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Dual Examinations for Identification of Urine as Being of Human Origin and for DNA-Typing from Small Stains of Human Urine

ABSTRACT: Concurrent methods for identification of urine as being of human origin, and for DNA-typing from small stains of human urine were examined. A urine stain was extracted with phosphate-buffered saline (PBS), and the extract was filtered using a Centricon-100 device. The filtrate was subjected to electrospray ionization liquid chromatography-mass spectrometry (ESI-LC-MS) for identification of human urine and a DNA-typing sample was obtained by dialfiltration of the residue using a DNA purification kit. After the purified residue was treated with an AmpflSTR[®] Profiler[™] PCR amplification kit, the DNA-types were analyzed by capillary electrophoresis using a Genetic Analyzer. It was possible to identify a urine stain as being of human origin, and complete DNA profiles could be successfully obtained from a urine stain which had been created by 50 μ L of female urine. Serial analyses of urine stains found at a crime scene provide effective information for forensic investigation. This method is recommended for stain identification and for DNA-typing from a urine stain.

KEYWORDS: forensic science, urine stain, forensic investigation, DNA typing

Forensic investigation of urine stains in a murder case is necessary in order to ascertain the exact location of the crime and the precise manner of death. Such investigation involves identification of urine as being of human origin, and personal identification of an individual from a urine stain. These two types of examination can be conveniently carried out in parallel with each other. Consequently, urine samples need to be divided into two samples for these examinations. For forensic purposes, it is desirable to unify the two tests for use on just one sample. To the best of our knowledge, no other reports dealing with the serial procedure of urine stain identification and DNA-typing have been published so far.

We carried out a parallel study on two different methods for identifying human origin and DNA-typing from a urine stain (1–3). The former method was profiling analysis of five 17-ketosteroid conjugates by high-performance liquid chromatographic (HPLC) analysis after extraction and derivatization, while the latter method was DNA-typing after using a DNA-purification kit following dialfiltration. In this study, we attempt to unify these two different techniques into serial procedures and to modify previously developed techniques.

Materials and Methods

Reagents

Androsterone glucuronide (AN-G), androsterone sulfate (AN-S), dehydroepiandrosterone sulfate (DHEA-S), etiocholanolone

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glucuronide (ETIO-G), and etiocholanolone sulfate (ETIO-S) were obtained as their sodium salts from Sigma Chemical Co. Ltd (St. Louis, MO). Oasis HLB Cartridges were purchased from Waters (Milford, MA).

Standard Solutions

AN-G, AN-S, DHEA-S, ETIO-G, and ETIO-S were respectively dissolved in methanol to obtain their stock standard solutions, each at a concentration of 1 mg/mL.

Samples

Urine and oral mucous membrane samples were collected from 20 volunteers (10 males, 10 females). Some of the urine samples were soaked onto pieces of bleached cotton cloth in order to create

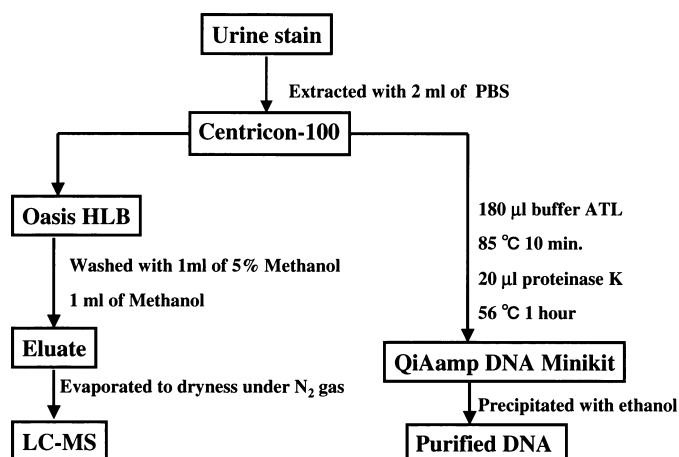


FIG. 1—Extraction procedure for 17-ketosteroid conjugates and DNA from a urine stain.

urinary stains. Urinary DNA-typing was independently confirmed by contrasting the profiles obtained from oral DNA-typing.

