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DEACTIVATION OF GAS CHROMATOGRAPHIC SYSTEMS FOR QUANTITATIVE ANALYSIS OF MO-TMS DERIVATIVES OF STEROIDS AT THE PICOGRAM LEVEL

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SUMMARY

Factors affecting the loss of MO-TMS derivatives of steroids in GLC. and GC–MS systems employing open-tubular glass capillary columns were studied. The losses were due primarily to the presence of untreated glass and steel surfaces, and the largest losses were observed with MO derivatives of 3-keto-4-ene steroids. The most effective deactivation was achieved when glass surfaces were treated with dimethyldichlorosilane and metal surfaces (including the jets of the molecule separator) were coated with water glass followed by treatment with benzyltriphenylphosphonium chloride. Following treatments by these procedures, MO-derivatives gave approximately linear responses from about 50–100 pg in the GLC system and from about 100 pg in the GC–MS system. The detection limit for the MO derivative of progesterone in the latter system was at least 50 times lower than in a previous study using packed columns and an untreated two-stage jet separator.

INTRODUCTION

When steroids are analyzed by gas chromatography (GLC) and gas chromatography-mass spectrometry (GC–MS), sensitivity and accuracy are often decreased by losses due to adsorption and decomposition on the column and in other parts of the systems. In the case of packed columns, the surface of support particles is an important site of adsorption [1, 2]. Various materials have been used and several methods have been employed for deactivation of these surfaces (see 3–5). By using silanized diatomite as support and thermostable, nonpolar derivatives, it has been possible to reduce the losses of steroids in the column [1, 6–8]. The introduction of open tubular glass capillary columns has further increased the sensitivity of the analyses. In this case, however, losses in the injection system, molecule separator, detector and connecting tubings may limit the sensitivity. To circumvent the problems of adsorption when only one or a few steroids are determined, particularly favourable derivatives have been selected or excess labelled carrier has been added [9]. Treatment of the column with the compound to be analyzed has also been used. However, these methods cannot be used for analysis of complex and partly unknown steroid mixtures. In this case a widely applicable derivative must be chosen and adsorption kept at a minimum. During the development of methods for the analysis of metabolic profiles of unconjugated steroids in plasma, it was necessary to minimize losses of *O*-methyloxime-trimethylsilyl ether (MO-TMS) derivatives in GLC and GC–MS systems using glass capillary columns [10, 11]. This paper describes a study of factors affecting the yields of steroids and the establishment of conditions suitable for analysis in the picogram range.

The terms pregnanolone and pregnanediol used in this paper indicate general structures and do not imply a specific stereochemistry.

EXPERIMENTAL

Chemicals

All solvents were of reagent grade and were distilled twice before use. Hexamethyldisilazane and trimethylchlorosilane (Applied Science Labs, State College, PA., U.S.A.) were redistilled and trimethylsilylimidazole (Supelco, Bellefonte, PA, U.S.A.) was used as supplied. Methoxyamine hydrochloride (Eastman Organic Chemicals, Rochester, NY, U.S.A.) was recrystallized from methanol. Dimethyldichlorosilane was from E. Merck AG (Darmstadt, Germany) and benzyltriphenylphosphonium chloride (BTPPC) from Merck–Schuchard (München, Germany). Water glass (40%) was obtained from Kebo AB (Stockholm, Sweden). Lipidex®-5000 (Packard, Downers Grove, IL, U.S.A.) was washed prior to use [12].

Steroids

Unlabelled steroids were purchased from Ikapharm (Ramat-Gan, Israel) and kindly donated by Dr. J. Babcock (Upjohn, Kalamazoo, MI, U.S.A.).

Preparation of MO-TMS derivatives

O-methyloximes were prepared as described by Thenot and Horning[13], using 10–20 μg of steroid

to which 5α-cholestane or 5α-pregnane had been added as internal standard. After completion of the reaction, the pyridine was evaporated under a stream of nitrogen, and the residue dissolved in water. The steroids were extracted with ethyl acetate. The solvent was removed, and TMS ethers were prepared with pyridine–hexamethyldisilazane–trimethylchlorosilane, 3:2:1 (by vol.). After 30 min at room temperature, the reagents were removed under a stream of nitrogen. The steroid derivatives were dissolved in hexane. When steroids with 17α-hydroxyl groups were to be derivatized, trimethylsilylimidazole was used for the silylation [13]. Reagents were removed by rapid filtration through Lipidex 5000 in hexane–pyridine–hexamethyldisilazane–dimethoxypropane, 97 : 1 : 2 : 10 (by vol) [11].

