Mini Review

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Bioanalytical methods for quantitation of levamisole, a widespread cocaine adulterant

Abstract

Levamisole is an anthelminthic that was first used as a de-worming agent in humans and animals. It has also been used to treat inflammatory conditions as well as certain types of cancer. Levamisole was discontinued for human use in the early 21st century due to toxic side effects including agranulocytosis and vasculitis. Recently, levamisole was discovered as a cocaine adulterant after reports emerged of drug users with the above disorders. As the prevalence of cocaine usage has grown in the last 15 years, measurement of levamisole in human samples has become increasingly important. This review focuses on the various bioanalytical methods available for the determination of levamisole in human plasma and urine. Earlier methods employed gas chromatography coupled with nitrogen-selective thermionic specific detection and nitrogen-phosphorus detection, as well as high performance liquid chromatography coupled with ultraviolet detection. In addition, gas chromatography-mass spectrometry (GC-MS) and high performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) have also been described. Currently, GC-MS appears to be the method of choice however recent developments in the area of LC-MS/MS make this technology an attractive alternative. The merits of both GC-MS and LC-MS/MS for the determination of levamisole are evaluated on the basis of sample preparation, chromatographic separation conditions, run time, and analytical performance. In addition, emerging methods in this area are also reviewed.

Keywords: bioanalytical methods; cocaine adulterant; gas chromatography-mass spectrometry; levamisole; liquid chromatography-tandem mass spectrometry; review.

Introduction

Levamisole (Figure 1) was discovered in 1966 by Janssen Pharmaceutica and approved as a broad spectrum anthelminthic for human and veterinary use [1]. It was initially prepared as tetramisole, a racemic mixture of levamisole and dexamisole, however it was later discovered that the levo isomer, levamisole, was the active molecule [2]. In subsequent years, levamisole was also recognized for its immunomodulatory properties and as such, was used in the treatment of inflammatory conditions, such as rheumatoid arthritis and nephrotic syndrome. It has also been used as an adjuvant to 5-fluorouracil for the treatment of colon cancer [3]. In 1976 several cases of leukopenia and agranulocytosis were reported in patients being treated with this drug; reversal of symptoms was observed upon drug discontinuation [4–6]. In 1978, the first cases of vasculitis secondary to levamisole therapy were reported. These included a patient who developed leukocytoclastic vasculitis secondary to levamisole therapy for rheumatoid arthritis [7] and another who developed severe cutaneous necrotizing vasculitis and neutropenia after a 3 month regimen of levamisole [8]. Following these initial reports, many other patients with toxic side effects including leukoencephalopathy were described [9, 10]. As a result, levamisole was withdrawn from the US and Canadian markets for human use in 2000 and 2003, respectively. Levamisole is still available, however, as an anthelminthic for livestock.

Pharmacokinetic studies have shown that levamisole is quickly absorbed in the gastrointestinal tract with peak plasma concentrations of 716.7±217.5 ng/mL achieved within 2 h of a single 150 mg oral dose [11]. It is eliminated from plasma with a half-life ranging from 4.0 [12] to 5.6 h [11]. Reid et al. demonstrated that only a small proportion (3.2%) of the parent drug was recovered unchanged in urine of cancer patients undergoing high-dose levamisole treatment [12]. Major products of levamisole metabolism include p-hydroxylevamisole and aminorex. Specifically, it was found that 12.6% of the parent drug is recovered as p-hydroxylevamisole [11, 13]. Of the p-hydroxylated metabolite, 11% was found conjugated to glucoronic acid and...
1.6% was in its free form. Although the equine conversion of levamisole to aminorex has long been known [14, 15], it was recently shown that human metabolism also results in formation of this metabolite [16].

