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NEW METHOD FOR THE PREPARATION OF HIGHLY STABLE POLYSILOXANE-COATED GLASS OPEN-TUBULAR CAPILLARY COLUMNS AND APPLICATION TO THE ANALYSIS OF HORMONAL STEROIDS

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SUMMARY

A new approach to the preparation of methylsiloxane-coated glass open-tubular capillary columns is described. A siloxane polymeric mixture is first prepared by hydrolysis of dimethyldichlorosilane. The polymer is then coated on to the previously etched glass wall by a base-catalyzed reaction to yield a non-polar system of high stability and showing satisfactory chromatographic properties. This type of column has been used for the high-resolution separations of complex hormonal steroid mixtures. The flexibility of this method should permit the preparation of capillary systems with a wide range of polarity.

INTRODUCTION

During the last 10 years, considerable efforts have been devoted to the development of glass open-tubular columns suitable for high-resolution separations in biochemical analysis[1]. However, the lack of stability at high temperature of wall-coated open-tubular (WCOT) columns was an early recognized problem attributed to the tendency of the liquid film to break up into droplets[2]. Two main approaches have been followed in order to improve the spreading and stability of the stationary phase film. The first solution aims to achieve greater wettability of the column wall by chemical modification of the glass surface[3] or by adding a surfactant to the coated phase[4]. Using either dynamic or static coating procedures, this approach has been successful for the separation of hormonal steroid metabolites[5–7]. The second approach consists in retaining the stationary phase by coating it together with a finely powdered support material[8]. Both non-polar and polar columns obtained by following this principle have been successfully applied to the separation of biochemical mixtures, including steroids[9–11].

In this work, we followed another approach for the coating of the capillary glass wall, using a siloxane polymer obtained by prior hydrolysis of dimethyldichlorosilane (DMCS). The polymeric product was then made to react *in situ* under base-

catalyzed conditions to yield a coating with a high stability probably due to chemical bonding to the glass surface.

The capillary columns prepared by this procedure were evaluated for their chromatographic properties, especially with regard to their application in hormonal steroid analysis.

EXPERIMENTAL

Glass capillary tubes

Borosilicate glass tubing (Pyrex, Sovirel, Paris, France) of 6 mm O.D. and 4 mm I.D. was used after rinsing it successively with water, methanol and acetone. A coiled capillary tube (13 cm in diameter) was obtained using a Sedere (Paris, France) drawing machine, yielding a capillary of 0.20–0.25 mm I.D. The capillary tube was filled with gaseous hydrogen chloride according to the method of Alexander and Rutten[5], sealed at both ends and heated at 350° for 24 h. After this etching step, the tube was flushed with a stream of nitrogen and was then ready for coating.

Siloxane polymer

To an ice-chilled flask containing 40 ml of DMCS (Pierce, Rockford, Ill., U.S.A.) 60 ml of 12.5% ammonia solution were added dropwise with continuous agitation. The oily product that formed during the reaction was left overnight in the aqueous medium. The polymeric product was then thoroughly washed with distilled water until neutral and the remaining water was removed by centrifugation. The final clear fluid obtained was dissolved in methylene chloride (20%, v/v).

Coating of the column

A plug (about one quarter of the column length) of the polymer solution was pushed through the capillary tube under a slight nitrogen pressure (5–10 cm/sec). This step was carried out very easily owing to the fluidity of the polymeric preparation. The column was then dried with a nitrogen stream for 2 h and filled with ammonia generated in a connected flask by adding 25% ammonia solution to solid sodium hydroxide. After sealing at both ends, the column was heated in an oven at 320° for at least 24 h. Thereafter, the coated column was installed in the gas chromatograph and conditioned by programming the temperature from 150° to 200° at 2°/min. The column was then ready for use.

Gas chromatography

Gas chromatography was carried out with Carlo Erba Model 2400 T and 2200 instruments with a flame-ionization detector and hydrogen as the carrier gas at a flow-rate of 0.8–1.5 ml/min. The instruments were equipped with an all-glass solid injector system[12] and a temperature programmer accessory.

