

# Pharmacokinetic Approaches to the Study of Drug Action and Toxicity

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## INTRODUCTION

Pharmacokinetics refers to the events that occur following drug administration to alter the availability of a drug by elimination or metabolism. These processes contribute to the effects of a drug not only by removal from the body but, in some instances, by forming pharmacologically or toxicologically active compounds. It is therefore critical to understand the pharmacokinetics of drugs when studying their effects *in vivo* and in cellular systems. Because pharmacokinetics is based on measurements of the concentration of drugs and metabolites in plasma and tissue, it requires bioanalytical chemical techniques. The concentrations involved in the studies are usually in the micromolar range, and when pharmacokinetic experiments are performed, the volumes involved are small, commonly 0.1 to 0.2 mL. In the authors' studies, we have found it advantageous to perform experiments at the *in vivo* and *in vitro* levels to direct subsequent experiments or interpret results from others. The *in vitro* incubation mixtures usually contain cellular components, whereas the *in vivo* samples are plasma or tissue homogenates so that the analytes are in complex matrices. As a result, the analytical procedures require high sensitivity and specificity. This chapter describes applications of these procedures to studies of the pharmacology and toxicology of methylenedioxymethamphetamine (MDMA)- and methylenedioxyamphetamine (MDA)-related compounds.

## PHARMACOKINETIC AND PHARMACODYNAMIC STUDIES OF MDMA

MDMA is a substituted amphetamine with actions on the 5-hydroxytryptamine (5-HT) and the dopamine (DA) systems (Schmidt 1987; Stone et al. 1988; Johnson et al. 1988). The compound causes an initial release of DA as well as 5-HT but under appropriate regimens also causes a long-term depletion of 5-HT and reduction in tryptophan hydroxylase activity (Stone et al. 1989). In addition to these chemical changes, there are morphological changes consistent with a loss of presynaptic terminals in different areas of the brain (Ricuarte et al. 1985;

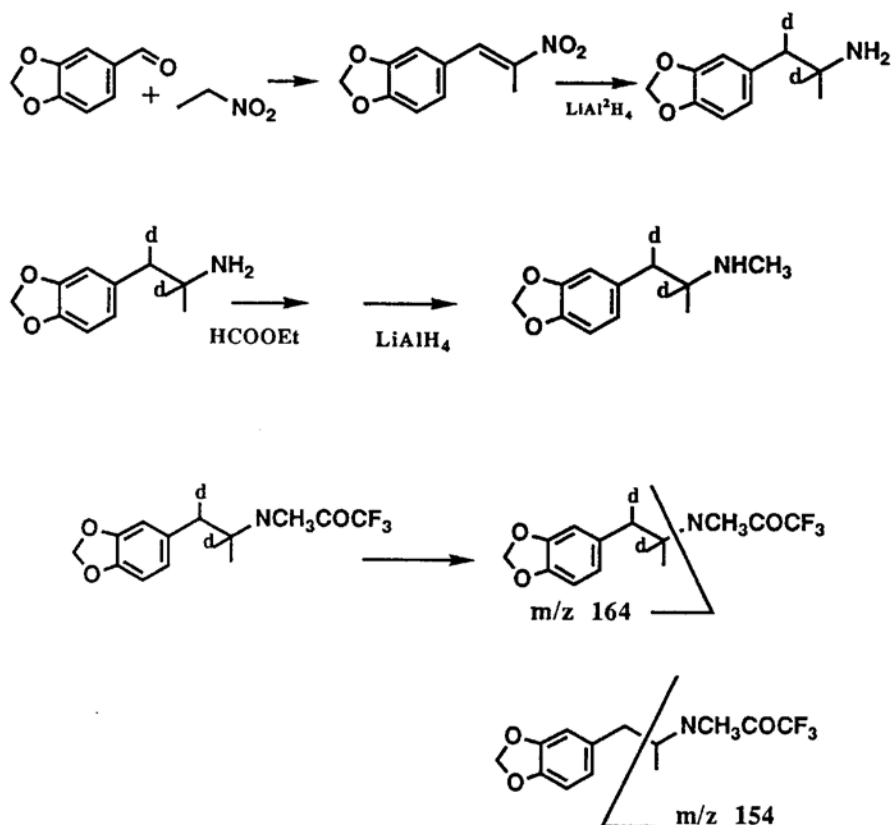
Wilson et al. 1989). Early studies focused on MDMA's temporal and metabolic disposition in the whole animal as it related to the pharmacodynamics of MDMA. Because MDMA is a lipid-soluble compound of moderate volatility, it was analyzable by gas chromatography (GC) techniques. The more polar catechol metabolites required liquid chromatography (LC). Therefore, the analyses utilized GC/mass spectrometry (MS) and high-performance LC/electrochemical detection (HPLC-ECD). MS is highly sensitive, and when it was coupled to a capillary gas chromatograph, high specificity also was achieved. Similarly, ECD, when coupled to a liquid chromatograph, also was able to achieve high sensitivity and specificity for electroactive compounds. The electrochemical detection was especially sensitive for easily oxidized substrates such as the catechol function.