Gas–liquid chromatography

GLC was carried out using a Pye 104 gas chromatograph housing an open tubular glass capillary column (25 m × 0.34 mm), prepared according to Rutten and Luyten [14]. The inner wall of the glass capillary tubing was deactivated with BTPPC and then coated with OV-1. Solid injection in a siliconized (see below) all-glass system was employed [15], and the capillary column was connected to the jet of the flame ionization detector (FID) *via* a steel capillary tubing (15 cm × 0.2 mm), deactivated with water glass and BTPPC as described below. No extra gas was added. Glass–glass and glass–metal connections were made with thermo-shrinkable Teflon tubing. Nitrogen was used as carrier gas at an inlet pressure of 0.5 kp/cm^2. The flow rate was about 1 ml/min and the flow velocity 30 cm/sec. The oven temperature was 250° unless otherwise noted. The retention time of 5α-cholestane was about 4 min.

Gas chromatography–mass spectrometry

GC–MS was carried out using a modified LKB 9000 instrument [16]. A 25 m open tubular glass capillary column coated with OV-1 [14] was connected to the ion source *via* a glass capillary resistance and a single stage adjustable jet separator. Glass tubing was used between the separator and the ion source [11, 17]. Glass and metal parts of the separator with which steroids were likely to come into contact were deactivated as described for the GLC system. The temperatures of the oven, separator and ion source were 275°, 240° and 290°, respectively. The energy of the bombarding electrons was 22.5 eV, ionizing current 60 μA and accelerating voltage 3.5 kV. The elution of steroids from the column was monitored by selected ion detection.

Treatment of glass and steel capillary tubings

Pyrex glass capillary tubing (0.2 mm i.d.) was drawn with a Shimadzu glass drawing machine. Seamless 316 stainless steel tubing, 0.2 mm i.d. was purchased from Superior Tube Co. (Norristown, PA, U.S.A.). The tubings were thoroughly washed with ethanol, acetone and hexane before treatment according to one or several of the following procedures.

Glass capillaries (100 cm × 0.3 mm) were treated with 1.0 M or 5.0 M hydrochloric acid. After washing with 30 ml of the acid, the capillary was filled with the acid and left over night at room temperature. It was then washed with 25 ml each of water, ethanol, acetone and hexane and was dried under a slow stream of N_2 for 5 h at room temperature.

Two conventional procedures for silanization were tested (see 3,6). The acid-washed capillary was filled with a mixture of pyridine–hexamethyldisilazane (HMDS)–trimethylchlorosilane (TMCS), 3:2:1 (by vol.) and was left to react over night at room temperature. Excess reagents were removed by suction, and the capillary was placed in an oven for 2 h at 200°. It was then rinsed with toluene and dried with N_2.

Dimethyldichlorosilane–toluene, 5:95 (v/v), was used in an analogous way in the second procedure. Following the toluene wash, the capillary was filled with ethanol and left for 1 h at room temperature. Finally, it was dried by a stream of N_2.

Treatment with BTPPC was carried out essentially as described by Rutten and Luyten [14]. The capillary was filled with a 1% (w/v) solution of BTPPC in dichloromethane and left to react over night at room temperature. It was then rinsed with dichloromethane and dried by a slow stream of N_2.

Stainless steel capillaries (30 cm × 0.2 mm) were treated either with 5 M hydrochloric acid or concentrated nitric acid, using the same procedure as that described for the acid treatment of glass. Treatment with BTPPC was also performed as above. Coating with 5% OV-1 in hexane (w/v) was carried out by filling the capillary with the OV-1 solution (deaerated in vacuum), which was then slowly (3 cm/sec) removed by suction. The hexane was then evaporated by a slow stream of N_2 for 5 h at room temperature.

Coating of untreated steel capillary with 10% aqueous water glass was carried out in an analogous way. However, 2 days were required for removal of the water. The capillary was then mounted in the oven of the gas chromatograph, and the temperature was increased at a rate of 1°/min to 200°, where it was kept for 3 h. Coating with a very thin layer of water glass and use of a long drying time at room temperature were essential for successful results. The coated capillary was finally treated with BTPPC as described above. The jets of the molecule separator in the GC–MS instrument could be treated essentially in the same way. Any excess of water glass was removed with a needle or razor blade and a wet cloth prior to treatment with BTPPC.