Infrared spectra

IR spectra were recorded with a Beckman IR-8 instrument, in methylene chloride as the solvent, at a 10% (v/v) concentration for the siloxane polymer and a 1% (v/v) concentration for the commercially available OV-1 phase (Supelco, Bellefonte, Pa., U.S.A.).

Biological samples

Steroids were purchased from Mann Labs. (New York, N.Y., U.S.A.). Methyloxime trimethylsilyl ethers (MO-TMS) were prepared according to Gardiner and Horning[13] and benzyloxime trimethylsilyl ethers (BO-TMS) as described by Devaux *et al.*[14]. Extraction and treatment of urinary steroid metabolites from human adult subjects or newborn infants were carried out as previously described[15,16].

RESULTS AND DISCUSSION

Chromatographic properties of the methylsiloxane-coated columns

Some fundamental chromatographic parameters were evaluated using straight-chain hydrocarbons and steroidal structures of biological interest as MO-TMS derivatives. The values obtained for different columns are listed in Table I. These parameters compared favourably with the data published for both WCOT and support-coated capillary systems[17], particularly with regard to their efficiency as reflected by the number of theoretical plates per metre. Although the capacity factors for the alkanes appeared satisfactory, further studies are needed in order to evaluate more precisely the amount of coated phase (*e.g.*, phase ratio) for the sake of comparison with established glass capillary systems[17].

TABLE I

CHARACTERISTICS OF METHYLSILOXANE-COATED OPEN-TUBULAR CAPILLARY COLUMNS

Property	*Column No.*		
	1	*2*	*3*
Length (m)	20	27	29
Internal diameter (mm)	0.20	0.20	0.20
Temperature (°C)	220	220	220
Capacity factor for:			
n-C_{24}	5.61	4.50	6.92
Androsterone	7.3	7.08	—
Etiocholanolone	7.7	7.46	—
Linear gas velocity (cm/sec)	23.8	23.48	24
Separation number (TZ), *n*-C_{24-26}	27.1	33.3	34.4
Plate height (mm)	0.41	0.28	0.31
Theoretical plates per metre for:			
n-C_{24}	2425	3524	3230
Androsterone	1620	3340	—
Etiocholanolone	1680	2712	—
Resolution: androsterone–etiocholanolone	6.46	13.09	—

The straightforward and easy procedure of preparation of these wall-coated columns yielded reproducible chromatographic characteristics and no failure due to practical difficulties such as clogging as in the preparation of support-coated material was encountered[17].

Nature of the coating

Various conditions have been described for obtaining a polymeric structure by

hydrolysis of DMCS[18]. Under our basic conditions (excess of ammonia solution) and in the absence of end-blocker[19], the reaction could be expected to yield both cyclic moieties of the type $(O\text{–}Si(CH_3)_2\text{–}O)_n$ (n being mostly 5–10) and linear chains bearing free terminal hydroxyl groups[18,19]. Infrared spectra of the prepared polymeric mixture and of a commercially available OV-1 phase were recorded and compared (Fig. 1). Similar general spectral features were obtained but an absorption band at 3600 cm^{-1} indicated that the siloxane mixture contained free hydroxyl groups whereas, as expected, the OV-1 did not.