### **GC/MS With Selected Ion Monitoring**

A mass spectrometer is more commonly used to collect data resulting from the fragmentation of the analyte in an electron beam. A series of charged fragments is generated, and the mass spectrometer identifies the charge-to-mass ratio of each fragment and determines its relative abundance. The fragmentation pattern is characteristic of the analyte and may be used to identify or partially identify its chemical structure. In the selected ion monitoring mode, the mass spectrometer is focused on two fragments, one characteristic of the analyte and the other for the internal standard, which is frequently an isotopic variant of the analyte (Jenden and Cho 1979). Because the instrument is focused on only two masses instead of scanning a range, the limits of detectability are much lower. In the assay for MDMA, a fragment with mass/charge ( $m/z$ ) 154 was used for the analyte and a fragment of  $m/z$  164 for the internal standard (figure 1). The peak at  $m/z$  154 is caused by  $\beta$ -cleavage of the trifluoroacetyl derivative and is the base peak or the most abundant fragment in the spectrum. The internal standard was a deuterium-substituted variant that contains two deuterium atoms in the ethyl side chain. On fragmentation, this variant has one peak at  $m/z$  155 and a second, less intense peak (50 percent) at  $m/z$  164. Although the  $m/z$  155 peak would be more sensitive, the nondeuterium-enriched ( $d_0$ ) analyte MDMA also has a small (5 percent) quantity of fragment at this mass as well. To avoid overlap, the signal at  $m/z$  164 with less than 1 percent overlap was chosen.

### **Synthesis of Internal Standards**

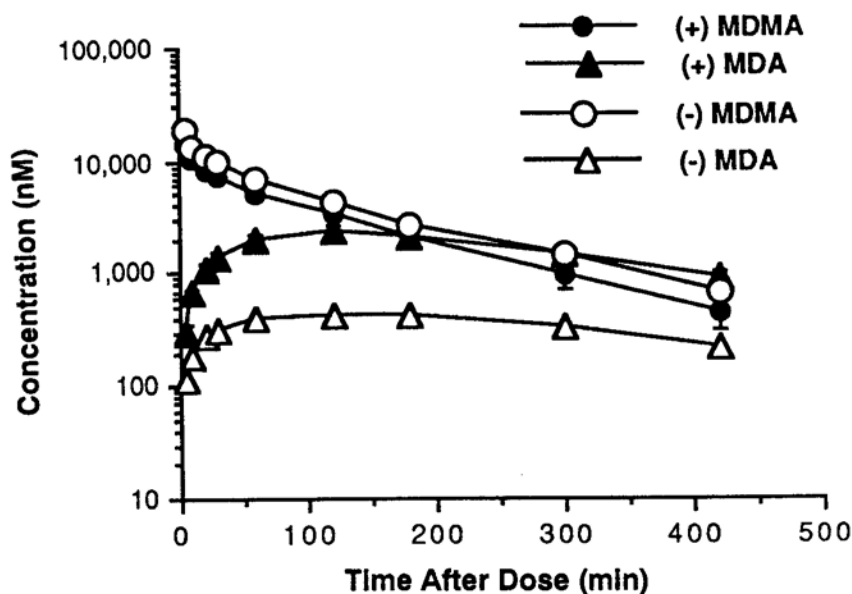
The internal standard was prepared by the route indicated in figure 1, utilizing conventional synthetic procedures with lithium aluminum deuteride instead of the hydride (Lindeke and Cho 1972).



