A SIMPLIFIED METHOXIME (MO) - TRIMETHYLSILYL (TMS) DERIVATIZATION FOR SIMULTANEOUS DETERMINATION OF KETOSTEROID HORMONES BY GC-MS

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Abstract

Ketosteroid hormones have been earned a great concern due to their adverse effects on the endocrine system of wildlife and human. GC-MS has become a preferred technology for simultaneous analysis of ketosteroid hormones because of its superior separation and identification capabilities. Derivatization of ketosteroids is usually done by methoxime (MO) - trimethylsilyl (TMS) derivatization which could stepwise derivatize the hydroxyl and ketone groups of ketosteroids. But, a tedious purification process to remove excess derivatization reagents was applied in most studies which will induce the loss of sample and hamper application of this approach in the trace analysis. Therefore, a simplified MO-TMS derivatization for the simultaneous determination of ketosteroid hormones, such as estrone (E1) and progesterone (PROG), by gas chromatography-mass spectrometry (GC-MS) was described in this study. The relatively tedious purification process on the derivatization was abandoned. The effects of reaction time, temperature and the solvent on the MO derivatization were investigated in detail. The followed TMS derivatization with 150 µL N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) were suggested as a necessary procedure to complete TMS derivatization of hydroxyl groups and resolve the problem of GC column damage caused by residual MOX. The optimized MO-TMS derivatization conditions were 50 µL MOX at 80 $^{\circ}$C for 40 min and straightly followed by 150 µL BSTFA at room temperature (around 20 $^{\circ}$C) for 10 min. Under the optimized conditions, hydroxyl and ketone groups in those ketosteroids were successfully stepwise derivatized. Furthermore, the contribution value of MO derivatization on the MO-TMS derivatization was indirectly evaluated by comparing the optimized MO-TMS derivatization with the single TMS derivatization. As a conclusion, it was demonstrated that the MO derivatization cannot increase the sensitivity of ketosteroid hormones and the followed TMS derivatization provided the main derivatization contributions in the optimized MO-TMS derivatization.

Introduction

In recent years, ketosteroid hormones have been earned a great concern due to their function of interfere the endocrine system of wildlife and human. The natural ketosteroid hormones estrone (E1) and progesterone (PROG) are the most important contributors to estrogenic activity in the environment[1-3]. Even at the extremely low concentration (ng/L), those compounds have adverse effects on the reproduction and development of organisms[4-5]. Nowadays, a wide variety of analytical techniques have been developed for ketosteroid hormones analysis. GC-MS has become a preferred technology for simultaneous analysis of ketosteroid hormones because of its superior separation and identification capabilities.

Derivatization of ketosteroids is usually done by methoxime (MO) - trimethylsilyl (TMS) derivatization which is based on the MO derivatization of keto groups and the TMS derivatization of hydroxyl groups[5-14]. But, a tedious purification process to remove excess derivatization reagents was applied in most studies, such as Suzuki[15] and Matsuzaki[12] used Sep-Pak silica gel column and Sephadex LH-20 column to remove excess derivatization reagent respectively. Those purification processes will induce the loss of sample and hamper application of this approach in the trace analysis. Furthermore, it is well known that the TMS derivatization will greatly increase the sensitivity of the target compounds[16-20], but the contribution value of MO derivatization on the MO-TMS derivatization was rarely evaluated because the MO derivatization was always couple with TMS derivatization.

