TABLE III

Relative Rates of Esterification of Acids in Excess Methanol

Conditions: H_2SO_4 catalyst, temperature
64.7 ± 0.2 C (reflux) (18,19)

Acid	Rate relative to stearic acid
Stearic	1
Isostearic	0.95
C_{13} acids	0.35
C_{26} acids	0.05

close to that expected for a C_{26} secondary acid from the caustic oxidation of the C_{26} aldol dimer alcohol. IR and PMR spectra of this fraction indicated only traces of ether functionality; it seems that ether alcohols, in contrast to simple ethers, must be either cleaved to acids and olefins or oxidized to ether acids which decarboxylate.

The total yield of distillable acids from the caustic oxidation of C_{13} oxo bottoms is thus about 65% (the distillation residue amounted to 11%). Depending upon the particular end-use contemplated, any one or combination of the three acid cuts can be used. All have colors of Gardner 1 or less and the C_{13} acid cut has a titer of approximately -50 C.

Esterification of Acid Products

Since the rate of formation of derivatives such as esters or amides is strongly dependent upon the structure of the acids, it was desirable to have a measure of the reactivities of the C_{13} acids and high boiling acids relative to straight-chain fatty acids. Table III shows that the C_{13} and high boiling acid products are considerably more hindered about the carboxyl group than stearic and isostearic acids.

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REFERENCES

1. Kinsman, D.V., JAOCS 56:823A (1979).
2. Benoit, G.F., Jr., U.S.Patent 3,110,673, 1963.
3. Falbe, J., "Carbon Monoxide in Organic Synthesis," Springer-Verlag, New York, 1970, p. 71.
4. Glauz, R.L., Chemical Economics Handbook, 609.5022, June 1977.
5. Bartlett, J.H., I. Kirshenbaum and C.W. Muessig, Ind. and Eng. Chem. 51:257 (1959).
6. Bartlett, J.H., A.J. Morway, L.A. Mikeska and S.G. Galto, U.S. Patent 2,801,971, 1957.
7. Mertzweiller, J.K., U.S. Patent 2,802,846, 1957.
8. Rehn, K. and G. Theilig, Ger. Prov. Patent 1,061,770, 1959.
9. Hill, P., U.S. Patent 2,766,267, 1956.
10. Diamond, H.L., and A.C. Whitaker, U.S. Patent 3,370,074, 1968.
11. Weedon, B.C.L., Techniques of Chemistry, 4:325 (1973).
12. Ashworth, P.J., U.S. Patent 3,227,737, 1966.
13. Ansell, M.F., I.S. Shepherd and B.C.L. Weedon, J. Chem. Soc. C:1840 (1971).
14. Bartlett, J.H., A.J. Morway and J.C. Munday, U.S. Patent 2,801,970, 1957.
15. Morway, A.J., and J.H. Bartlett, U.S. Patent 2,801,969, 1957.
16. Bartlett, J.H., A.J. Morway and L.A. Mikeska, U.S. 2,801,972, 1957.
17. Private communication from J. Reich and C. Savini of Exxon Research and Engineering Corp.
18. Smith, H.A., J. Am. Chem. Soc. 61:254 (1939).
19. Loening, K.L., Ibid. 74:3929 (1952).

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High Resolution Glass Capillary Columns with Chemically Bonded Stationary Phases: Application to the Gas Chromatographic Analysis of Sterols and Steroids in Biological Extracts

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ABSTRACT

A new approach has been developed for the preparation of highly stable glass capillary columns using irreversible bonding of polysiloxane polymers to the glass surface. The general procedure involves (a) synthesis of reactive linear polysiloxanes from variously (alkyl, aryl) substituted dichlorosilanes through homologous or heterologous polymerization under alcaline aqueous conditions, and (b) covalent condensation of the polymers to a properly prepared glass capillary surface at high temperature. The principle has been first applied to the successful preparation of apolar gas chromatographic systems (methylpolysiloxanes), then extended to the obtention of polar systems (methyl phenyl polysiloxanes). The flexibility of the approach is demonstrated by the possibility of obtaining stationary phases of various and controlled polarity (i.e., extent of phenyl substitution), tailor-made to a given analytical problem. These gas chromatographic systems appear to be remarkably stable both with time and temperature up to 300 C and compare favorably to existing systems for their high resolution properties. These columns have been satisfactorily used in the last years for the analysis of sterols and steroids of biological origin and most extensively in the study of urinary steroid metabolite in humans under pathological conditions.

INTRODUCTION

In the last ten years, glass capillary gas chromatography has been widely accepted as one of the most powerful analytical tools in biochemical separations. However, manufacture of long-lived, thermostable high resolution systems remains a challenging problem, especially if polar stationary phases are needed. The major recognized problem is the wettability of the glass surface which limits the production of a regular and stable coating. Commercially available polymers have generally been used as stationary phases and

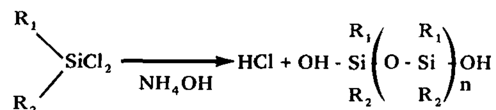
most of the efforts in the field have been to increase the specific coating surface of the glass wall, either by physicochemical treatment such as etching (1,2), dehydration (3) whisker growth (4) or by stabilization of the phase as a colloidal coating suspension with microparticles (5-11).

