T. Nakazono,[1,2] B.A.; S. Kashimura,[1] M.D.; Y. Hayashiba,[2] M.D.; T. Hisatomi,[2] B.A.; and K. Hara,[1] M.D.

Identification of Human Urine Stains by HPLC Analysis of 17-Ketosteroid Conjugates

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ABSTRACT: A new method for identifying human urine stains utilizing high-performance liquid chromatographic (HPLC) analysis of five major 17-ketosteroid conjugates: dehydroepiandrosterone sulfate, etiocholanolone sulfate, etiocholanolone glucuronide, androsterone sulfate, and androsterone glucuronide was examined. Samples of urine stains were extracted with borate buffer solution (pH 9.3) and the extracts were applied onto a Sep-Pak tC18 cartridge. The analytes were eluted from the cartridge with methanol. The eluates were prelabeled with 2,4-dinitrophenylhydrazine in trichloroacetic acid-benzene solution and were separated by HPLC on a reversed-phase ODS column using a mobile phase of 80% methanol in a buffer consisting of 25 mM sodium acetate in 2% acetic acid. The eluates were monitored by a spectrophotometer at 380 nm. While all five 17-ketosteroid conjugates were clearly detected in the human urine stain samples, traces of only some of these conjugates were detected in the animal samples. Therefore, the presence of all five 17-ketosteroid conjugates indicated human specificity. In addition to the above finding, the properties of those five 17-ketosteroid conjugates were confirmed by electrospray ionization liquid chromatography-mass spectrometry (ESI-LC-MS).

KEYWORDS: forensic science, forensic investigation, urine stains, 17-ketosteroids, HPLC

Identification of human urine stains is of great importance when attempting to ascertain the exact location of a crime, and when attempting to ascertain the method of death in forensic investigations. Several methods for identifying urine stains have been reported to date, such as the detection of urea and/or creatinine (1–6), but because these compounds are commonly found in animal urine, detection of urea and/or creatine does not constitute human urine specificity. In forensic investigations, it is important to identify whether urine stains found indoors are human urine stains or those of household pets, such as dogs or cats. The uricase method, in which the presence of uric acid is demonstrated by photometry after enzymatic digestion, has often been used to conveniently identify human urine stains (7,8). However, there are some obstacles with regard to ultraviolet (UV) detection because the urine stains of some animals, as well as bird droppings, also respond positively to the test. For these reasons, urine-like stains adhering to carpets and clothes sometimes produce ambiguous results following enzyme testing. Other methods of identifying human urine have been attempted, such as utilizing immunological techniques (9–14), estimating the ratio of uric acid to urea nitrogen (15), or analyzing 17-ketosteroids using gas chromatography (16). When utilizing these methods, however, a large piece of stained material measuring 1–4 cm^2 and/or time-consuming procedures are required.

17-ketosteroids such as androsterone, dehydroepiandrosterone, and etiocholanolone, which are steroid hormone derivatives, are excreted to a significant extent as conjugates, especially in human urine. The main components comprise five 17-ketosteroid conjugates, these being dehydroepiandrosterone sulfate, androsterone glucuronide, androsterone sulfate, etiocholanolone sulfate, and etiocholanolone glucuronide (17). Most of the androgens are metabolized and conjugated in the liver and are excreted as urine via the adrenal glands and urinary bladder (18–20). Accordingly, it can be assumed that most of these steroid conjugates are contained in urine and plasma. Some of the five 17-ketosteroid conjugates contained in urine were not detected from 0.2 mL of serum by HPLC analysis (21). Therefore, the presence of all five conjugated 17-ketosteroids in a small stain would suggest that it was a urine stain. Kreutzmann et al. developed an HPLC method for the routine analysis of urinary 17-ketosteroid conjugates for the study of androgen metabolism (22). If HPLC with spectrophotometric detection of the conjugates treated with 2,4-dinitrophenylhydrazine were available, then the procedure for identifying human urine would be simple and rapid. In this study, we attempted to apply the HPLC method to the practical identification of human urine stains.

Materials and Methods

Reagents

Androsterone glucuronide (AN-G), androsterone sulfate (AN-S), dehydroepiandrosterone sulfate (DHEA-S), etiocholanolone glucuronide (ETIO-G), and etiocholanolone sulfate (ETIO-S), were obtained as their sodium salts from Sigma Chemical Co. Ltd. (St. Louis, MO). Trichloroacetic acid (TCA) and 2.4-dinitrophenylhydrazine (DNPH) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Sep-Pak tC18 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.

[1] Department of Forensic Medicine, Fukuoka University School of Medicine, 7-45-1 Nanakuma, Jonan-ku, Fukuoka, 814-0180, Japan.
[2] Forensic Science Laboratory, Fukuoka Prefectural Police, 7-7 Higashikoen, Hakata-ku, Fukuoka, 812-0903, Japan.
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Reagent Solution for Derivatization

One quarter of a gram of DNPH was dissolved in 5 mL of ethyl acetate prior to the derivatization. TCA was dissolved in benzene to obtain its solution at a concentration of 3 mg/mL.