Testing procedure for evaluation of adsorption

A procedure analogous to that used for tests of the properties of packed columns was used [1]. Different amounts (30 pg–50 ng) of a mixture of the steroid to be tested and 5α-cholestane or 5α-pregnane were injected on the glass capillary column. The ster-

oid hydrocarbons were used as internal standards since they gave linear responses within the concentration range studied. The yield of a steroid was calculated from the ratio between peak heights or peak areas given by the steroid and the internal standard. The effects of surface treatments were then evaluated by insertion of untreated and treated capillaries between the end of the column and the steel capillary leading to the FID. In the following text the term adsorption is used when loss of steroids is observed. However, it is realized that losses may also be due to chemical transformations.

RESULTS AND DISCUSSION

Relationships between steroid structure and adsorption

In a preliminary survey, the properties of different steroid derivatives were studied. Recoveries of enol TMS and enol heptafluorobutyrate derivatives of 3-keto-4-ene steroids appeared to be better than recoveries of MO derivatives. However, the enol ethers and esters were considered less suitable since several isomers were formed in the derivatization reactions. MO-TMS derivatives have been shown to be widely applicable for multicomponent analysis [18, 19] and methods to improve the yield of these derivatives were therefore studied.

Table 1 shows a comparison of losses of MO-TMS and TMS derivatives and of underivatized steroids in a deactivated GLC system with and without insertion of an untreated piece of steel capillary tubing (30 cm × 0.2 mm). It is evident that the steel surface caused marked losses both of free and derivatized ketosteroids. This is in agreement with previous experience in the use of steel capillary columns for analysis of steroids [20, 21].

As seen in Table 1, the extent of adsorption is determined by the nature and position of the functional groups. The TMS ethers of pregnanolone, pregnanediol and estradiol behaved more like steroid hydrocarbons than the other steroids tested. MO derivatives were markedly adsorbed in the steel capillary but only to a minor extent in the GLC column. In contrast, steroids with underivatized keto groups were lost both in the steel capillary and the GLC system. Another difference between ketosteroids and their MO derivatives was that the peaks of the latter compounds remained symmetrical irrespective of the amounts injected, whereas peaks of ketosteroids showed a tailing that was more pronounced when small amounts were injected. This was accompanied by an increase of the retention time and was particularly marked for 3-ketosteroids. Thus, the losses of ketosteroids and their MO derivatives are probably due to different processes, and it is possible that the losses of MO derivatives are partly due to degradation *via* a Beckmann cleavage reaction as described by Thenot and Horning[22].

With the steroids tested, losses in the steel capillary were larger for 3-MO than for 17-MO or 20-MO derivatives. This may be related to the observation that *O*-methyloximes and unsubstituted oximes have a positive charge in organic solvents, and that the affinity of oximes to a strong cation exchanger is stronger when the substituent is at C-3 than at C-17 or C-20 [12].

Factors influencing loss of MO derivatives on steel surfaces

As shown in Fig. 1 the losses in the steel capillary were relatively constant when larger amounts of steroid derivative were injected. In the case of progesterone *bis-O*-methyloxime the loss was about 1.5–2 ng for amounts larger than 3 ng. This indicates the presence of a limited number of active sites. These are saturated for a very short time since the loss was

Table 1. Percentage loss* of steroids in the deactivated GLC system with and without insertion of an untreated steel capillary, 30 cm × 0.2 mm, between the end of the column and the FID

Steroid†	Derivative	Amount of steroid injected, ng					
		Without steel capillary			With steel capillary		
		10 %	1 %	0.1 %	10 %	1 %	0.1 %
P^4-3,20-one	MO	3	9	17	15	85	100
P^4-3,20-one	None	31	71	100	49	100	100
P^4-20α-ol-3-one	MO-TMS	1	6	5	4	78	100
P^4-20α-ol-3-one	TMS	11	53	68	27	83	100
P^4-17α-ol-3,20-one	MO-TMS	1	2	18	10	75	100
P^4-11β,17α,21-ol-3,20-one	MO-TMS	3	5	7	28	100	100
5αP-3,20-one	MO‡	4	7	8	7	69	100
5αP-3,20-one	None	3	27	51	16	100	100
5αP-3α-ol-20-one	MO-TMS	0	1	2	0	18	53
5αP-3α-ol-20-one	TMS	0	1	10	0	8	31
5αP-3β,20α-ol	TMS	1	1	9	3	20	60
A^5-3β-ol-17-one	MO-TMS	0	1	5	4	55	100
$E^{1,3,5(10)}$-3,17β-ol	TMS	0	0	8	0	8	42

* Zero loss is defined as the peak area ratio obtained with about 50 ng of steroid in the absence of a steel capillary (see Fig. 1). † A = androstane, P = pregnane, E = estrane; superscript indicates position of double bond(s), greek letters denote configuration of hydroxyl groups. ‡ Amounts refer to one of the syn/anti isomers.