**Levamisole as a cocaine adulterant**

In 2003, levamisole was reported as a novel cocaine adulterant [17], adding it to the growing list of compounds identified for this specific use. Other known adulterants include hydroxyzine, lidocaine, procaine, benzocaine, caffeine, boric acid, and phenacetin [18]. In 2009, the first cases of toxicity due to use of levamisole-contaminated cocaine were reported [19]. In this paper, five cocaine users were hospitalized with agranulocytosis as well as other symptoms including fever, anemia, and a variety of infectious complications. All five patients also tested positive for lupus anticoagulant. Urine specimens from each patient were positive for either cocaine or its metabolite, benzoylecgonine, as well as levamisole. Each patient fully recovered with the use of filgrastim (a granulocyte colony-stimulating factor analog), intravenous antibiotics, and careful monitoring. Since these initial cases of levamisole-induced agranulocytosis in cocaine users were first described, over 200 additional cases of toxicity have been reported. As neither condition is very common, a diagnosis should initiate the search for levamisole-adulterated cocaine use. As such, measurement of levamisole is becoming increasingly important in the clinical laboratory.

**Analytical methods for quantitation of levamisole**

Numerous methods have been described in the literature for the determination of levamisole in both animal and human specimens. Earlier methods include gas chromatography coupled with nitrogen-selective thermionic specific detection [21] and nitrogen-phosphorus detection [11], as well as high performance liquid chromatography coupled with ultraviolet detection (HPLC-UV) [22, 23]. These procedures suffered from lack of sensitivity and specificity with detection limits ranging from 2 to expanded in recent years. One of many proposed hypotheses is that levamisole may increase peripheral sympathetic activity and central neurotransmission through stimulation of nicotinic acetylcholine receptors thereby augmenting the euphoric effects of cocaine [20].

The United Nations Office on Drugs and Crime estimated in 2009 that between 14.3 and 20.5 million people aged 15–64 years used cocaine at least once in the preceding year [18]. This is of particular importance to European countries, as the number of cocaine users doubled between 1998 and 2006 although usage appears to have stabilized in more recent years (Figure 2). Interestingly, over 80% of cocaine users in Europe live in just five countries including the UK, Spain, Italy, Germany, and France [18]. As levamisole is now readily found in the cocaine supply, it is likely that the prevalence of both agranulocytosis and cutaneous vasculopathy will increase both within Europe and globally. As neither condition is very common, a diagnosis should initiate the search for levamisole-adulterated cocaine use. As such, measurement of levamisole is becoming increasingly important in the clinical laboratory.

**Figure 1** Structure of levamisole.

**Figure 2** Annual prevalence of cocaine use among individuals aged 15–64 years in EU and EFTA countries (adapted from [18]).
For this reason, they have largely been replaced with mass spectrometry based detection. Currently, samples are typically screened by immunoassay for cocaine metabolites (benzoylecgonine) and those that are positive undergo confirmatory measurement using mass spectrometry that includes determination of levamisole. A downfall of current immunoassays is that they do not detect levamisole in human samples; hence, confirmation of levamisole-induced toxicity in cocaine users requires mass spectrometric techniques. Gas chromatography-mass spectrometry (GC-MS) has been the technique of choice in recent years for identification and quantification of various drugs however the advent of high performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) in forensic toxicology has resulted in simpler sample preparation, superior sensitivity, and shorter run times. As such, the use of LC-MS/MS for quantitation of levamisole is becoming increasingly popular.

GC and LC mass spectrometry techniques are reviewed in the following section for measurement of levamisole in human urine and plasma. Moreover, each methodology is evaluated on the merits of analytical performance and ease of sample preparation. In addition, up-and-coming methodologies including differential mobility spectrometry-mass spectrometry (DMS-MS), chiral capillary gas chromatography-flame ionization detection (GC-FID), and biochip array technology are presented and assessed for their potential clinical application. A literature search was conducted using PUBMED for methodology articles published in the last 10 years describing the determination of levamisole in human specimens (whole blood, plasma, and urine) in detail. A review of the articles matching these criteria is presented below as well as two additional articles describing the application of novel techniques for levamisole measurement in different matrices.

### Gas chromatography-mass spectrometry

Although gas chromatographic methods have been used with nitrogen-selective thermionic specific detectors as well as nitrogen-phosphorus flame ionization detectors as mentioned above, most laboratories are currently using GC-MS for the detection of levamisole [24–26]. Two recent publications describe the quantitation of levamisole in urine using GC-MS [16, 27] and are summarized in Table 1. Both methods demonstrate acceptable precision and sensitivity. As is common with GC-MS methods, however, run times are quite long.