Mobile Phase for HPLC

The stock buffer (pH 3.5), which consisted of 25 mM of sodium acetate in 2% acetic acid, was mixed with 4 volumes of methanol for use in the HPLC mobile phase.

Urine Stains

Human urine samples were collected from 74 volunteers (34 males and 40 females). Animal urine samples were collected from 10 dogs, 7 cats, 5 beef cattle, 5 pigs, and one weasel. Samples were prepared by soaking urine on pieces of bleached cotton. A urine stain of approximately 25 cm^2 was obtained when 1 mL of urine was placed on the bleached cotton. Samples of bird droppings were prepared by adhering a few droppings to the bleached cotton.

Extraction Procedure

An extract of the urine stain (1 cm^2) with 1 mL of 0.1 M borate buffer solution (pH 9.3) was applied onto a Sep-Pak tC18 cartridge, and the cartridge was washed with 5 mL of distilled water. Then the analytes were eluted with 3 mL of methanol. Finally, the eluate was evaporated until dry under a nitrogen stream at 40°C. Before the sample was loaded, the cartridge was solvated with 10 mL of methanol and then flushed with 10 mL of water and 10 mL of borate buffer solution.

Ten microlitres of the DNPH solution and 100 μl of the TCA solution were added to the dried eluate. The mixture was incubated in a water bath at 60°C for 20 min, and then evaporated until dry under a nitrogen stream at 40°C. The residue containing 2,4-dinitrophenylhydrazine derivatives of 17-ketosteroid conjugates was dissolved in 25 μl of the HPLC mobile phase.

Preparation of Standard Curves

Standard samples for making standard curves were prepared from 1 mL of borate buffer solution containing 17-ketosteroid conjugates at certain concentrations: 40, 80, 120, 160, or 200 ng/mL. The peak area of the tracing absorbance at 380 nm was plotted against the corresponding concentration.

Instrumentation

Conditions of HPLC with Spectrophotometric Detection

The HPLC system used in this study comprised of a Waters 600E Multisolvent Delivery System and a Waters 486 Tunable Absorbance Detector controlled and integrated with a Waters 805 Data Station. The absorbance at 380 nm was monitored for detection. For the separation column, a reversed–phase Shim-pack CLC-ODS (Shimadzu, 6.0 mm i.d. × 150 mm) was used. The solvent system was used at a flow rate of 1.0 mL/min at room temperature.

Conditions of HPLC-MS

For mass spectral confirmation, a Micromass Platform II mass spectrometer (Cheshire, UK) equipped with a Jasco Model PU980 high-performance liquid chromatograph (Tokyo, Japan) was used. For ionization, an electro-spray ionization mode was used under conditions of capillary temperature at 120°C, spray needle voltage at–3.5 kV and cone voltage at–45 V. For a separation column, a reversed–phase NOVA-PAK C_{18} (Waters, 4.6 mm i.d. × 150 mm) was used. For the solvent system, the buffer (pH 3.7) consisting of 40 mM ammonium acetate in 2% acetic acid/methanol (1:4), was used at a flow rate of 0.75 ml/min.

Results

Using standard reference samples, 17-ketosteroid conjugates were examined and detected under various conditions. Five 17-ketosteroid conjugates were independently identifiable by their respective retention times and ESI mass spectra, as shown in Table 1. There were no serious interfering peaks during detection. Accordingly, regular analysis of the conjugates was performed by HPLC with spectrophotometric detection at 380 nm. The linearity of detection for each conjugate was obtained within a range of 40–200 ng.

Using this method of HPLC with spectrophotometric detection, 74 samples of human urine stains were analyzed. In every human specimen, all five conjugates were clearly detected, as indicated in Table 2. An example of an HPLC chromatogram obtained from a human urine stain is shown in Fig. 1. Since traces of only some of these 17-ketosteroid conjugates were

TABLE 1—*Retention times and mass spectral information of DNPH derivatives of 17-ketosteroid conjugates by HPLC and LC-MS.*

Compound (M.W.)	Retention Time (min)	Remarkable Ion (m/z)
Dehydroepiandrosterone sulfate derivative (548)	25.7	547
Etiocholanolone sulfate derivative (550)	32.3	549
Etiocholanolone glucuronide derivative (646)	34.2	645
Androsterone sulfate derivative (550)	35.8	549
Androsterone glucuronide derivative (646)	40.3	645

M.W. = molecular weight.