**FIGURE 1.** The synthesis of deuterium-substituted MDMA and its conversion to the trifluoroacetyl derivative. The fragmentation resulting in the ions used for selected ion monitoring is shown.

#### Analytical Procedure

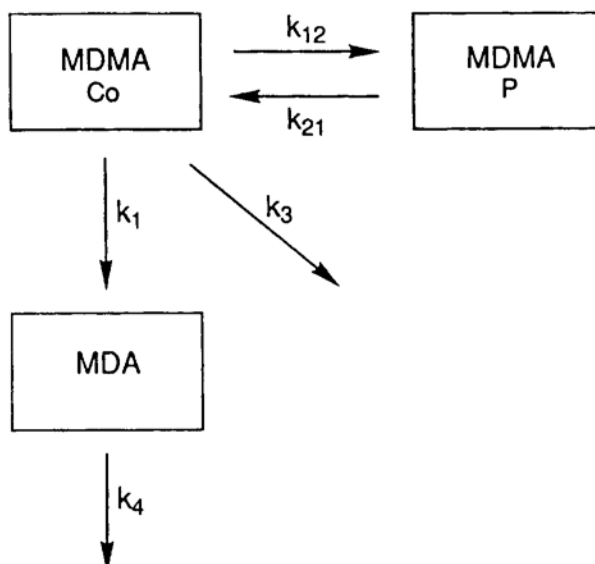
In the analysis, the internal standard was added to the sample; and the analyte, MDMA, and the internal standard were extracted into an organic solvent. The extract was concentrated by evaporation of solvent; the residue was treated with trifluoroacetic anhydride and injected into the GC/MS. The mass spectrometer was focused on the preset masses, and the appropriate gas chromatographic peak was analyzed for ratios of signal from the analyte and internal standard. Quantitation was achieved with a standard curve generated from mixtures of fixed concentrations of internal standard and varying concentrations of analyte. Results of the initial studies determining the pharmacokinetics of MDMA after equimolar intravenous doses of (+) and (-) MDMA are shown in figure 2.



**FIGURE 2.** Time course of plasma MDMA and MDA after intravenous administration of (+) and (-) MDMA (10 mg/kg)

#### In Vivo Pharmacokinetics

The MDMA was administered to groups of at least five rats that had been implanted with Vascular Access Ports (Model SLA, Norfolk Medical Products, Skokie, IL) (Cho et al. 1990). These ports permit drawing of blood from unrestrained and conscious animals. Volumes of 0.5 mL of blood were collected, and the plasma (0.2 mL) was separated and analyzed by the procedures described above. The times chosen for sample collection were based on preliminary experiments and the time course of plasma decay. The object was to have data points that follow drug concentration changes with time so that parameters for the function describing the plasma time curve could be estimated. The function could then be used to (1) estimate the concentrations at time points not analyzed, (2) obtain rate constants for intercompartment transfer, and (3) estimate the area under the plasma time curve. (Intercompartment transfer refers to processes such as absorption from the site of administration, distribution throughout the body compartments, and elimination, either by excretion or by metabolism.) The data were fitted to the pharmacokinetic model shown in figure 3, an open two-compartment model, reflecting the movement of drug from the central to the peripheral compartment ( $k_{12}$ ,  $k_{21}$ ), through metabolism to MDA ( $k_1$ ,  $k_4$ ) and other pathways of elimination ( $k_3$ ). The fitting was



**FIGURE 3.** *Compartment model used to analyze the pharmacokinetic data of figure 2*

accomplished by nonlinear regression procedures (Motulsky and Ransnas 1987) using Dixon's BMDP software program on a microcomputer (Dixon 1985). In this regression procedure, the data are interactively fitted to an exponential function that is the solution to the differential equation describing the plasma decay curve. The function can be set up on a spreadsheet and the concentrations at any time calculated by substituting the desired time. This method of data analysis provides estimates of rate constants (table 1) together with estimates of the errors in their values. The significance of the constants can be evaluated using the F value (Motulsky and Ransnas 1987). The values of the individual rate constants or microparameters are useful in evaluating the relative importance of different metabolic processes. For example, the ratio of  $k_1$ , the rate constant for MDA formation, to  $k_3$ , the rate constant representing all other pathways of elimination, is about 10. This means that MDA formation represents only a small proportion of the total drug elimination process (~10 percent). The relative values of  $k_3$  for the (+) and (-) isomers indicate a substantial enantiospecificity in MDA formation. The effect of pharmacological manipulation of the rate constants is shown in table 1 also. Phenobarbital pretreatment, which commonly induces cytochrome P450 isozymes, did not appear to alter the rates of demethylation but had a significant effect on  $k_3$ , the alternate pathways of elimination. At the