Extraction Procedure

A flow chart of the extraction procedure is shown in Fig. 1. The extractions were carried out on the urine stains created from 50, 100, and 200 μ L of urine. A piece of cloth containing a urine stain was cut into sections, and the sections were steeped in 2 mL of

phosphate-buffered saline (PBS; pH 7.4) at room temperature for about 1 h. The PBS extract containing DNA and proteins, etc. was condensed with a Centricon-100 device (Amicon Inc., Beverly, MA). The condensed extract was subjected to DNA-typing after

TABLE 1—Retention time and mass spectral information of 17-ketosteroid conjugates by LC-MS.

Compound (M.W.)	Retention Time (min)	Remarkable Ion (m/z)
Dehydroepiandrosterone sulfate (368)	8.50	367
Etiocholanolone sulfate (370)	13.40	369
Etiocholanolone glucuronide (466)	11.10	465
Androsterone sulfate (370)	14.60	369
Androsterone glucuronide (466)	12.45	465

M.W. = molecular weight.

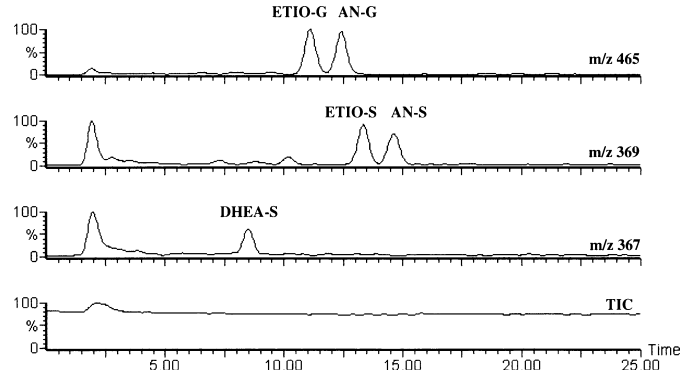


FIG. 2—Example of mass chromatograms of 17-ketosteroid conjugates from a urine stain. Urine stain: 50- μ L sample of urine soaked on bleached cotton.