1

Therefore, the objective of this work was to investigate and develop the MO-TMS derivatization technique, which using O-methylhydroxylamine hydrochloride (MOX) and BSTFA as the MO and TMS derivatization reagent respectively, for the simultaneous detection of two typical ketosteroid hormones (E1 and PROG) by GC-MS. The relatively tedious purification process on the derivatization was abandoned on the purpose of simplifying the experiment. The effects of reaction time, temperature and the solvent on the MO derivatization were systematical investigated. Furthermore, the contribution value of MO derivatization on the MO-TMS derivatization was indirectly evaluated by comparing the optimized MO-TMS derivatization with the single TMS derivatization.
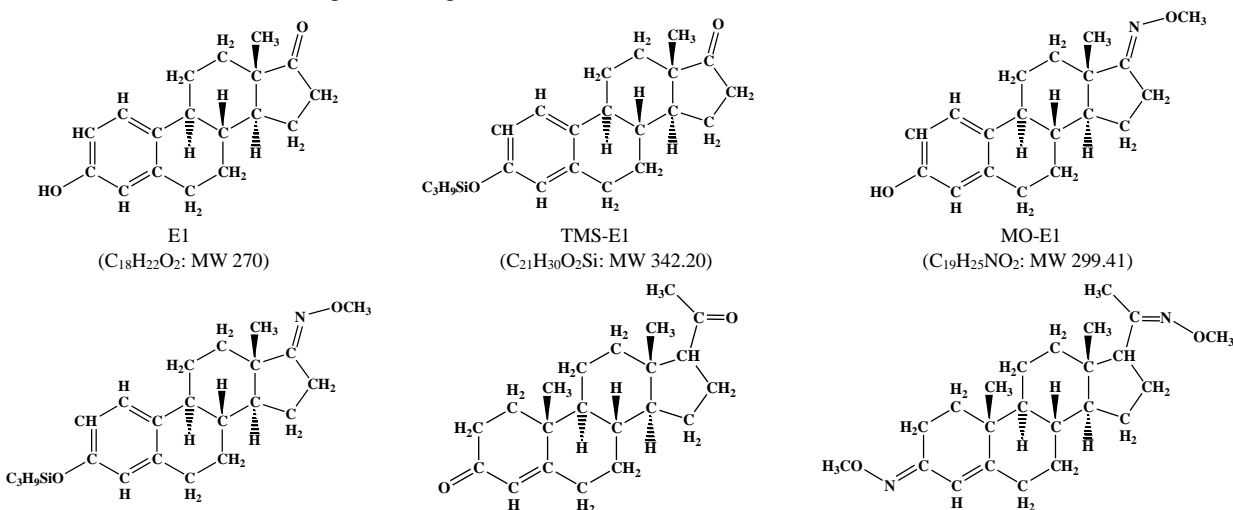
Materials and Methods

Chemicals

Ketosteroid standards (E1, PROG) and internal standard (I.S.) 5α-androstane with a purity of 97 % or higher were supplied by Sigma-Aldrich (USA). Derivatization grade MOX was obtained from Sigma-Aldrich (USA). Derivatization grade BSTFA, GC grade anhydrous methanol, acetone and pyridine were obtained from Fluka (USA).

Solutions

Individual standard stock solutions of E1, PROG and I.S. were prepared at 1 μg/μL in anhydrous methanol, from which appropriate dilutions were made in methanol according to need. A mixture of working standards solution containing each ketosteroid compounds (E1, PROG) at 10 ng/μL was prepared weekly by diluting the stock solution in methanol. The inject concentration of the I.S. was 1 ng/μL. All the solutions should be stored in a freezer at -20 °C and protected from light.

Derivatization

The systematic study was carried out through derivatization of standards. The derivatization procedure as follows: 100 μL of a prepared woking standard solution (each ketosteroid at 10 ng/μL) was pipeted into a 2 mL amber vial and evaporated to dryness under a gentle nitrogen stream at room temperature (around 20 °C), addition of 50 μL MOX (10 mg/ml in anhydrous methanol) into the vial, and then capped, vortexed and placed at 80 °C for 40 min to create MO-derivatives. After the reaction, it was evaporated to dryness under a gentle nitrogen stream at room temperature (around 20 °C). 150 μL BSTFA were added to the residual, capped, vortexed and placed at room temperature (around 20 °C) for 10 min to derivatize the hydroxyl groups. After reaction, it was evaporated to dryness under a gentle nitrogen stream at room temperature (around 20 °C). With addition of 10 μL I.S. (10 ng/μL) and 90 μL acetone, then capped and vortexed, 1 μL of the mixtures were analyzed by GC-MS. Chemical structures of E1, PROG and their derivatives are given in Fig. 1.