**TABLE 1.** *Microscopic rate constants—MDMA*

| Treatment              | $k_1$ (min <sup>-1</sup> ) | $k_3$ (min <sup>-1</sup> ) | $k_4$ (min <sup>-1</sup> ) |
|------------------------|----------------------------|----------------------------|----------------------------|
| (+)MDMA, control       | .0025                      | .0240*                     | .0058                      |
| (-)MDMA, control       | .0006                      | .0190                      | .0062                      |
| (+)MDMA, phenobarbital | .0026                      | .0354 <sup>†</sup>         | .00103                     |
| (-)MDMA, phenobarbital | .0011                      | .0378                      | .0093                      |
| (+)MDMA, SKF 525A      | .0022                      | .0202*                     | .0058                      |
| (-)MDMA, SKF 525A      | .0004                      | .0207 <sup>†</sup>         | .0059                      |

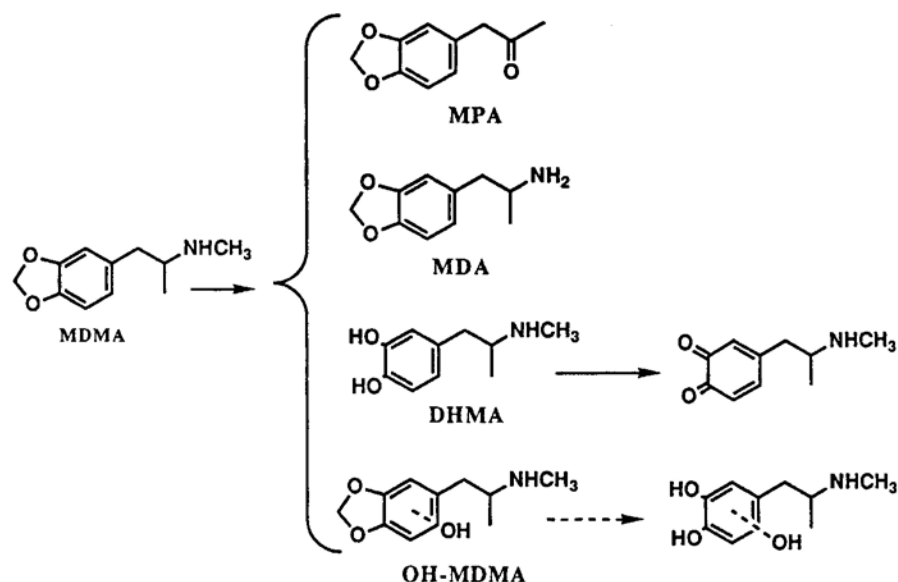
\*  $p < .01$  vs. (-) control<sup>†</sup>  $p < .01$  vs. (+) control

doses used, the cytochrome P450 inhibitor SKF 525A appeared to decrease the values of  $k_3$  for the (+) but not the (-) isomer.

The results show that the outcome of pretreatment is not always predictable. Thus, phenobarbital induction did not increase levels of MDA as might be expected. Instead of increasing the rate of MDA formation, induction increased the rate of MDA elimination.

#### **In Vitro Metabolism**

The pharmacokinetic data showed that MDA formation is a relatively minor pathway in the overall elimination pathways of MDMA (Hiramatsu et al. 1990). To evaluate other pathways, in vitro experiments were conducted to obtain quantitative data of regiospecificity (Kumagai et al. 1991a). These studies required the analysis of highly polar catecholamines and their derivatives (figure 4). To study the overall metabolism, the aforementioned GC/MS assay was used along with an additional HPLC/ECD procedure (Hiramatsu et al. 1990). The results, shown in table 2, reveal that the catecholamine dihydroxy methamphetamine (DHMA) is the dominant metabolite, occurring at levels 10 times greater than the others (Kumagai et al. 1991a). In spite of the extensive demethylenation in vitro, there was very little if any DHMA present in any of the plasma samples assayed, including the early time points. An explanation for this discrepancy was found in direct pharmacokinetic evaluation of  $\alpha$ -methyldopamine ( $\alpha$ -MeDA) because the N-methyl analog was not available in sufficient quantity to study in this manner. The pharmacokinetic parameters obtained after equimolar, intravenous doses of  $\alpha$ -MeDA and MDA are shown in table 3. The value of  $k_2$  for  $\alpha$ -MeDA, the rate constant for elimination, was found to be 10 times greater