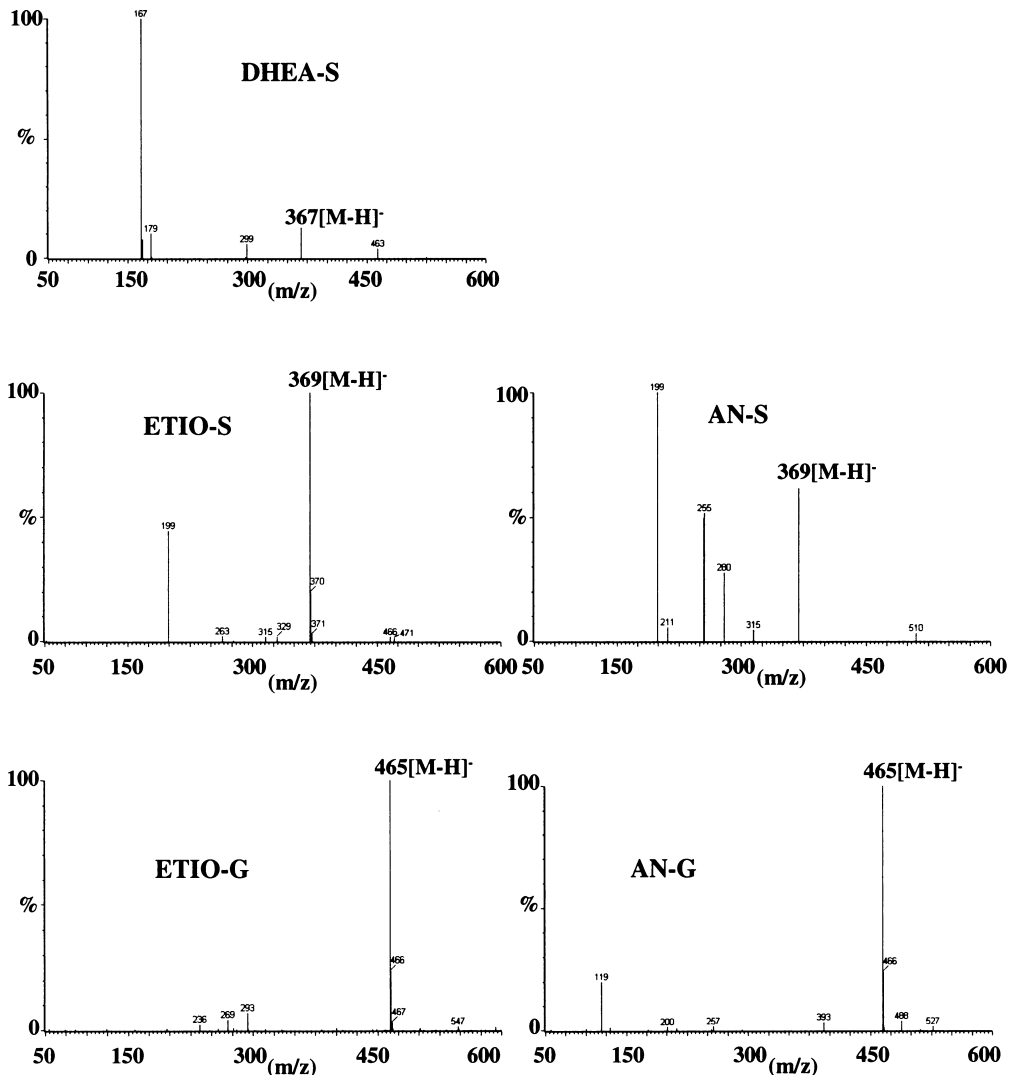


FIG. 3—ESI mass spectra of 17-ketosteroid conjugates obtained from a human urine stain by LC/MS. (1) DHEA-S, (2) ETIO-S, (3) AN-S, (4) ETIO-G, (5) AN-G; Urine stain: 50- μ L sample of urine soaked on bleached cotton.