After spreading of the siloxane polymeric mixture on to the column wall, the second step of the procedure involved heating of the preparation at 320° under alkaline conditions (gaseous ammonia generated from an aqueous medium). Under these conditions, one might expect rearrangements of the cyclic siloxane structures as well as hydrolysis of straight chains[18,19]. Under heating one might suppose that covalent bonding would occur between siloxane moieties and the glass surface. The

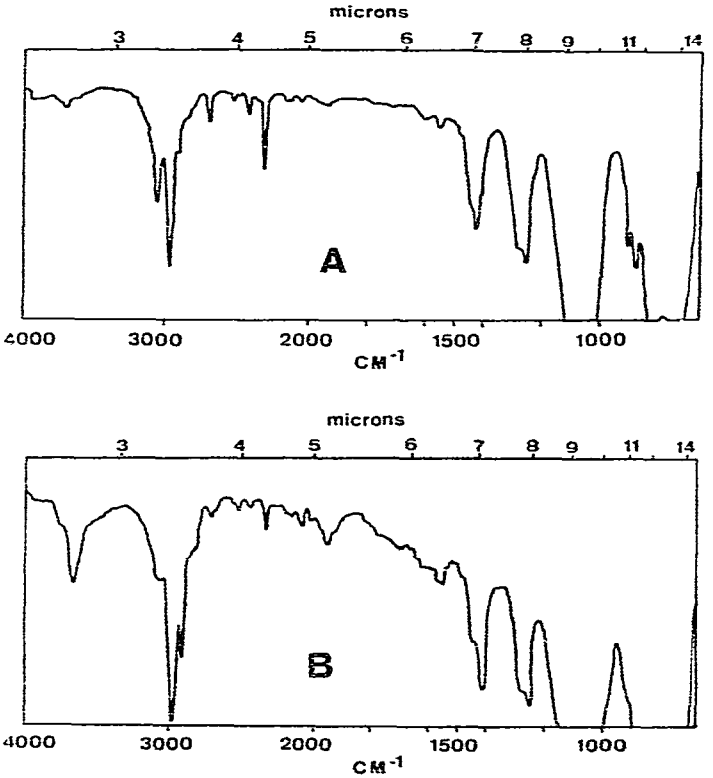

Fig. 1. Comparative infrared spectra (800–4000 cm^{-1}) of (A) commercially available methylpolysiloxane OV-101 in methylene chloride and (B) siloxane polymer, obtained by hydrolysis of DMCS, in methylene chloride.

latter had previously been etched and should present a large number of accessible free hydroxyl groups. Silica-bound siloxanes have been obtained under similar conditions by several research groups and attributed to dehydration reactions[20–22].

To check the extent of chemical bonding of the siloxane phase to the glass surface, a column was extracted overnight with dichloromethane. After elimination of the solvent, unchanged chromatographic properties were found for this extracted column. However, this result may not provide definite proof of chemical bonding as very stable ("non-extractable") association of a commercial polysiloxane phase with diatomaceous support has been reported[21]. In this instance, the association was explained by physical interactions favoured by a heating step at 350°[21]. However, under our conditions, chemical bonding with the participation of end-chain functional groups was further suggested by the fact that the presence of ammonia in the coating step was essential for satisfactory results. In addition, preliminary results showed that satisfactory capillary coating could be achieved by following the same procedure using phenyl-substituted silanes instead of DMCS as the starting monomer. This result suggested that chemical bonding is a major common mechanism leading to a stable phase film.

Separation of hormonal steroid metabolites

The retention parameters of several major human steroid metabolites as MO-TMS derivatives were determined on two different methylsiloxane-coated capillary columns. The values obtained could be compared with those given by a conventional 1% OV-1 packed column (Table II). The figures were mostly identical in all instances, showing that the capillary coating had very similar chromatographic properties compared with the widely used methylpolysiloxane OV-1, at least in the temperature range employed (190–280°).