TABLE 2—*Detection levels of 17-ketosteroid conjugates in 78 human urine stains on bleached cotton.*

Compound	ng/cm^2
Dehydroepiandrosterone sulfate	0.49–587.39
Etiocholanolone sulfate	8.86–651.83
Etiocholanolone glucuronide	6.97–301.13
Androsterone sulfate	10.05–205.33
Androsterone glucuronide	2.58–621.05

NOTE: Each of the conjugates was detected in each of the samples. All the peaks of conjugates were clearly detected (signal to noise ratio (S/N)≥ 10). The estimates of conjugates were calculated, even though the linearity of detection for each of the conjugates was obtained within 40–200 ng.

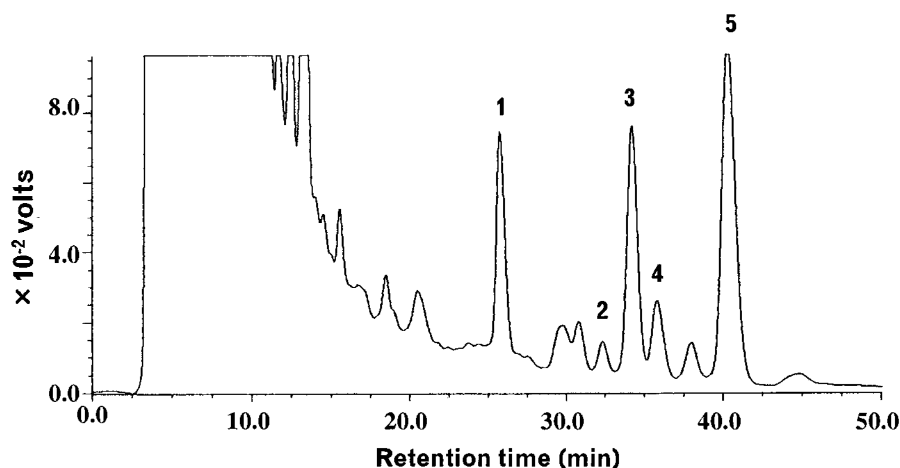

FIG. 1—*Example of an HPLC chromatogram obtained from a human urine stain on a piece of bleached cotton (1 cm^2). 1: DHEA-S; 2: ETIO-S; 3: ETIO-G; 4: AN-S; 5:AN-G.*

TABLE 3—*Detection of 17-ketosteroid conjugates in stains from animal urine or bird droppings.*

Compound	Human*	Dog	Cat	Beef Cattle	Pig	Weasel	Bird
Dehydroepiandrosterone sulfate	+	–	–	–	–	–	–
Etiocholanolone sulfate	+	–	–	–	–	–	–
Etiocholanolone glucuronide	+	–	–	±	±	–	–
Androsterone sulfate	+	–	–	–	–	±	–
Androsterone glucuronide	+	–	–	–	±	–	–

* See Table 2. ±: trace.

detected in the animal urine stains or bird dropping stains (Table 3); it is possible through comparison of human results with animal results to verify whether or not a urine stain shows human specificity.

ESI mass spectra of 17-ketosteroid conjugate derivatives obtained from a human urine stain are shown Fig. 2. The spectra coincide with the results obtained from the original five 17-ketosteroid conjugates.

Discussion

Urine can be characterized through the demonstration of urea or uric acid, excreted in urine as a terminal metabolite of the ornithine cycle or purine catabolism. However, urea or uric acid excretions are common among many species of animals. In forensic investigations, it is of course critical to ascertain human specificity. This method for identifying urine stains utilizing HPLC analysis of 17-ketosteroid conjugates is quite different from previous methods based on the detection of urea or uric acid. The five conjugates described in this report can be detected by their structure and molecular size. We were able to demonstrate that the analytical profiles of 17-ketosteroid conjugates were clearly distinguishable between humans and animals. Accordingly, the analysis of 17-ketosteroid conjugates in urine stains would appear to be a satisfactory method of human urine identification.

Even from extremely small human urine samples, all five major 17-ketosteroid conjugates were clearly detected as 2,4-dinitrophenylhydrazilones using HPLC with spectrophotometric detection. Moreover, these conjugates could be confirmed by ESI-LC-MS. Watanabe et al. analyzed the free form of 17-ketosteroids following hydrolysis using GC-MS for the purpose of identifying urine stains (16). In the current method, we directly analyzed 17-ketosteroid conjugates without hydrolysis, because these steroids are mostly excreted as conjugates in urine. Accordingly, we consider this method to be more suitable for the identification of human urine stains. It is important to bear in mind that these steroids are present in other body fluids, because androgens are widely distributed throughout the human body. However, if this method is combined with the detection of urea (5,6), or uric acid (7,8), then it can become the established method of identifying human urine stains in forensic investigations.