Trehy et al. [27] recently described a novel application of GC-MS for the determination of levamisole in urine that included validation at two laboratories. For method development, an Agilent DB-5MS UI column (30 m × 0.25 mm, 0.25 μm film thickness) was used as part of an Agilent 6890 GC/5975B MS. The source was operated in electron impact mode. For sample extraction, 1 M sodium hydroxide and 95:5 hexane/isoamyl alcohol extraction solution was added to 5 mL of urine after addition of the internal standard, cyheptamide. The molecular ion m/z 204 was used for quantitation and fragment ion m/z 148 was used as a qualification ion. Using these parameters, recovery was 99% at 8 ng/mL and 117.5% at 61 ng/mL. Precision was 1.6%–2.4% and linearity was verified between 6 and 223 ng/mL. The limit of detection (LoD) was determined to be 1 ng/mL.

Bertol et al. [16] also recently published a GC-MS method used for the measurement of both levamisole and its metabolite, aminorex, in urine. The method described was adapted from a routine amphetamines procedure. Urine samples were collected from eight patients (4 males

<table>
<thead>
<tr>
<th>Specimen type</th>
<th>Trehy et al. [27]</th>
<th>Bertol et al. [16]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample extraction</td>
<td>LLE (1 M NaOH+95:5 hexane/isoamyl alcohol)</td>
<td>LLE (diethyl ether at pH 9)</td>
</tr>
<tr>
<td>Column</td>
<td>Agilent DB-5MS UI (30 m × 0.25 mm, 0.25 μm film thickness)</td>
<td>Agilent phenyl-methylpolysiloxane 5% column (30 m × 0.25 mm, 0.25 μm film thickness)</td>
</tr>
<tr>
<td>Internal standard</td>
<td>Cyheptamide</td>
<td>Mephentermine</td>
</tr>
<tr>
<td>Ions monitored, m/z</td>
<td>204 and 148</td>
<td>73, 101, 148, 203, and 204</td>
</tr>
<tr>
<td>Precision, %CV</td>
<td>&lt;2.4%</td>
<td>&lt;8.6%</td>
</tr>
<tr>
<td>Accuracy/recovery</td>
<td>&gt;84.1% (recovery)a</td>
<td>&lt;9.1%</td>
</tr>
<tr>
<td>Linear range</td>
<td>6–223 ng/mL</td>
<td>0–100 ng/mL</td>
</tr>
<tr>
<td>LoD</td>
<td>1 ng/mL</td>
<td>0.15 ng/mL</td>
</tr>
<tr>
<td>Levamisole retention time</td>
<td>~10.1 min</td>
<td>11.04 min</td>
</tr>
</tbody>
</table>

Table 1 Summary of two GC-MS methods published for the determination of levamisole in urine specimens.

*aAverage recovery determined for spiked urine samples ranging from 0.80 to 29.7 ng/mL levamisole. GC-MS, gas chromatography-mass spectrometry; LLE, liquid-liquid extraction; LoD, limit of detection.
and 4 females) at baseline as well as 3 and 6 h after oral administration of 47 mg (females) and 58 mg (males) of levamisole enabling the authors to investigate the metabolic fate of this analyte in humans. As previously mentioned, this was the first study that identified aminorex as a metabolite of levamisole in vivo in humans, as had previously been shown in horses [14, 15]. For chromatographic separation, a phenylmethylsilicone 5% capillary column (30 m×0.25 mm, 0.25 μm film thickness) was used as part of an Agilent 5975C Series GC/MSD. Prior to measurement, 1 mL of urine underwent a liquid-liquid extraction with diethylether at pH 9. Mephentermine was used as an internal standard. Identification of levamisole and aminorex was performed by monitoring five ions for each compound (levamisole, m/z 73, 101, 148, 203, and 204; aminorex, m/z 56, 91, 118, 145, and 162) and comparing scan spectra to known libraries. The authors did not state which ion was used for quantification. Accuracy was determined by spiking known amounts of levamisole and aminorex into drug-free urine and was observed to be ≤9.06%. Precision was determined to be 3.34%–8.56% and 4.05%–8.47% for concentrations ranging from 20 to 100 ng/mL of levamisole and aminorex, respectively. Linearity was also verified up to 100 ng/mL. The LoD was lower than the method described above at 0.15 ng/mL for both analytes.