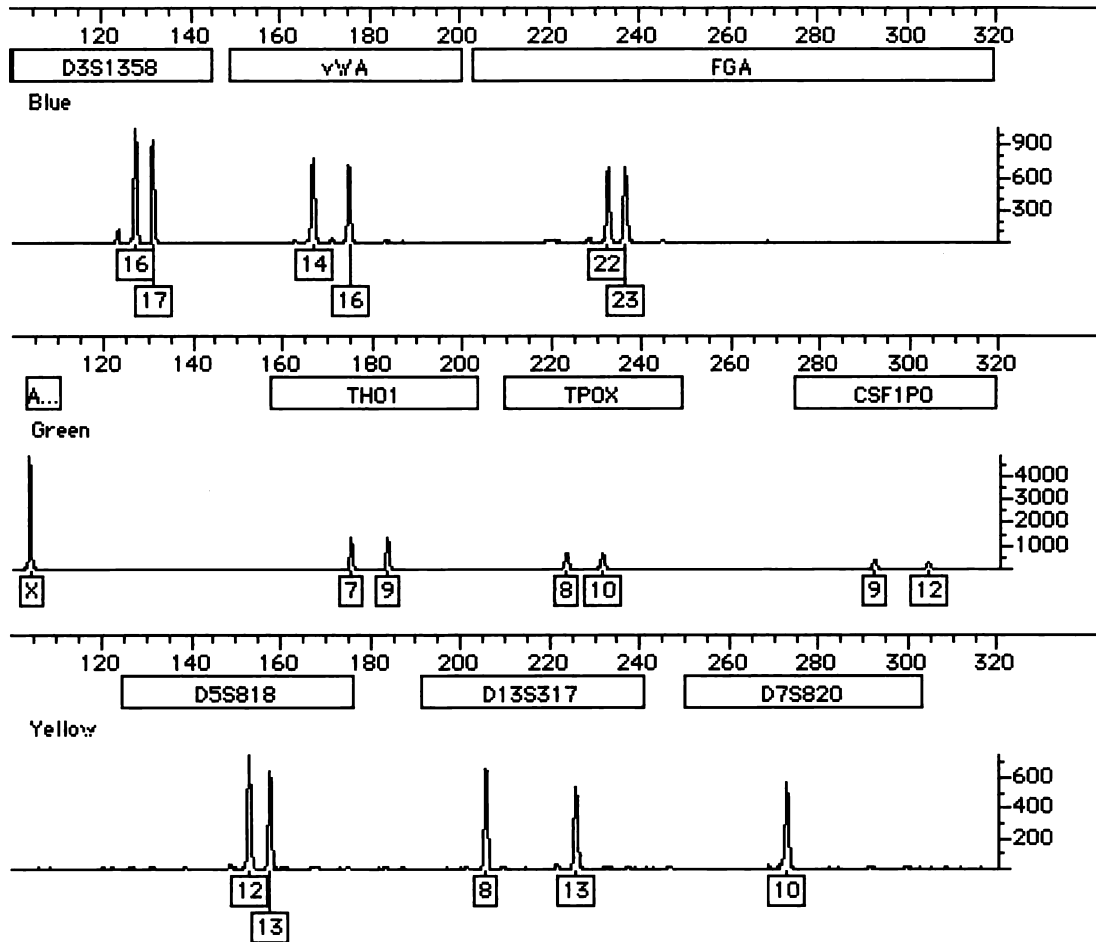


FIG. 4—An electropherogram of PCR products from a urine stain. Urine stain: 50- μ L sample of urine soaked on bleached cotton. The vertical scale is the fluorescence intensity in relative fluorescence units (RFU), while the horizontal scale is the length of the amplified fragments in nucleotide bases. The numbers under the peaks refer to the allelic designation at the individual loci. The genetic loci indicated are from left to right, D3S1358, vWA, and FGA (top: Blue); amelogenin, TH01, TPOX, and CSF1PO (middle: Green); and D5S818, D13S317, and D7S820 (bottom: Yellow).

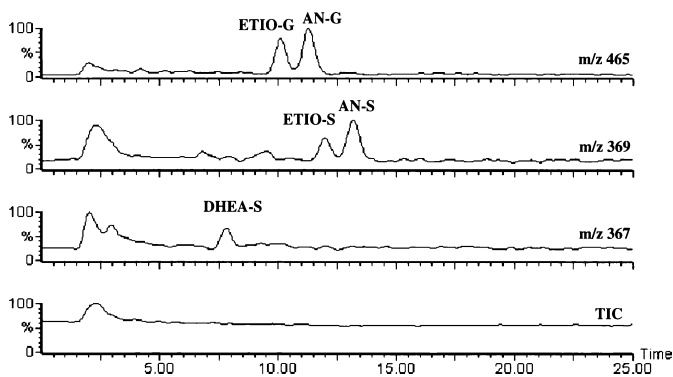


FIG. 5—Mass chromatograms of extract from a stain on a waterproof sheet.

dilution with 180 μ L of Buffer ATL (Qiagen, Hilden, Germany), and filtrate from the Centricon-100 was treated for LC/MS analysis in the following manner.

Sample Preparation for LC/MS—The filtrate was applied onto an Oasis HLB Cartridge, and the cartridge was then washed with 1 mL of 5% methanolic water. Then the analytes were eluted with 1 mL of methanol. The eluate was evaporated until dry under a

nitrogen stream and the residue was dissolved in methanol for LC/MS analysis.

Sample Preparation for DNA-Typing—DNA isolation was performed with a procedure for dried blood spots as described in the pamphlet provided with the QiAamp DNA Minikit (Qiagen). The DNA was eluted with 400 μ L of Buffer AE, and precipitated with ethanol. The precipitate was collected, dried, and re-suspended in 10 μ L of TE buffer for DNA-typing.

Conditions of HPLC-MS

For mass spectral confirmation, a Micromass Platform LCZ mass spectrometer (Manchester, U.K.) equipped with a Waters 2690 separation module high-performance liquid chromatograph and a Waters 997 photodiode array detector (Waters, Milford, MA) was used.

For ionization, an electro-spray ionization mode was used under the following conditions: capillary temperature, 100°C; capillary voltage, -3.0 kV; and cone voltage, -30 V. For the separation column, a reversed-phase CAPCELL PAK C₁₈ AQ (Shiseido, Tokyo, 3.0 mm i.d. \times 150 mm, 3 μ m), was used and maintained at 30°C. For the solvent system, buffer consisting of 25 mM ammonium acetate was mixed with methanol (45:55, v/v), and this was used at a flow-rate of 0.5 mL/min.