E1
(C$_{18}$H$_{22}$O$_2$: MW 270)

TMS-E1
(C$_{21}$H$_{30}$O$_2$Si: MW 342.20)

MO-E1
(C$_{19}$H$_{25}$NO$_2$: MW 299.41)

MO-TMS-E1	PROG	di-MO-PROG
($C_{22}H_{33}NO_2Si$: MW 371.59)	($C_{21}H_{30}O_2$: MW 314.46)	($C_{23}H_{36}N_2O_2$: MW 372.54)

Fig. 1. Chemical structures of E1,PROG and their derivatives

GC-MS analysis

Trace DSQ quardrupole mass spectrometer coupled to a Trace GC (Thermo Fisher Scientific, USA) fitted with a DB-5MS (30 m $\times$ 0.25 mm $\times$ 0.25 μm) column (J&W Scientific, USA) and an autosampler Triplus AS were used. The GC was fitte with a split/splitless injector operated in the splitless mode. The injector temperature was set at 280 ^{o}C and the column temperature program was as follows: the initial temperature was 50 ^{o}C for 2 min, ramped at 12 ^{o}C /min to 260 ^{o}C and maintained at this temperature for 8 min, then ramped at 3 ^{o}C /min to 280 ^{o}C and held for 5 min. The GC-MS interface temperature was set at 280 ^{o}C. The carrier gas was helium with a constant flow rate of 1 mL/min. Mass spectra were obtained in full scan mode from 50-600 *m/z* mass range for qualitative analysis or selected ion monitoring (SIM) mode for quantitative analysis. Electron impact ionization energy was 70 eV. All GC and MS parameters were implemented using Xcalibur version 1.4 software.

Results and discussion

Resolve the problem of GC column damage

The excessive MOX was needed to complete the MO derivatization of the keto groups to create MO-derivatives. But, the tedious purification process on the derivatization was abandoned in this study. The GC column would be gravely damaged by the residual MOX without any further treatment, as shown in Fig. 2A. The peak of I.S. was not observed because the interference of the residual MOX. The peaks of MO-E1 and di-MO-PROG were appeared, but only low relative abundance of those peaks was obtained. Two peaks of di-TMS-PROG which caused by syn- and anti- addition during MO reaction were also appeared. When studied the MO-TMS derivatization, it was discovered that BSTFA not only could derivatize hydroxyl groups, but also had the ability to consume the residual MOX, as shown in Fig. 2B. Clear chromatogram can be received when the amount of BSTFA was increased to 150 μL. The MO-TMS-E1 and di-MO-PROG had the syn- and anti- isomers with respect to the nitrogen of the oxime group and singal to noise of PROG was greatly reduced because the proportion of two isomers was nealy 2:1.

In order to investigate the effect of BSTFA on protection the GC column, derivatization was conducted with 50 μL MOX (10 mg/ml in anhydrous methanol) at 80 ^{o}C for 40 min following different quantity of BSTFA (50, 100, 150, 200, 250, 300 μL) at room temperature (around 20 ^{o}C) for 10 min. The peaks of MO-TMS-E1 and I.S. were emerged along with the increase of BSTFA. The RRFs of MO-TMS-E1 and di-MO-PROG reached the maximum when the amount of BSTFA increased to 150 μL, as shown in Fig. 3. In conclusion, the followed TMS derivatization with 150 μL BSTFA were suggested as a necessary procedure to complete TMS derivatization of hydroxyl groups and resolve the problem of GC column damage caused by residual MOX.
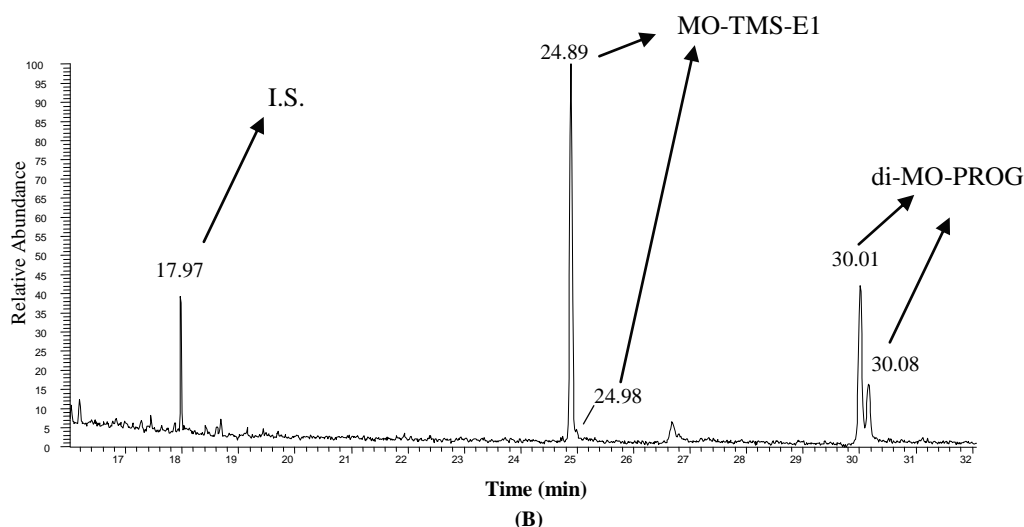