**FIGURE 4.** *Metabolic pathways of MDMA*

**TABLE 2.** *Metabolism of MDMA by liver microsomes*

| Species  | DHMA | MDA  | MPA  | OHMDMA            |
|----------|------|------|------|-------------------|
| Rat (+)* | 5.37 | 0.21 | 0.12 | .025 <sup>†</sup> |
| Rat (-)* | 3.35 | 0.31 | 0.05 | .010 <sup>†</sup> |

\* Nmols of product formed in 5-minute incubation of 30 nmols MDMA

<sup>†</sup> approximate

KEYS: MPA=methylenedioxy phenylacetone;

OHMDMA=hydroxymethylenedioxy methamphetamine

than that for MDA. The rate constant for  $\alpha$ -MeDA is equivalent to a half-life of about 2 minutes, compared with 22 minutes for MDA. Thus, even with a large rate of formation, the high rate of elimination precludes accumulation of significant levels of the catechol in plasma.

#### In Vitro Toxicity

Methods also are being developed to study the chemical mechanisms of neurotoxicity utilizing a neuroblastoma glioma clonal cell line, NG 108-15

**TABLE 3.** Microscopic rate constants—MDA and  $\alpha$ -MeDA

| Compound       | Vd<br>(l/kg) | $k_{21}$ | $k_2$<br>(min <sup>-1</sup> ) | $k_{12}$ | $t_{1/2}$<br>(min) |
|----------------|--------------|----------|-------------------------------|----------|--------------------|
| $\alpha$ -MeDA | 0.16         | .059     | .360                          | .048     | 1.92               |
| MDA            | 0.76         | .079     | .032                          | .219     | 21.65              |

NOTE: The open, two-compartment parameters are: Vd is the volume of distribution of the central compartment;  $k_2$  is the rate constant for elimination;  $k_{12}$ ,  $k_{21}$  are rate constants for fluxes to and from the central compartment. The half-life,  $t_{1/2}$ , was calculated from  $k_2$ .

(Patel et al. 1991). This cell line has been described by Furuya and associates (1985) as having features associated with the 5-HT nerve terminal such as the ability to store, release, and accumulate 5-HT. The intention is to use such a model to test metabolites of MDMA and other neurotoxins. These cells contain high levels of glutathione (GSH), a peptide that protects cells against insult by electrophiles, free radicals, and metals by undergoing addition, oxidation, and complexing reactions, respectively (Reed 1985). The high levels of GSH in this cell line and the availability of depleting agents permits the role of this agent in protection against toxins to be examined experimentally.

The proposed metabolism of MDMA to potentially toxic metabolites is shown in figure 4. In this sequence, MDMA can undergo demethylenation to the catechol DHMA (Kumagai et al. 1991a) or hydroxylation to the Ar-hydroxy compound. In studies reported elsewhere (Kumagai et al. 1992), a ring-hydroxylated metabolite of MDMA has been characterized, but the location of the hydroxyl group has not been established. DHMA can be generated directly by a cytochrome P450-dependent reaction or by a hydroxyl radical-dependent oxidation (Kumagai et al. 1991b). Catechols are readily autoxidized to the o-quinone, and this reaction, together with a rapid addition of GSH, has been reported (Hiramatsu et al. 1990). However, in low-GSH conditions, the o-quinone can react with nucleophilic centers in biological molecules and cause a biochemical lesion (Graham et al. 1978; Slaughter and Hanzlik 1991). Alternatively, the proposed Ar-hydroxy metabolite can be generated, which also can undergo demethylenation to form the highly reactive trihydroxy derivative. The position of ring hydroxylation of MDMA has since been identified as the 6-position based on comparison of HPLC retention times and GC/MS data with authentic material. These functions can cause cellular toxicity by reduction of oxygen to superoxide catalytically, acting as an electron transfer agent between reduced nicotinamide adenine dinucleotide phosphate and oxygen (Graham et al. 1978). Superoxide rapidly