Amplification and Electrophoresis of DNA

The DNA sample suspended in TE buffer was subjected to DNA amplification with an AmpflSTR® Profiler™ PCR amplification kit (Applied Biosystems, Foster City, CA) on a GeneAmp system 9700 thermal cycler (Applied Biosystems). Amplification reactions were carried out according to the manufacturer's manual (Applied Biosystems). PCR amplification was performed at a final volume of 25 μ L, composed of 10.5 μ L of the PCR reaction mix, 5 μ L of the primer set, 0.5 μ L of the AmpliTaq Gold DNA polymerase, and 10 μ L of the concentrated sample.

Capillary Electrophoresis for DNA-Typing—An ABI Prism 310 Genetic Analyzer (Applied Biosystems) was used for capillary electrophoresis in this study. The PCR products were run as described in the protocol for the AmpflSTR® Profiler™ PCR amplification kit. Data were analyzed using GeneScan Analysis 3.7.1 software (Applied Biosystems) and allele typing was carried out by Genotyper 3.7 software (Applied Biosystems).

Results

Using standard reference samples, 17-ketosteroid conjugates were examined. Five 17-ketosteroid conjugates were independently

identifiable by their ESI mass spectra and their retention times on mass chromatograms, as shown in Table 1.

An example of a mass chromatogram obtained from a human urine stain created from 50 μ L of urine is shown in Fig. 2. The chromatogram was clearly detected from a small urine stain. The ESI mass spectra of five major conjugated 17-ketosteroids obtained from a urine stain at the same time are shown in Fig. 3. In the electro-spray ionization, deprotonated molecular ions were clearly observed at all the mass spectra of these conjugated 17-ketosteroids. The conjugates were confirmed by their respective retention times and the ESI mass spectra of standard reference samples.

An example of DNA-typing of concentrated extract with dialfiltration from a urine stain is shown in Fig. 4.

Case Report

The examination process provided in this study was applied to a murder case where a urine stain was found on a waterproof sheet in a motel bedroom. The sheet was covering a bed, and was underneath a cotton sheet. Part of the pale yellow stain on the sheet was subjected to the examination. At first, the stain was surmised to be urine based on the findings of a urea pretest. Then, the stain was treated for 17-ketosteroid conjugate analysis and DNA-typing, as described above.