(A)

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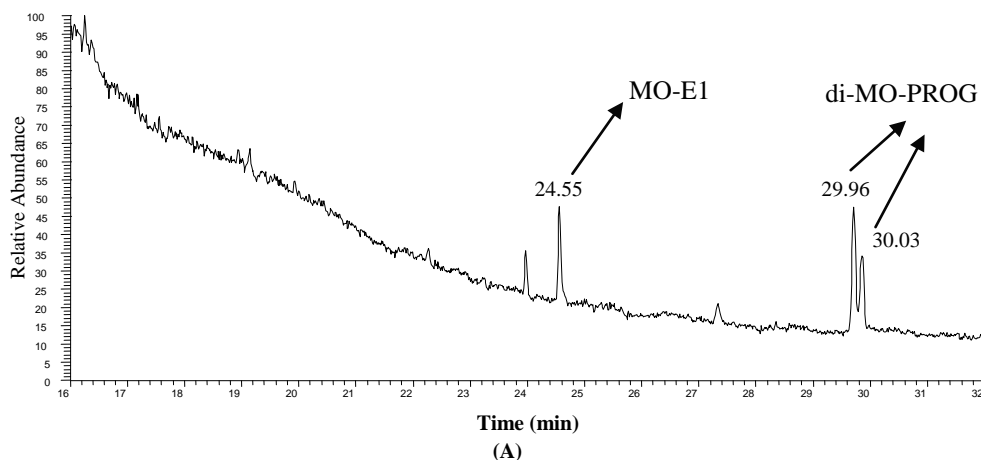
Fig. 2. Full scan chromatogram of ketosteroid derivatives (10 ng/µL) and I.S. (1 ng/µL)
(A) MO derivatization performed without TMS derivatization
(50 µL MOX at 80 °C for 40 min)
(B) MO derivatization performed followed by TMS derivatization.
(50 µL MOX at 80 °C for 40 min, followed by 150 µL BSTFA at room temperature (around 20 °C) for 10 min)
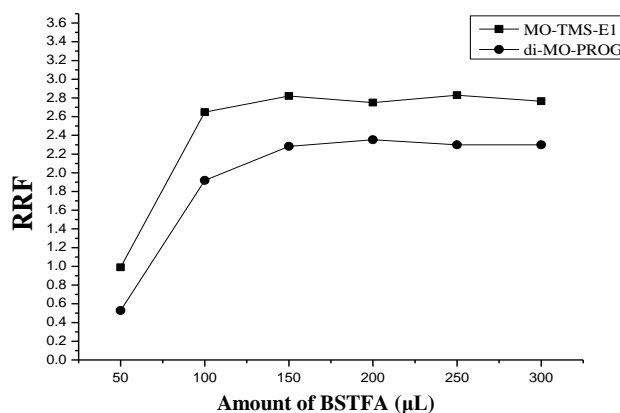

Fig. 3. Effect of amount of BSTFA on the derivatization.

Effect of reaction solvent, temperature and time

From the previously studies, the reaction solvent of MO derivatization was pyridine because MOX had always dissolve in pyridine. The effect of reaction solvent on the MO derivatization was evaluated by using 50 µL MOX (10 mg/ml in pyridine or methanol) at 80 °C for 40 min and followed TMS derivatization with 150 µL BSTFA at room temperature (around 20 °C) for 10 min. It was illuminated that unsatisfied results was obtained when used pyridine as the reaction solvent because there was a little peak of TMS-E1 which indicated the incomplete derivatization of keto groups of E1. The complete derivatization results were achieved with reaction in methanol. Therefore, methanol was the better reaction solvent in MO derivatization.