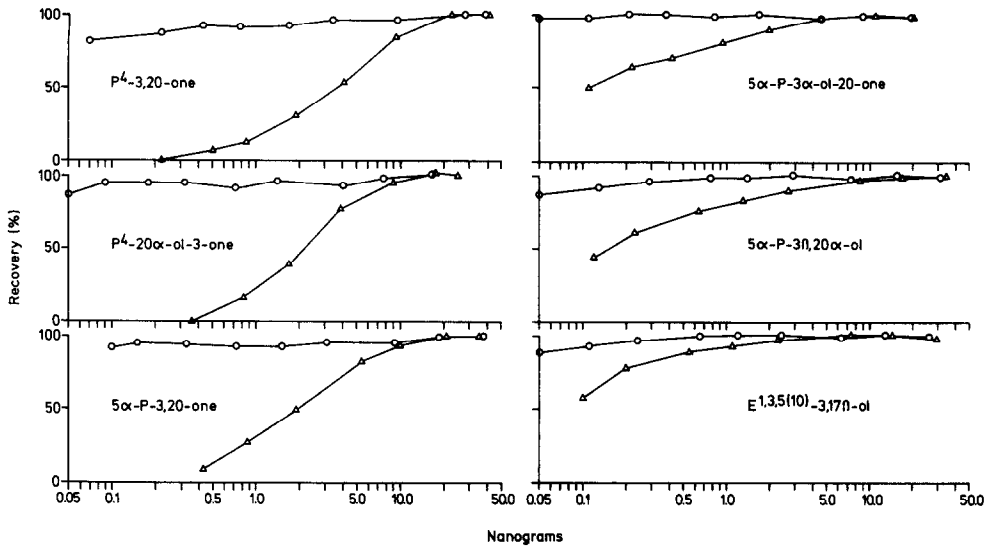

Fig. 1. Recoveries of MO-TMS derivatives of steroids with different sample sizes in the deactivated GLC system, with (△——△) and without (○——○) insertion of an untreated steel capillary, 30 cm × 0.2 mm, between the end of the column and the FID. (For abbreviations and definition of recovery see Table 1).

not influenced by a 200 ng sample injected 2 min earlier. The losses were also found to be proportional to the length of the steel capillary.

When smaller amounts were injected, the absolute loss of steroid decreased. Thus, 0.85 ng and 0.54 ng were lost when 1 and 0.6 ng were injected, respectively. This may be related to the decreasing concentration of steroid derivative in the mobile phase. If this is the case, losses in the molecule separator of a GC–MS instrument might be decreased by addition of extra carrier gas at the end of the GLC column. This method is often used to optimize flow rates when molecule separators for packed columns are used in the coupling of capillary columns to the mass spectrometer [23].

Attempts were made to influence the loss of steroid derivative by changes of temperature, carrier gas and flow rates. Temperature variations between 230° and 270° and use of He instead of N_2 did not influence the recoveries. Three different inlet pressures (0.25, 0.5 and 1.0 kp × cm^{-2}) were tested, giving linear velocities through the steel capillary of about 30, 60 and 120 cm × s^{-1}. When 5 ng progesterone *bis-O*-methyloxime were injected the recoveries were 52, 62 and 70% under the respective conditions. This shows that the time of exposure of the steroid to the active surface is of importance, but the possibility of increasing carrier gas velocity is obviously limited.

Table 2. Percentage loss of 1 ng of progesterone *bis-O*-methyloxime in glass and steel capillaries treated by different procedures and inserted into the deactivated GLC system

Surface treatment*	Percentage loss in Glass†	Steel‡
Wash with solvents	30	85
1 M hydrochloric acid	51	—
5 M hydrochloric acid	76	100
1 M hydrochloric acid / Pyridine–HMDS–TMCS, 3:2:1	29	—
1 M hydrochloric acid / Dimethyldichlorosilane	7	—
5 M hydrochloric acid / Dimethyldichlorosilane	3	—
BTPPC	17	59
OV-1 dynamic coating	—	69
Conc. nitric acid		62
Conc. nitric acid + BTPPC		55
Water glass		40
Water glass + BTPPC	—	7

* See Experimental. † 100 cm × 0.3 mm, about 2 h after insertion. ‡ 30 cm × 0.2 mm, about 24 h after insertion.