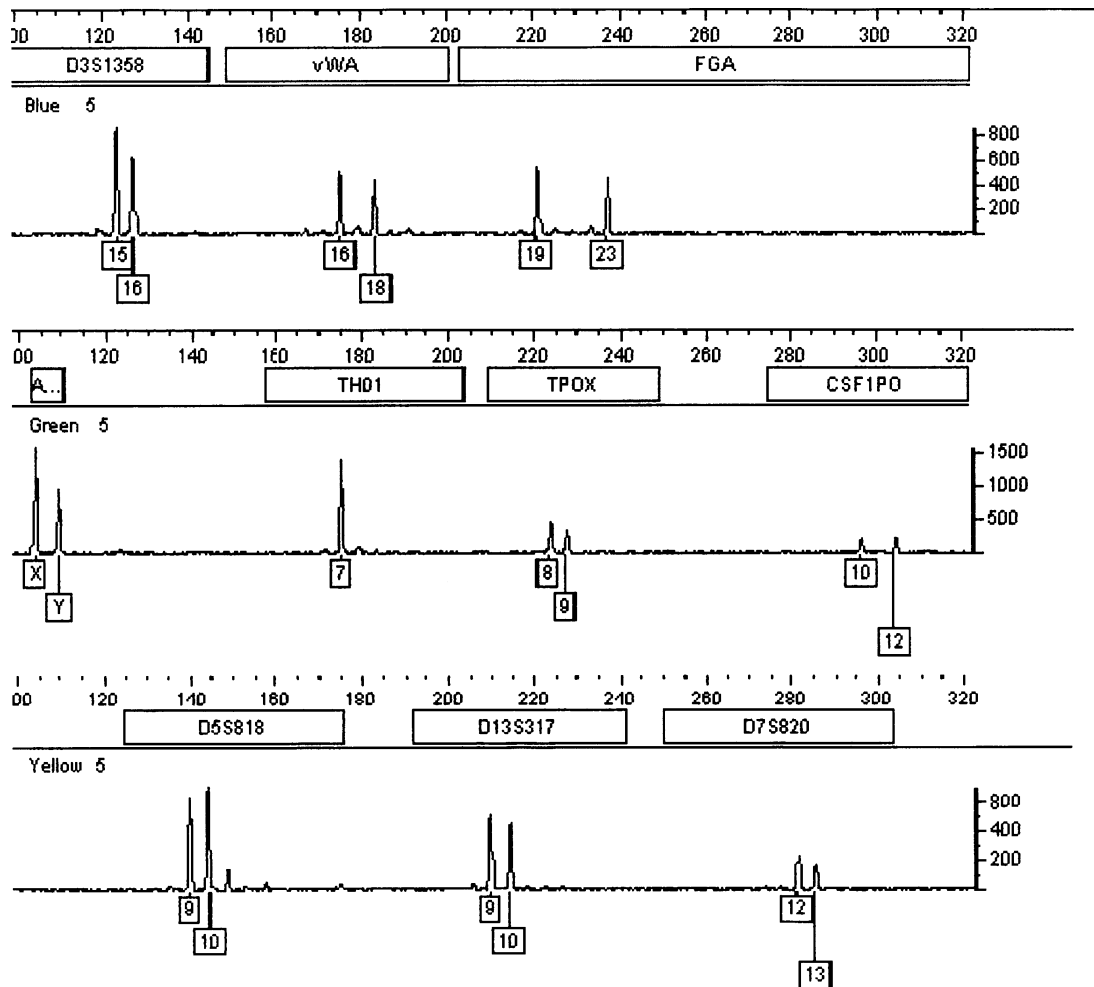


FIG. 6—An electropherogram of PCR products from a urine stain on a waterproof sheet. The vertical scale is the fluorescence intensity in relative fluorescence units (RFU), while the horizontal scale is the length of the amplified fragments in nucleotide bases. The numbers under the peaks refer to the allelic designation at the individual loci. The genetic loci indicated are from left to right, D3S1358, vWA, and FGA (top: Blue); amelogenin, TH01, TPOX, and CSF1PO (middle: Green); and D5S818, D13S317, and D7S820 (bottom: Yellow).

As shown in Fig. 5, all five 17-ketosteroid conjugates were clearly detected from the stain found on the waterproof sheet. Accordingly, it could be estimated that the stain had originated from human urine. Furthermore, DNA-typing indicated that the urine was excreted from a male human and nine STRs were clearly detected, as shown in Fig. 6.

Discussion

The purpose of dialfiltration is the collection of both cells and the free high-molecular DNA which is released from those cells. Meanwhile, it also removes low-molecular-weight materials from within the samples. In the current study, for stain identification, we attempted to apply 17-ketosteroid conjugate analysis to filtrates containing low-molecular-weight materials. By this means it was possible to unify the two tests for use on just one sample.

The analysis of 17-ketosteroid conjugates in urine stains is useful for identification of a human urine stain, since 17-ketosteroids are mostly excreted as conjugates in urine and we have been able to demonstrate that the analytical profiles of 17-ketosteroid conjugates are clearly distinguishable between humans and animals (1). Conjugated 17-ketosteroids analysis by HPLC demanded somewhat complicated steps since it is essential that the conjugates are prelabeled with 2,4-dinitrophenylhydrazine for spectrophotometric detection. In the current method, we attempted to directly analyze 17-ketosteroid conjugates after extraction from filtrates with solid-phase cartridges by LC/MS without prelabeling. Accordingly, the analysis method of 17-ketosteroid conjugates has become simple and has high sensitivity. Furthermore, no loss of conjugates was caused by dialfiltration.

Identification of human urine and DNA profiling were carried out with this serial procedure using both experimental urine stains, and a urine stain found at a crime scene, and good results were obtained. When carrying out stain identification and DNA typing from urine stains found at a crime scene, we were able to conclude that if there are urine samples, then it is possible to identify a urine

stain as being of human origin but not to detect a male DNA profile unless the stain is large. This is because there is a difference between male and female urine samples as regards the quantity of DNA available. When 200 μ L of male urine was used to create urine stains, a complete DNA profile could be obtained from just some of the stains. However, when only 100 μ L of female urine was used complete profile could be successfully obtained from all of the stains (3). To detect DNA typing it would be helpful if a relative cell count were made in the sample prior to extraction, as well as determination of the amount of DNA in the sample, using either real-time PCR (4), or some other method.

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