To investigate the effect of reaction temperature on the MO derivatization, it was conducted with 50 µL MOX (10 mg/ml in methanol) at different temperature (room temperature (around 20 °C), 40, 60, 80, 100, 120 °C) for 40 min and followed by 150 µL BSTFA at room temperature (around 20 °C) for 10 min. The results were shown in Fig. 4A.

4

The keto groups of E1 only can be completely derivatized at higher temperatures. The RRFs of MO-TMS-E1 and di-MO-PROG reach the maximum when the reaction temperature increases to 80 $^{\circ}$C. As a result, 80 $^{\circ}$C was the optimized reaction temperature at this derivatization procedure.

The effect of reaction time on the MO derivatization was performed with 50 µL MOX (10 mg/ml in methanol) at 80 $^{\circ}$C for different reaction time (10, 20, 30, 40, 50, 60, 70 min), and followed by 150 µL BSTFA at room temperature (around 20 $^{\circ}$C) for 10 min. The results were shown in Fig. 4B. The RRFs of di-MO-PROG were increased rapidly with addition of the reaction time from 10 to 40 min, but RRF of MO-TMS-E1 had little sensitivity of the MO derivatization reaction time. In conclusion, 40 min was the optimized reaction time in this study.
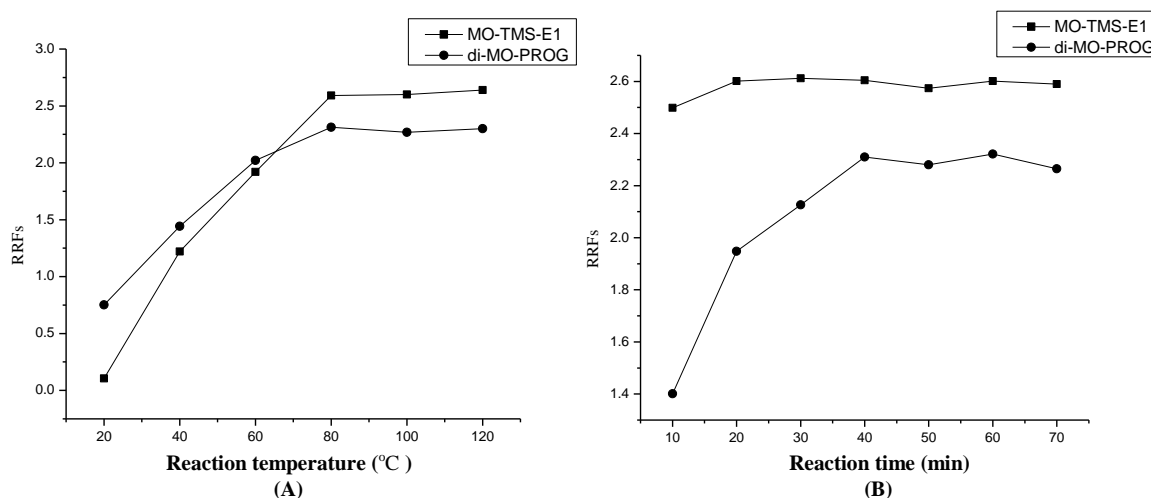

Fig. 4. Effect of reaction temperature and time on the MO derivatization of steroid hormones at 10 ng/µL
(A) Effect of reaction temperature on the MO derivatization of steroid hormones.
(50 µL MOX at room temperature (around 20 $^{\circ}$C), 40, 80, 60, 80, 100, 120 $^{\circ}$C for 40 min, followed by TMS derivatization)
(B) Effect of reaction time on the derivatization of ketosteroids.
(50 µL MOX at 80 $^{\circ}$C for 10, 20, 30, 40, 50, 60, 70 min, followed by TMS derivatization)

Evaluate the contribution value of MO derivatization
The contribution value of MO derivatization was indirectly evaluated by comparing the optimized MO-TMS derivatization with the single TMS derivatization, as shown in Fig. 5. The analysis of the mixture standard solution (each ketosteroid at 10 ng/µL) and I.S. at 1 ng/µL was also preformed as the background value. The single TMS derivatization as follows: 100 µL of a prepared woking standard solution (each ketosteroid at 10 ng/µL) was pipeted into a 2 mL amber vial and evaporated to dryness under a gentle nitrogen stream at room temperature (around 20 $^{\circ}$C), addition of 90 µL BSTFA at room temperature (around 20 $^{\circ}$C) for 10 min, 10 µL internal standard was added, then capped and vortexed, 1 µL of the mixtures were analyzed by GC-MS.