Table 1 summarizes the methodology and analytical performance of each of these procedures. Although both methods describe acceptable accuracy/recovery, the method developed by Bertol et al. has the advantage of requiring significantly less sample compared to the method described by Trehy et al. In addition, this method also demonstrated greater sensitivity. Retention time of levamisole was similar for both methods in the range of 10–11 min. The method by Trehy et al. benefits from an extended linear range and superior precision. Although each method has its analytical advantages, the development of an LC-MS/MS method to quantify levamisole in urine, as is discussed below, improves on accuracy, dynamic range, and run time compared to these GC-MS methods while maintaining similar sensitivity.

### Liquid chromatography-tandem mass spectrometry

Although GC-MS has historically been used for detection of drugs of abuse, LC-MS/MS methods are becoming increasingly popular, largely due to their ease of use and improvements on analytical performance. In general, LC-MS/MS is advantageous over GC-MS for drug analysis as it does not require derivitization of the analyte and thermolabile drugs can be analyzed directly. In addition, sample preparation is usually more straightforward and run times are shorter. For this reason, there has been an increase in the number of LC-MS/MS methods described in the literature in recent years. In particular, three articles describe the application of LC-MS/MS for the measurement of levamisole in both urine and plasma/whole blood samples [28–30] and are summarized in Table 2. The first of these describes an assay used for the quantitative screening of levamisole in cocaine positive urine samples [28]. Samples were initially screened using the CEDIA® cocaine assay with a cut-off value of 300 ng/mL. Cocaine was then confirmed, along with measurement of levamisole, using LC-MS/MS. In this method, urine

<table>
<thead>
<tr>
<th>Specimen type</th>
<th>Lynch et al. [28]</th>
<th>Tong et al. [29]</th>
<th>Dowling et al. [30]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample preparation</td>
<td>1:10 dilution with 0.05% formic acid</td>
<td>LLE (ethyl ether)</td>
<td>Mixed mode SPE</td>
</tr>
<tr>
<td>Column</td>
<td>Phenomenex Kinetex™ C18 (50 mm×2.1 mm, 2.6 μm)</td>
<td>Agilent HC-C₈ (150 mm×4.6 mm, 5 μm)</td>
<td>Phenomenex HYPURITY C₈ (100 mm×4.6 mm, 5 μm)</td>
</tr>
<tr>
<td>Internal standard</td>
<td>Aminorex</td>
<td>Mebendazole d₁-Codeine</td>
<td></td>
</tr>
<tr>
<td>Ion transitions monitored, m/z</td>
<td>205.2→178.1</td>
<td>205.1→178.2</td>
<td>204.6→178</td>
</tr>
<tr>
<td>Precision, %CV</td>
<td>&lt;6.0%</td>
<td>&lt;8.5%</td>
<td>&lt;7.0%</td>
</tr>
<tr>
<td>Recovery</td>
<td>&gt;95.8%</td>
<td>&gt;88.3%</td>
<td>&gt;97%</td>
</tr>
<tr>
<td>Linear range</td>
<td>2–2500 ng/mL</td>
<td>0.1–30 ng/mL</td>
<td>0–2000 ng/mL</td>
</tr>
<tr>
<td>LoD</td>
<td>0.5 ng/mL</td>
<td>0.1 ng/mL</td>
<td>12 ng/mL</td>
</tr>
<tr>
<td>Levamisole retention time</td>
<td>1.1 min</td>
<td>4.9 min</td>
<td>6.48 min</td>
</tr>
</tbody>
</table>

Table 2 Summary of three LC-MS/MS methods for the determination of levamisole in urine, plasma, and whole blood specimens.

