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DIETHYLHYDROGENSILYL-CYCLIC DIETHYLSILYLENE DERIVATIVES IN GAS CHROMATOGRAPHY/MASS SPECTROMETRY OF HYDROXYLATED STEROIDS. V. ANALYSIS OF CORTISOL AND 6β-HYDROXYCORTISOL IN HUMAN URINE

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A gas chromatography/selected ion monitoring (GC/SIM) method for a simultaneous determination of cortisol (Kendall's compound 'F': F) and its hydroxylated metabolite, 6β-hydroxycortisol (6OHF) in human urine has been developed using [$^2H_3$]cortisol as an internal standard. These compounds were analyzed as their methyloxime-diethylhydrogensilyl-cyclic diethylsilylene derivatives. Gas chromatography of the F derivative showed a fairly broad single peak, whereas the 6OHF derivative exhibited a well-resolved doublet peak corresponding to the structural syn- and anti-isomers. The mass spectra were characterized by their intense molecular ion peaks and by their inherent ions of [M-OCH_3]$^+$, [M-diethylhydrogensilanol(DEHSOH)]$^{+\cdot}$, and [M-OCH_3-DEHSOH]$^+$. When GC/SIM was carried out using their base peak ions at m/z 559 for F and 557 for 6OHF, the sensitivity for F was about 10 pg with signal-to-noise ratio of more than 10, and this was about a 5 times higher response factor than that of 6OHF. The calibration graphs were found to be linear in the observed range covering the concentrations found in healthy human urine.

KEYWORDS —— cortisol; 6β-hydroxycortisol; cyclic diethylsilylene derivative; mass spectrum; microanalysis; GC/MS; GC/SIM; DEHS-BSTFA

The urinary excretion ratio of cortisol (Kendall's compound 'F': F) and its metabolite 6β-hydroxycortisol (6OHF) has recently been given attention in the field of drug metabolism as an index of the induction of hepatic drug-metabolizing enzymes.[1] This is a valuable index because the hydroxylation of F can be stimulated by foreign compounds which induce the hydroxylating enzyme in hepatic microsomes. F and 6OHF may be determined by various analytical methods, including radioimmunoassay, enzyme immunoassay, high performance liquid chromatography, Gas chromatography (GC) or gas chromatography/selected ion monitoring (GC/SIM). Of these, GC/SIM is the most specific and reliable method for the microanalysis, as it provides both structural information and GC retention time. The methyloxime (MO)-trimethylsilyl (TMS) ether derivative of F has been used exclusively as a derivative suitable for GC/SIM. However, on a fused silica capillary column cross-linked with methylsilicone or 5%-phenylmethylsilicone, F elutes together with 6OHF. This causes an overestimation of

the F levels in biological fluids, especially in urine. Consequently, the influence of urinary 6OHF levels during GC/SIM analysis cannot be disregarded. To resolve this problem, a new cyclization reaction suitable for the simultaneous analysis of F and 6OHF was investigated.

F and 6OHF were converted to their methyloxime (MO)-diethylhydrogensilyl (DEHS)-cyclic diethylsilylene (DES) derivatives by treating them with O-methylhydroxylamine HCl and then with N,O-bis(diethylhydrogensilyl)trifluoroacetamide. This avoided any problem in the derivatization reaction due to the steric hindrance of 17α-hydroxyl group. The reaction products were analyzed using a 25 m fused silica capillary column cross-linked with methylsilicone (Ultra 1, Hewlett Packard Co., PA., USA.). The resulting derivative of F showed a fairly broad single peak, whereas that of 6OHF exhibited a well-resolved doublet peak corresponding to the structural syn- and anti-isomers. The same phenomenon has also been observed in these MO-TMS ether derivatives, indicating that the substitutional group at C-6 in 6OHF derivative made a large contribution to the appearance of the well-resolved doublet. The MU-value of the F derivative was 35.47. This was about 2.3 higher than that of the corresponding TMS ether derivative (33.11). The MU-values of the two components of the 6OHF derivative were 37.14 and 37.26, respectively. These were about 4.2 higher than the corresponding TMS ether derivatives (32.94 and 33.13). Consequently, a difference in retention times of about 1.6 MU-values results in the complete separation of F and 6OHF. In contrast, their corresponding MO-TMS ether derivatives overlapped each other.