By comparison of the RRFs, the results as follows: (1) The RRF of E1 was greatly increased through MO-TMS derivatization. But, the RRF of TMS-E1, which was created by the single TMS derivatization, was a little higher than MO-TMS-E1. It was illuminate the single TMS derivatization already reached the good sensitivity and the MO derivatization was not enhanced the effect. By the contrary, the residual MOX might interfere in the GC column lead to lower sensitivity. (2) The single TMS derivatization was ineffective on PROG which only had keto groups. But the RRF of di-MO-PROG which was created by the MO-TMS derivatization was lower than the standard's. It was also attributed to the interference of residual MOX. Furthermore, the low concentration of PROG and

5

di-MO-PROG were also detected. The standard of PROG had a good sensitivity at 60 pg/μL, but the di-MO-PROG was not detected below 200 pg/μL. The lower detection limit of di-MO-PROG could be explained by the influence of the isomers and MOX. It was illuminated the sensitivity of PROG was reduced by the MO derivatization. As a conclusion, the MO derivatization cannot increase the sensitivity of ketosteroid hormones and the followed TMS derivatization provided the main derivatization contributions in the optimized MO-TMS derivatization.
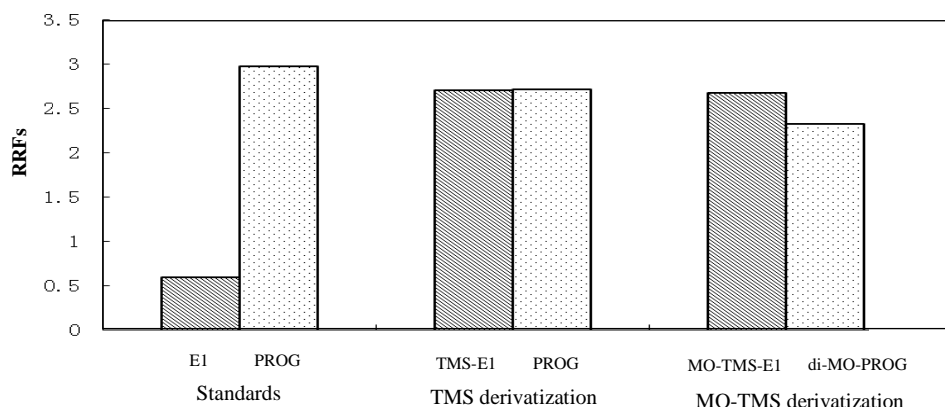

Fig. 5. Comparison the RRFs of standards and their derivatives

Conclusion

In this work, a simplified MO-TMS derivatization for the simultaneous determination of ketosteroid hormones, such as estrone (E1) and progesterone (PROG), by gas chromatography-mass spectrometry (GC-MS) was described. The relatively tedious purification process on the derivatization was abandoned. The followed TMS derivatization with 150 μL *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) were suggested as a necessary procedure to complete TMS derivatization of hydroxyl groups and resolve the problem of GC column damage caused by residual MOX. Hydroxyl and ketone groups in those ketosteroids were successfully stepwise derivatized with 50 μL MOX at 80 °C for 40 min and straightly followed by 150 μL BSTFA at room temperature (around 20 °C) for 10 min. The contribution value of MO derivatization on the MO-TMS derivatization was indirectly evaluated by comparing the optimized MO-TMS derivatization with the single TMS derivatization. It was demonstrated that the MO derivatization cannot increase the sensitivity of ketosteroid hormones and the followed TMS derivatization provided the main derivatization contributions in the optimized MO-TMS derivatization.

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6

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7