LC-MS/MS, liquid chromatography-tandem mass spectrometry; LLE, liquid-liquid extraction; LoD, limit of detection; SPE, solid phase extraction.
The other two articles describe LC-MS/MS protocols for determination of levamisole in plasma and whole blood. In the first publication, Tong et al. [29] describe a simple, efficient method using an Agilent 1200 series HPLC coupled to an Agilent G6410 B tandem quadrupole mass spectrometer. Separation was performed on an HC-C<sub>8</sub> column (150×4.6 mm, 5 μm) with isocratic elution using acetonitrile and 10 mM ammonium acetate solution (70:30 v/v). Plasma samples underwent liquid-liquid extraction with ethyl ether, after the addition of the internal standard, mebendazole. The authors investigated a number of additional organic extraction solvents (n-hexane, ethyl acetate, and dichloromethane under differing pH) however found the greatest recovery was observed with ethyl ether (>87%). For levamisole, ion transition m/z 205.1 → 178.2 was monitored for quantitation. The retention time for levamisole and the internal standard were 4.9 and 4.0 min, respectively, while total run time was 6 min. Total imprecision was between 5.5% and 8.1% and bias ranged from −3.4% to 7.4% for three concentrations of spiked samples (0.2, 2.0, and 25 ng/mL). Linearity was observed up to 30 ng/mL and LoD was 0.1 ng/mL. In addition to these analytical parameters, Tong et al. also investigated the stability of levamisole in human plasma. They found that levamisole was stable in plasma for 5 h at room temperature and 6 weeks at −20°C. As well, stability was not affected by three freeze-thaw cycles.

The last paper reviewed differs from the above studies in two respects: 1) levamisole was detected as part of a broad screen used to detect 18 drugs and 2) use of a more complex sample extraction procedure (mixed mode cation exchange solid phase extraction). Nonetheless, Dowling et al. [30] describe a fairly efficient method for determination of levamisole in human blood with a run time of 12.5 min. Drug separation was performed using a Phenomenex HYPURITY C<sub>8</sub> column (4.6×100 mm, 5 μm) using gradient elution (mobile phase A, water and methanol 95:5 v/v and 25 mM ammonium acetate; mobile phase B, methanol:propan-2-ol 97:95:2 v/v and 0.05 mM formic acid). The internal standard, d<sub>9</sub>-codeine, was added to samples prior to the addition of ammonium acetate (adjusted to pH 6 using concentrated formic acid). Samples were then centrifuged and the supernatant was injected into mixed mode cation exchange solid phase extraction cartridges (Evolute™ CX SPE) prior to HPLC separation. Detection was performed using a QTRAP 4000 from Applied Biosystems. Two transitions were monitored for quantitation of levamisole: m/z 204.6 → 178 and 204.6 → 123. Confirmation was carried out by comparison to an in-house library based on product ion spectra. Total imprecision ranged from 4.7% to 7.0% for concentrations between 50 and 100 ng/mL and accuracy (measured as recovery of spiked samples) varied between 97% and 100%. Linearity was confirmed up to 2000 ng/mL. The LoD was 12 ng/mL and limit of quantitation was 20 ng/mL.

Although the goal of the work done by Dowling et al. using SPE was to achieve a lower limit of detection compared to methods using LLE for sample extraction, they were not successful in this attempt. The method described by Tong et al. demonstrated better sensitivity and a shorter run time, although at the expense of a limited linear range. Nonetheless, SPE is a laborious extraction procedure requiring a bigger time commitment and resulting in higher consumable costs. As LLE is much simpler, it would appear that the method described by Tong et al. is more amenable to implementation in a clinical or forensic lab.
Aside from the articles reviewed above, numerous other studies describe the quantitation of levamisole using LC-MS/MS in non-human specimens including alfalfa plants [31], aquaculture fish samples [32], and animal feed [33]. Interestingly, the method described by Lopes et al. utilizes ultra high performance liquid chromatography coupled to mass spectrometry, a relatively recent development involving smaller column particle size (<2 μm) that has led to improvements in sensitivity and resolution over traditional HPLC. The reader is directed toward these references for full details of the methodologies used.

### Emerging methods for measurement of levamisole

Although LC-MS/MS methods are gaining popularity for use in clinical toxicology laboratories, alternative methods are continuously being developed. For example, three articles published in the past year describe very different approaches to the measurement of levamisole including the use of DMS-MS, chiral capillary GC-FID, and biochip array technology. Although still in their infancy, each method attempts to overcome some of the pitfalls of GC-MS and LC-MS/MS methods. Each of these articles will be reviewed in the following section.