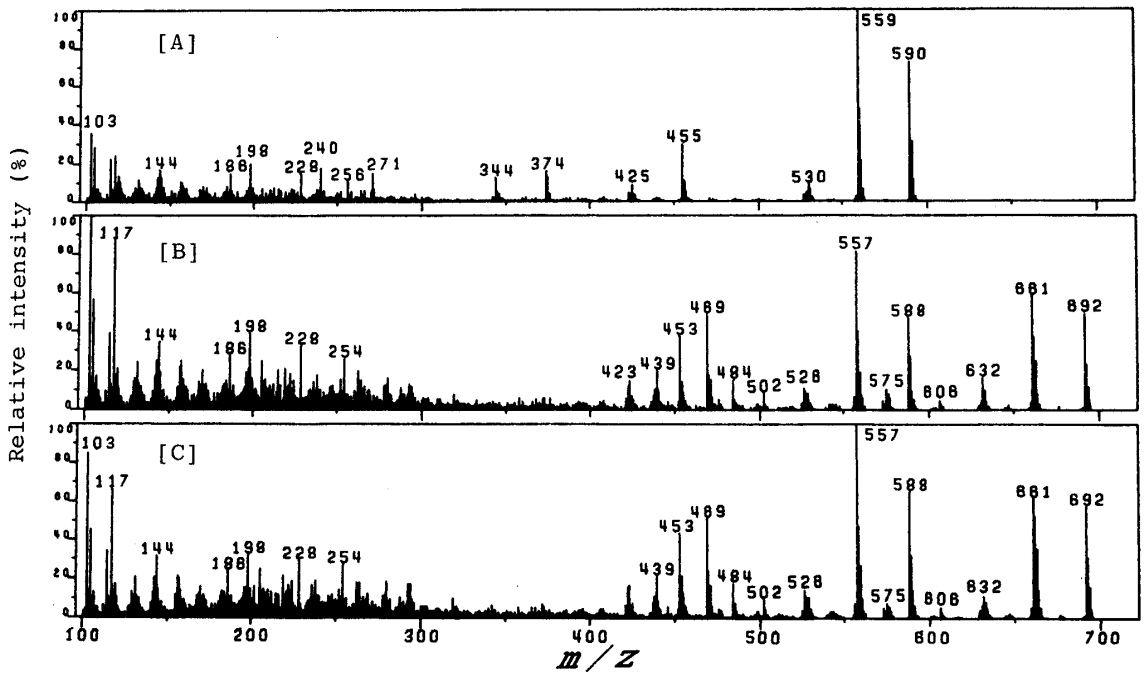

Fig. 1. Mass Spectra of the MO-DEHS-DES Derivatives of Cortisol [A] and 6ß-Hydroxycortisol ([B]: First-Eluted Component, [C]: Second-Eluted Component)

GC/MS was performed using a VG ZAB-HF mass spectrometer (VG Analytical Ltd., Manchester, UK) interfaced to a shimadzu 9AP gas chromatograph (Shimadzu Co., Kyoto, Japan) and a VG DS-2000 data processing system. GC conditions: column; 25 m x 0.32 mm, methylsilicone cross-linked fused silica capillary column, column temp; 250°C (1 min), programmed to 320°C at 5°C/min; carrier gas, helium. MS conditions: ion source temp, 250°C; ionization energy, 70 eV; trap current, 200 μA; accelerating voltage, 8 kV.

The mass spectrum of the F MO-DEHS-DES derivative is shown in Fig. 1-A. The appearance of the molecular ion at m/z 590 with prominent intensity was sufficient to confirm the formation of the expected derivative. The molecular ion lost a methyloxy radical (CH_3O: 31 amu) from the methyloxime moiety to give rise to the ion of $[M-OCH_3]^+$ (m/z 559) as a base peak. Loss of the diethylhydrogensilanol molecule (DEHSOH: 104 amu) from this ion produced the ion at m/z 455. The ions characteristic of the steroidal moiety such as m/z 375, 374, 271, 256 and 240 were observed with moderate intensity. The fragmentation pathway and structures of these were well-documented by Tokes et al.[2] and Brooks et al.[3] The ion at m/z 228, characteristic of the DES group, was produced by the fission of the D-ring with a migration of the hydrogen atom, and was assigned to correspond to the ion of m/z 287 in the mass spectra of the DEHS-DES derivatives of pregnane-17,20,21-triols.[4]

The mass spectra of the structural isomers of the MO-DEHS-DES derivative of 6OHF are shown in Fig. 1-B and 1-C, giving the same mass spectral pattern. Almost all of the ions in the mass spectrum of the first-eluted component were also observed as the characteristic ions common in the second-eluted component. In comparison to the mass spectra of the MO-DEHS-DES derivatives of F and 6OHF, the shift of the molecular ion from m/z 590 to 692 indicates the incorporation of a DEHS group into the 6β-hydroxyl