Effect of surface treatments on losses of MO derivatives

Preinjections of 0.5 μl of trimethylsilylimidazole or pyridine did not affect the loss of progesterone *bis-O*-methyloxime in steel capillaries. Pieces of glass and steel capillaries were therefore treated in different ways prior to insertion into the GLC system, and the effect of the treatments on the loss observed for 1 ng of the progesterone derivative was studied. The results are summarized in Table 2. As expected, untreated and acid-washed glass gave considerable losses and the yields were improved by treatment with BTPPC [14] or silanization [1, 6, 7]. On the basis of previous studies [6, 7] and the results shown in Table 2, silanization with dimethyldichlorosilane was selected as the method for deactivation of glass surfaces (e.g. the injection system).

The studies of loss of steroids on steel surfaces described in the preceeding section were made with steel capillaries that had been inserted in the GLC system

for 24 h (Table 1, Fig. 1). When losses were measured 1 h after insertion of a new piece of steel capillary, more than 20 ng of progesterone *bis-O*-methyloxime was adsorbed. This indicates a time-dependent change, possibly an oxidation of the steel surface. This change was not reversed by washing with solvents, showing that it was not due to coating by the bleed from the column. Although a coating could be achieved when the steel capillary was kept at a temperature lower than that of the column, this coating was readily removed by heating.

Treatment of the steel with nitric acid, BTPPC or water glass decreased the adsorption of the progesterone derivative. However, none of these procedures were satisfactory (Table 2). The surface of water glass could be treated further and good results were obtained when the steel capillary was first coated with water glass and then treated with BTPPC. Therefore the steel capillary connecting the column to the FID was treated in this way. As shown in Fig. 1 this results in approximately linear responses for MO-TMS derivatives of the steroids tested down to the detection limit (30–100 pg). The stability of the water glass treatment was satisfactory, since continuous use for more than 6 months did not result in increased adsorption.

The procedures described above were used for deactivation of the glass parts and separator jets of the GC–MS system. The recovery of the MO derivative of progesterone was studied in the same way as for the GLC system, using single ion detection of the molecular ions of this derivative and cholestane (*m/e* 372). The recovery was about 80% at the 100 pg level (Fig. 2). The relative loss increased with smaller amounts, but it was still possible to detect 15 pg (Fig. 2). This may be compared with results obtained using packed columns and an untreated two-stage jet separator when amounts smaller than 500 pg could not be detected [16].

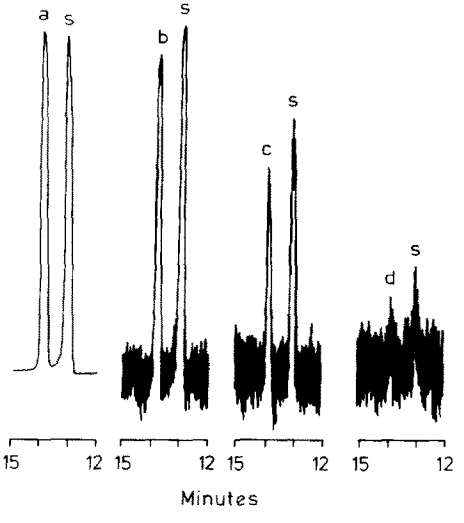

Fig. 2. Molecular ion (*m/e* 372) current chromatograms, obtained in four analyses of different amounts of a sample containing progesterone *bis-O*-methyloxime and 5α-cholestane (s). The peaks of the progesterone derivative, a, b, c and d, represent injected amounts of 130 ng, 500 pg, 100 pg and 15 pg, respectively.

The reason for the remaining nonlinearity of the GC–MS system has not been established. However, untreated steel surfaces are still present with which the steroids may come into contact if the gas flow is turbulent. Thus, the recoveries were highest when the entrance to the first separator jet had a conical shape. Some adsorption may also occur at the untreated wall of the ion source.

The silanization and treatment with BTPPC of parts in the GC–MS system appeared to be less stable than the corresponding treatments of the GLC system. This might be due to the high vacuum and temperatures. The treatments were effective for a few months and then had to be repeated. However, the water glass coating of the separator jets appeared to be stable.

The results of the present study indicate that the nonlinear response of MO-TMS derivatives of steroids in GLC and GC–MS systems is primarily due to the presence of untreated steel and glass surfaces. For satisfactory analyses of steroids in the picogram range such surfaces should be kept at a minimum and be deactivated. Glass capillary columns treated with BTPPC and coated with OV-1 according to Rutten and Luyten apparently give minimal losses of MO-TMS derivatives of steroids. For maximum recovery of steroids, carrier gas flow should be high and the ratio of steroid to carrier gas should be kept low. However, selection of these conditions will probably have to be governed by the nature of the sample and the need for optimum column efficiency.

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