TABLE II

RETENTION PARAMETERS OF STEROID METABOLITES AS MO-TMS (METHYLENE UNITS)

Compound	*Siloxane-coated capillary*		*1% OV-1 packed column (4 m)*
	27 m	*24 m*	
Androsterone	25.01	25.00	25.01
Etiocholanolone	25.20	25.21	25.22
Dehydroepiandrosterone	25.63	25.61	25.63
11-Ketoandrosterone	26.03	26.00	26.00
11-Ketoetiocholanolone	26.14	26.13	26.10
11-Hydroxyandrosterone	26.97	26.98	26.93
11-Hydroxyetiocholanolone	27.14	27.15	27.12
Pregnanediol	27.57	27.56	27.58
Pregnanetriol	27.89	27.89	28.00
Tetrahydrodesoxycortisol	28.63	28.71	28.61
Tetrahydrocortisone	29.60	29.69	29.60
Tetrahydrocortisol	30.21	30.29	30.23
allo-Tetrahydrocortisol	30.34	30.42	30.36
Cortolone	30.45	30.51	30.50
β-Cortolone	30.74	30.83	30.79

The separation of a model mixture of steroid metabolites as MO-TMS derivatives is shown in Fig. 2. The resolution was such as to yield a complete separation between metabolites that are usually difficult to resolve, *e.g.*, isomeric 11-oxoandrosterone and 11-oxoetiocholanolone. The very low bleeding level permitted the use of routine operating conditions using injection of 5 ng for each compound with the flame-ionization detector. In addition, very good response coefficients with regard to the internal standard were obtained, especially for the main corticosteroid metabolites. This indicated a limited adsorption level and satisfactory quantitative properties at the nanogram level.

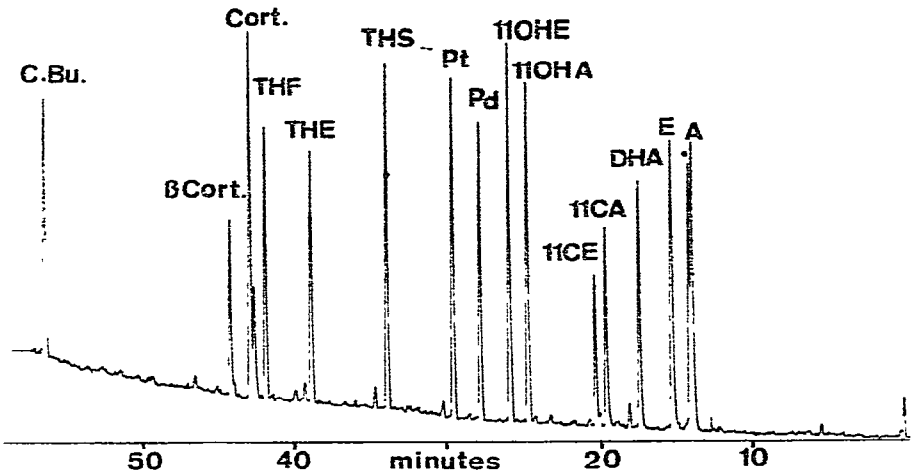

Fig. 2. GLC separation on a methylsiloxane-coated open-tubular column of a steroid mixture as MO-TMS derivatives. Temperature programming from 170° to 280° at 2°/min; 5 ng of each compound were injected. Peaks: A = androsterone; E = etiocholanolone; DHA = dehydroepiandrosterone; 11CA = 11-ketoandrosterone; 11CE = 11-ketoetiocholanolone; 11OHA = 11β-hydroxyandrosterone; 11OHE = 11β-hydroxyetiocholanolone; Pd = pregnanediol; Pt = pregnanetriol; THS = tetrahydro-11-deoxycortisol; THE = tetrahydrocortisone; THF = tetrahydrocortisol; Cort. = cortolone-20α; βCort. = cortolone-20β; C.Bu = cholesteryl butyrate (internal standard).

The chromatogram given by the neutral urinary steroid extract from a 2-year-old patient with a congenital adrenal cortex hyperplasia (21-hydroxylase defect) is given in Fig. 3. The isomeric pregnanetriols of clinical interest in such a case were completely resolved, as well as the related ketonic metabolites.

Fig. 4 illustrates the result obtained after administration of metyrapone in a normal adult subject. In such cases, conventional packed columns would give erroneously high values for the major metabolite of cortisone (THE), falsely suggesting a lack of effect of the drug on the adrenal hydroxylating capacity. The high resolution attained by the capillary column permitted the corresponding peak to be resolved into two components.