disproportionates to oxygen and hydrogen peroxide, and its continuous formation by the trihydroxy compound can result in hydroxyl radical formation through the Haber Weiss reaction. Hydroxyl radical is highly reactive and thought to be the basis for toxicity of a variety of oxidizing agents (Cohen 1984). GSH would protect the cells against the electrophile- and radical-based insults shown above so that its depletion should alter the toxicity of agents whose action proceeded by this mechanism.

In initial studies, the interaction of the metabolite of MDA,  $\alpha$ -MeDA, was compared with DA for its toxic actions on the cell line (Patel et al. 1991). Because both compounds contain the catechol moiety, toxic actions based on o-quinone formation should be very similar, reflective of the availability of the quinone. Toxicity was measured as cellular destruction and required fairly high concentrations of the compounds. The results are shown in table 4 as  $EC_{50}$  values, the concentration necessary to kill 50 percent of the cells. The value for DA was almost four times higher than that for  $\alpha$ -MeDA. The total quantities of catechol accumulated, on the other hand, were much greater after DA (Patel 1990). When the GSH in the cells was depleted by the synthesis inhibitor, buthionine sulfoximine (BSO), the toxicity of the two compounds increased, but the increase was much greater for  $\alpha$ -MeDA. Thus, the results of the study showed that, although DA is accumulated by the cells much more extensively, it is much less toxic than its  $\alpha$ -methyl analog. Furthermore, its toxicity does not increase in the same proportion on GSH depletion.

One explanation for this discrepancy is in the intracellular disposition of the two compounds (Patel et al. 1991). DA is a neurotransmitter, and its removal from cytoplasm by storage and monoamine oxidase-initiated metabolism resulted in low availability. In support of this notion, DA accumulation was found to

**TABLE 4.** *Interaction of catecholamines and NG 108-15 cells*

| Compound       | $EC_{50}^*$<br>(mM) |                          |
|----------------|---------------------|--------------------------|
|                | Control             | BSO Treated <sup>†</sup> |
| DA             | 24.5                | 10.5                     |
| $\alpha$ -MeDA | 6.8                 | 0.6                      |

\* Concentration required to kill 50 percent of the cells after a 2-hour exposure

<sup>†</sup> Cells treated with buthionine sulfoximine (BSO, 2 mM) for 24 hours prior to drug exposure (Patel et al. 1991)

decrease substantially in reserpine pretreated cells, whereas the accumulation of  $\alpha$ -MeDA was not affected (Patel 1990). The metabolism of DA by monoamine oxidase to dihydroxyphenylacetic acid and homovanillic acid is not available to  $\alpha$ -MeDA, although the latter compound may be converted to its 3-methyl derivative by catechol o-methyltransferase. The major transformation of  $\alpha$ -MeDA appears to be the GSH adduct, originally found in incubates of liver preparations (Hiramatsu et al. 1990). Although the GSH adducts are formed from both DA and  $\alpha$ -MeDA, the early levels of the latter are much higher. The intracellular concentration of GSH is in the millimolar range and far exceeds the concentration of the catecholamines so that the rate of formation of GSH adduct is dependent on catecholamine availability. Therefore, the concentration of  $\alpha$ -MeDA and its quinone oxidation product must be higher and available for binding to cellular nucleophiles. The GSH adducts appear to be excreted by the cell, and analysis of the culture medium after incubation has shown that there are micromolar levels of the GSH adduct of  $\alpha$ -MeDA present, whereas, similar to DA, GSH concentration was below detectable limits. Thus,  $\alpha$ -MeDA is extensively converted to the GSH adduct of the o-quinone, whereas DA appears to have limited conversion. If the concentration of this adduct reflects that of the toxic o-quinone, the greater toxicity of  $\alpha$ -MeDA can be accounted for by its greater availability.

#### ACKNOWLEDGMENT

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## DISCUSSION

Due to technical difficulties, there is no transcription available of the discussion that followed Dr. Cho's presentation.

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