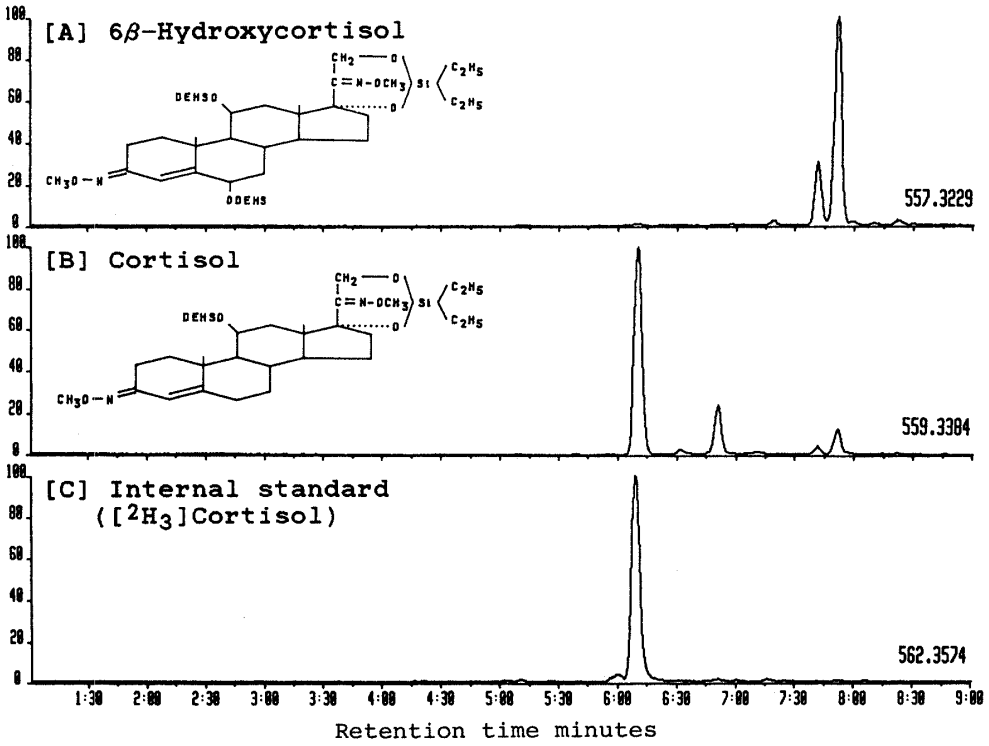

Fig. 2. Selected Ion Recordings of the MO-DEHS-DES Derivatives of Cortisol [A], 6β-Hydroxycortisol [B] and Internal Standard $[^2H_3]$Cortisol [C] in the Extract from Male Adult Urine

GC/SIM was performed using a VG 70-SE mass spectrometer (VG Analytical Ltd.) interfaced to a Hewlett Packard 5890A gas chromatograph and a VG 11-250J+ data processing system. GC conditions: column, 12 m x 0.20 mm; methylsilicone cross-linked fused silica capillary column, column temp, 250°C (1 min), programmed to 320°C at 5°C/min; carrier gas, helium. MS conditions: ion source temp, 250°C; ionization energy, 40 eV; trap current, 200 μA; accelerating voltage, 8 kV. For SIM at resolution of 10000, ions at m/z 557.3229, 559.3384 and 562.3574 were focused by reference to the ion at m/z 554.9665 derived from perfluorokerosene, which was independently introduced into the ion source.

group. A similar series of ions in the mass spectrum of the F derivative appeared in that of the 6OHF derivative, together with additional fragment ions due to the elimination of the DEHSOH molecule from the molecular ion and the $[M-OCH_3]^+$ ion. It is clear from these GC/mass spectrometric results that the reaction product of F was the expected 3,20-bis-MO-11-DEHS-17,21-DES derivative and that of 6OHF was a mixture of the structural isomers of the expected 3,20-bis-MO-6,11-bis-DEHS-17,21-DES derivative.

In order to examine the suitability of the present MO-DEHS-DES derivatives of F and 6OHF for the microanalysis in biological fluids, GC/SIM was carried out, using the characteristic ions of $[M-OCH_3]^+$ (m/z 559) for F and $[M-OCH_3\text{-DEHSOH}]^+$ (m/z 557) for 6OHF. The sensitivity for F was about 10 pg with signal-to-noise ratio (S/N) of more than 10. This was about a 5 times higher response factor than that of 6OHF. This significant decrease in sensitivity for 6OHF may be explained by the formation of the structural syn- and anti-isomers and the extensive fragmentation that accompanied the successive elimination of the DEHSOH molecule. Calibration graphs were prepared from GC/SIM analysis of increasing amounts of unlabeled F and 6OHF added to a constant amount of $[^2H_3]$cortisol (ICN Biomedicals, Inc., MA., USA.). They were found to be linear in the observed range covering the concentrations found in healthy human urine.

Male adult urine, collected in the early morning, was submitted to the chromatographic sample preparations according to the method of Ono et al.[5] The extract was treated by the derivatization method described above, and analyzed by GC/SIM. Fig. 2 illustrates typical selected ion recordings (SIRs) of the urine sample, and shows that the interfering substances from the urine matrix were almost eliminated during their microanalysis. F and 6OHF could be obserbed with good S/N ratio. The peaks appearing on the SIRs of the urine sample corresponded to about 4.6 ng for F and 11.2 ng for 6OHF.

Use of the present derivatives raises the possibility for the simultaneous detection of picogram levels of F and 6OHF with a high degree of confidence. The biomedical application of their quantitation in human urine by this technique will be reported elsewhere.

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