Fig. 5 shows the analysis of the urinary steroid metabolites extracted as sulpho-conjugates from a pool of 3-day-old normal human newborn urines. The use of BO-TMS derivatives is of great interest in this case for structural studies of unknown newborn steroid metabolites[14,23]. However, these bulky derivatives greatly increase

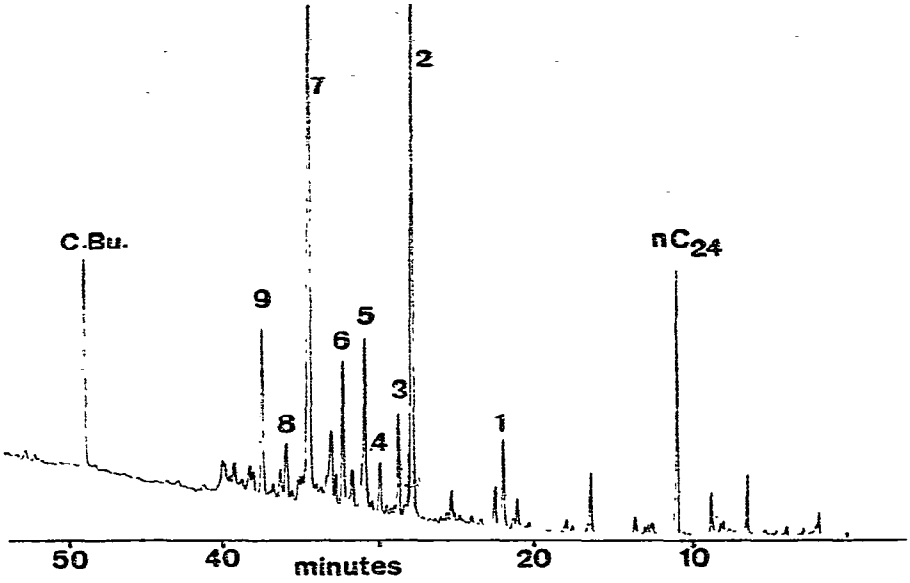

Fig. 3. GLC analysis of the neutral urinary steroid metabolites (MO-TMS) in a case of 21-hydroxylase defect. Analytical conditions as in Fig. 2. Peaks: 1 = androsterone; 2 = pregnanediolone; 3 = pregnanediolone; 4 = pregnanetriol; 5 = pregnanetriol; 6 = pregnanediolone; 7 = pregnanediol-dione; 8, 9 = unknown metabolites; C.Bu = cholesteryl butyrate (internal standard).

