496 ± 0.034	6.9	99.2
Inter-day assay			
(*R*)-FLX hydrochloride			
0.025	0.0262 ± 0.0021	8.1	104.8
0.5	0.520 ± 0.042	8.1	104.0
(*S*)-FLX hydrochloride			
0.025	0.0242 ± 0.0025	10.3	96.8
0.5	0.494 ± 0.048	9.7	98.8

assays using human serum samples (Table 2), the range of standard deviation of the mean for (*R*)-FLX hydrochloride and (*S*)-FLX hydrochloride was within 4.1 to 11.8%. The recoveries of (*R*)-FLX hydrochloride and (*S*)-FLX hydrochloride were within 93.6 to 110.0%. In the intra- and inter-day assays using human urine samples (Table 3), the range of standard deviation of the mean for (*R*)-FLX hydrochloride and (*S*)-FLX hydrochloride was within 6.6 to 10.3%. The recoveries of (*R*)-FLX hydrochloride and (*S*)-FLX hydrochloride were within 96.8 to 106.4%. The assay exhibited satisfactory precision and accuracy. Since the validation parameters are acceptable, our method should be suitable for pharmacokinetic studies in serum and urine.

Interference

As shown in Table 4, the retention times of other antidepressant derivatives were investigated. The relative retention times of fluvoxamine,

TABLE 4 Interference by Other Antidepressants

Derivatives	Relative Retention Time
Fluvoxamine	0.66
NFLX	0.74 and 0.83
Paroxetine	0.84
IS	1.00
Desipramine	1.00
Maprotiline	1.10
(*R*)-FLX	1.11
Nortriptyline	1.17
(*S*)-FLX	1.20

paroxetine, desipramine, maprotiline, and nortriptyline derivatives were 0.66, 0.84, 1.00, 1.10, and 1.17, respectively. The peaks of the desipramine and maprotiline derivatives almost entirely overlapped with the IS and (*R*)-FLX derivative peaks, respectively. In addition, the NFLX derivative interfered with paroxetine.

CONCLUSION

Although the determination and identification of NFLX enantiomers were not possible, an isocratic HPLC method was developed for quantitation of (*R*)-FLX and (*S*)-FLX in human serum and urine by means of fluorescence detection after pre-column derivatization with (*R*)-(+)-NBD-Pro-COCl. While the sensitivity of our method is less than has been obtained with other methods, the derivatization procedure is much quicker in our case.[5,12] Validation data (precision, accuracy, and recovery) were satisfactory for simultaneous (*R*)-FLX and (*S*)-FLX determination. Among related compounds examined, desipramine and maprotiline interfered with the IS and (*R*)-FLX derivative peaks. The established method appears to be suitable for routine therapeutic drug monitoring of FLX enantiomers during treatment for depression in patients not concomitantly receiving desipramine and maprotiline, as well as for pharmacokinetic studies in experimental animals.

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