In this study, when comparing human urine samples with animal urine samples, urine samples from only five animal species were examined. Perhaps a future study should examine urine stains from a greater range of species, including rodents, which are present indoors as household pets, and primates, which are more closely related to humans. Furthermore, there are some reports showing that the amount of total 17-ketosteroids contained in urine and the ratio of the components of each steroid change in accordance with age and gender (23,24). We intend to clarify whether or not these changes can be observed in urine stains, and whether or not age and gender can be identified from urine stains found at a crime scene.

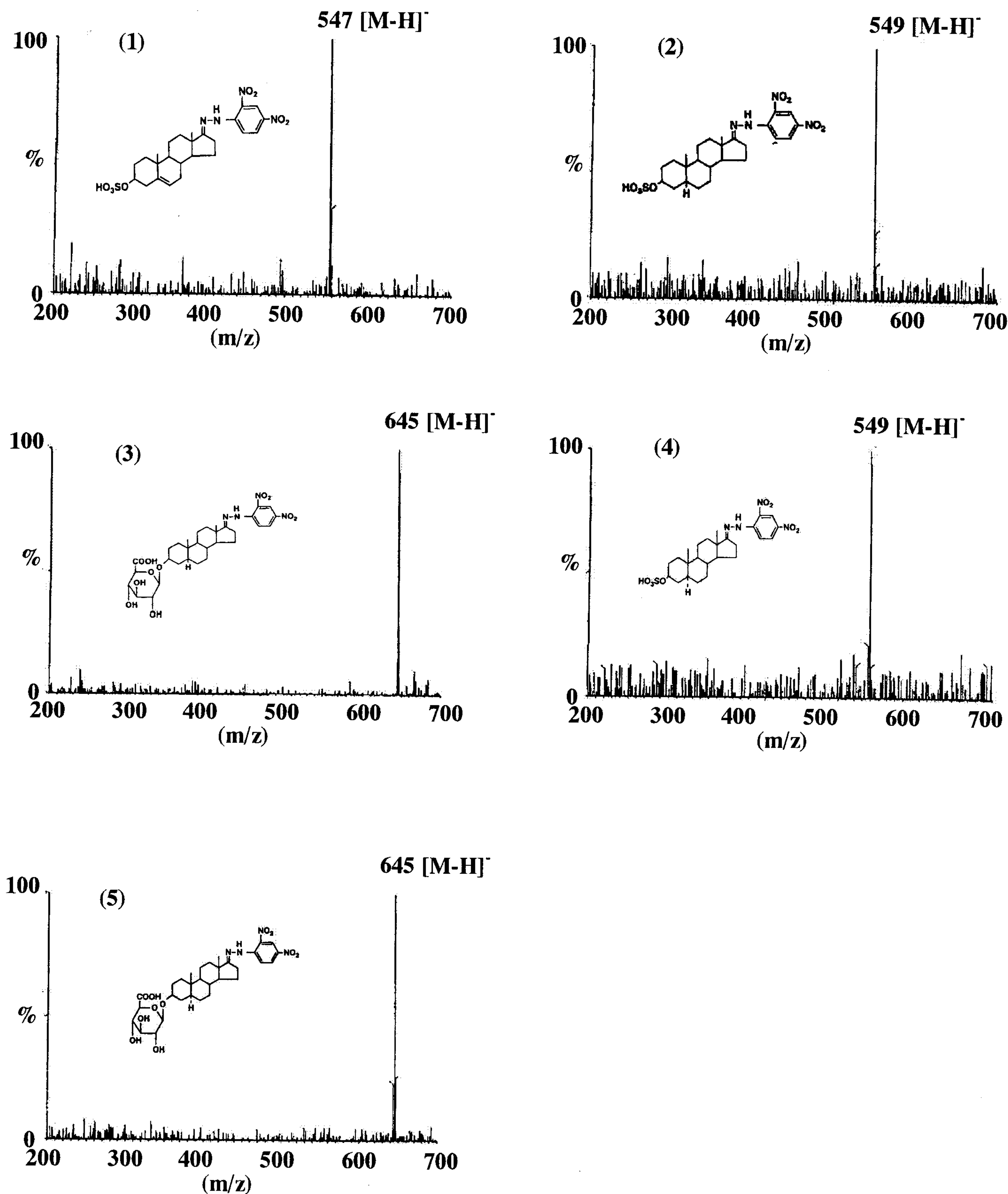

FIG. 2—*ESI mass spectra of conjugated 17-ketosteroid derivatives obtained from a human urine stain by LC-MS. (1): DHEA-S; (2): ETIO-S; (3): ETIO-G; (4): AN-S; (5): AN-G.*

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Additional information and reprint requests:
S. Kashimura
Department of Forensic Medicine
Fukuoka University School of Medicine
7-45-1 Nanakuma
Jonan-Ku, Fukuoka, 812-0903
Japan
Phone: +81-92-801-1011, ext. 3330
Fax: +81-92-801-4266