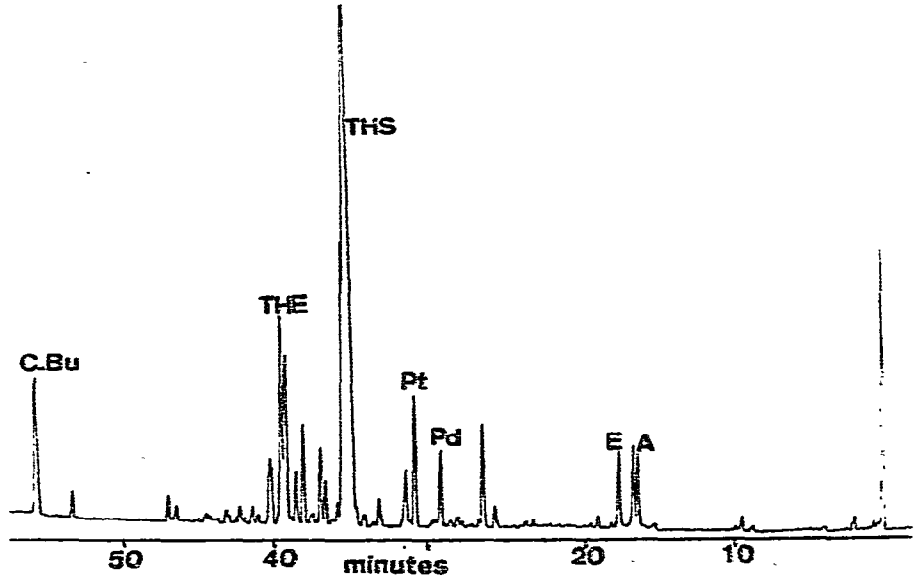

Fig. 4. Neutral urinary steroid extract (MO-TMS) from a normal subject after administration of metyrapone. Temperature programmed from 168° to 260° at 2°C/min. Abbreviations as in Fig. 2.

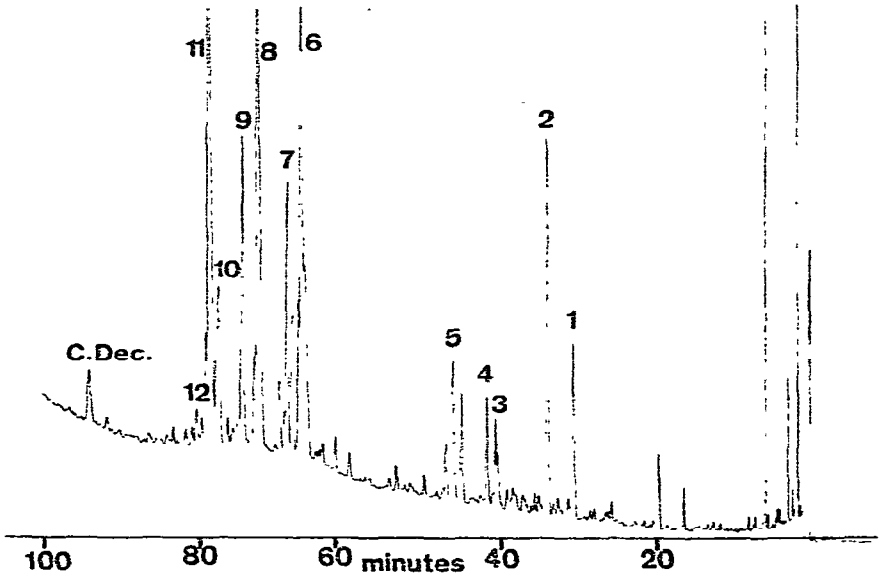

Fig. 5. Methylsiloxane-coated capillary column separation of the steroids excreted as sulphates in a urine pool from 3-day-old human newborns. BO-TMS derivatives; temperature programming from 190° to 310° at 2°/min. Peaks: 1 = androstenetriol; 2 = androst-5-ene-3β,16α,17β-triol; 3 = androstenetetrol; 4 = androstenetetrol; 5 = pregnanetriol; 6 = 3β,16α-dihydroxyandrost-5-ene-17-one; 7 = androstenetriolone; 8 = 3β,17β-dihydroxyandrost-5-ene-16-one + pregnanediolone; 9 = pregnenetriolone; 10 = androstenetriolone; 11 = 3β,16α-dihydroxypregn-5-ene-20-one; 12 = 3β,21-dihydroxypregn-5-ene-20-one; C.Dec. = cholesteryl decylate (internal standard).

the retention values and elution temperatures. The siloxane-coated capillary system appeared to remain very satisfactory both concerning its chromatographic properties and its stability under repeated routine temperature programming conditions up to 310°.

CONCLUSION

Although further studies are needed in order to characterize fully the molecular nature of the glass-coated siloxane material, our proposed procedure provides a new approach to the preparation of wall-coated glass open-tubular capillary columns. In addition to the simplicity of the procedure, which appeared devoid of practical pitfalls, preliminary evidence suggests that it may also be employed for the preparation of polar coatings. These columns have proved satisfactory for hormonal steroid analysis; their potential use for the separation of other types of biological mixtures, including prostaglandins, is currently being investigated.

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