In an effort to increase throughput for forensic purposes, Hall et al. developed a novel method for quantitation of levamisole in forensic drug samples that utilizes an ion-mobility-based separation combined with mass spectrometry [34]. DMS-MS has been demonstrated to show enhanced selectivity and specificity as well as higher throughput compared to GC- and LC-based methods [34]. Moreover, it eliminates the need for extensive and time-consuming sample extraction as required by GC-MS and some LC-MS/MS methods. Although Hall et al. did not perform a detailed method evaluation in their study, they did show that DMS-MS was capable of separating levamisole from a cocaine mixture containing 11 other known adulterants in 25 s. In this regard, a large number of samples could be analyzed by DMS-MS in the amount of time it would take for one GC- or LC-based chromatographic separation. Due to its ability to characterize complex mixtures in seconds, DMS-MS is a promising method that holds great potential for drug analysis where a high throughput is required. Furthermore, differential ion mobility systems have been successfully interfaced to a number of mass spectrometers, including ion trap, single quadrupole, and triple quadrupole mass analyzers, facilitating implementation into the clinical laboratory.

Also published in 2012, Casale et al. [35] describe an innovative use of GC-FID to differentiate between the two enantiomers, levamisole and dexamisole, in both cocaine bricks and urine samples, something that the majority of previously published methods had failed to accomplish. With this method, urine samples were extracted with chloroform/isopropanol (9:1), evaporated and reconstituted in trichloromethane/methanol (9:1). Analysis was performed using an Agilent model 7890A GC equipped with a Restek fused-silica column (30 m×0.25 mm) coated with Rt-β-DEXsm chiral film (25 μm thickness). Total run time was 20 min. Using this method, levamisole and dexamisole were successfully identified with a resolution factor of 2.37–2.75. Interestingly, the authors found that the majority of cocaine samples contained either levamisole only or the racemic mixture, tetramisole. In line with these findings, the authors found that the majority of urine extracts also contained levamisole only. Detection of both isomers in cocaine and urine samples has both clinical and forensic implications and applications. Differences in enantiomer composition in cocaine may provide information regarding the source of this illicit drug, as the formulation of adulterant used may differ between suppliers, although this is a complex issue as levamisole is typically added to cocaine prior to widespread distribution. Furthermore, the differential toxicity between the two enantiomers remains unresolved. Although it has been suggested in the past that levamisole is the active constituent, studies have shown that both enantiomers demonstrate comparable toxicity [36, 37]. Moving forward, it may become important to differentiate both enantiomers in the clinical laboratory.

Although the last article to be reviewed does not describe quantitation of levamisole in human samples, the innovative use of biochip array technology for the semi-quantitative measurement of anthelminthic drugs in milk and beef muscle is noteworthy [38]. With the advent of this technology, immunoanalytical determination of levamisole, as well as six other groups of anthelminthic drugs, was determined in 54 samples simultaneously. Briefly, a number of competitive immunoassays corresponding to different analytes are performed in defined test sites on each bio-chip. Total run time is approximately 90 min (not including sample extraction), however since 54 samples can be measured at once, this equates to 1.67 min per sample. Imprecision was <1% and average recovery was 74% in milk and 95% in beef muscle. LoD was 2.00 and 6.50 ppb in milk and beef muscle, respectively. Perhaps most important, the bio-chip exhibited excellent agreement with an LC-MS/MS method. As can be seen, these results are promising and warrant further
investigation regarding the applicability to human plasma and urine samples.

Conclusions

Once used as an anthelminthic drug, levamisole has recently been identified as a novel cocaine adulterant with severe side effects including agranulocytosis and cutaneous vasculopathy. As the incidence of cocaine use is increasing, this has become a large public health concern. Therefore, measurement of levamisole is becoming increasingly important. The goal of this review was to educate readers on both current and novel approaches for levamisole quantitation. At present, GC-MS and LC-MS/MS are the dominant technologies employed owing to their enhanced specificity and sensitivity over earlier methods. These technologies have been applied to quantitation of levamisole in both urine and blood however a random urine sample (within 2–3 days of exposure) is the desired specimen owing to the short half life of this molecule in blood and the ease of urine collection. Based on methods currently published and reviewed here it would appear that LC-MS/MS has some advantages over GC-MS including simple sample preparation and shorter run time. Further studies are warranted that directly compare the level of agreement and analytical performance between GC-MS and LC-MS/MS to conclusively determine which method is most suitable for levamisole quantitation.

Conflict of interest statement

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