We have undertaken a different approach with the aim of obtaining a covalent bonding between the stationary phase and the capillary glass wall, with the hope that this principle would yield particularly stable chromatographic systems (12). This approach implies the preparation of a reactive polymer which can then be chemically bonded to a conveniently prepared glass surface (12,13). The principle has been shown to be workable using apolar polymers (i.e., methylpolysiloxane) leading to apolar stationary phases (13,14). In this paper, we will review the basic methodology that we have developed to obtain such systems and we will show that it can be adapted to the preparation of chromatographic systems of a wide polarity range. These systems have been found well suited to routine biochemical analysis, particularly in the field of sterols and steroids of biological interest. One of the main advantages of the method is that the stationary phase polarity can be controlled in order to be adapted to a given analytical problem.

EXPERIMENTAL PROCEDURES

General Principle

(a) Pyrex glass capillary is treated with hydrochloric acid with the aim of making surface hydroxyl groups accessible. (b) Reactive polymers are prepared by hydrolysis of substituted dichlorosilane under conditions yielding linear polysiloxanes ended by free hydroxyl groups (15). In alkaline aqueous medium, the reaction will be (16):



According to the substitution of the starting dichlorosilane (R_1 and R_2), two kinds of polymerization may be obtained: homologous polymerizations, from a single type of monomer, where R_1 and R_2 can be identical or not, and heterologous polymerizations, from a mixture in various proportion of two silanes with differents substituents. This approach opens the possibility to synthesize polymers of controlled polarity. (c) The polymer is covalently bound to the glass surface under conditions favoring the condensation of hydroxyl groups by dehydration.

Materials and Methods

Dimethyldichlorosilane (DMCS), diethyldichlorosilane (DECS), diphenyl dichlorosilane (DPCS) were from Silar Laboratories (USA). Commercially available polysiloxanes were purchased from Applied Science (USA). All other reagents were from Merck, Sigma or Prolabo (France).

Preparation of siloxane polymers. The previously described procedure (12) for preparation of siloxane polymers by hydrolysis of substituted chlorosilanes was used. Homologous or heterologous chlorosilanes in the desired proportion, usually for a total volume of 20 cm^3, were mixed with constant stirring. To this mixture was added dropwise about 60 cm^3 7 mol dm^{-3} NH_3 at room temperature. Disappearance of acidic gas emission indicated the end of the reaction. A polymer of variable viscosity (depending upon the starting proportion of dichlorosilanes) resulted and was decanted and washed with distilled water until neutral. Each partition step was completed by a brief centrifugation. Handling of highly viscous preparations was facilitated by dilution with dichloromethane. The polymer was finally collected after a 20,000 g centrifugation for 15 min and stored in dark bottles at 5-10 C.

Glass capillary preparation. Pyrex (Schott, Mainz, Germany) glass tubes (6 mm od, 2 mm id) were introduced in a glass drawing machine (Sedere, Paris, France) to yield coiled capillary tubes of 0.20-0.25 mm id. This diameter was evaluated on several pieces of each capillary with a microscope equipped with a micrometer. The glass capillary (20 to 25 m long) was then filled with gaseous HCl according to Alexander (1), sealed at both ends and heated in an oven at 380 C for 2 hr.

Coating and irreversible bonding of the polymer to the capillary. After flushing the capillary with a nitrogen stream, the polymer solution (10% in dichloromethane) was introduced as a plug (about 1/4 of the column length) and pushed through the tube under a slight nitrogen pressure. As the polymer plug emerged, the nitrogen flow was increased and maintained for 18 hr. The dry column was then filled with gaseous ammonia (12-14), sealed and introduced into a gas chromatography oven at 70 C. The temperature was raised at 0.4 C/min up to 200 C and then maintained for 24 hr.

After flushing with a nitrogen stream, the column was placed in the gas chromatograph and conditioned by programming the temperature from 100 to 320 C at 2 C/min. After 24 hr at 320 C, the column was ready to use.

Infrared spectra were recorded using a Perkin-Elmer model 521 spectrometer; the polymer was disposed between two NaCl pellets sufficient to yield a light transmission of 10-60%.

Gas chromatographic procedures using a dry injection device and all ancillary techniques including steroid derivative preparation were as previously described (17).

TABLE I

Chromatographic Parameters of Two Glass Capillary Columns with Irreversibly Bonded Methyl Polysiloxane (MPS) Stationary Phase (220 C)

	Column	
	1	2
Length (m)	27	29
Internal diameter (mm)	0.20	0.20
Capacity factor		
for tetracosane	5.61	6.92
for androsterone (MO-TMS)	7.3	7.08
Gas linear velocity (cm/s)	23.4	24
Separation number TZ (tetracosane-hexacosane)	33.3	34.4
No theoretical plates/m for tetracosane	3524	3230

RESULTS AND DISCUSSION

Chemically Bonded Apolar Stationary Phase

A methylpolysiloxane (MPS) was the first polymer obtained after hydrolysis of dimethyldichlorosilane (DMCS) and successfully used to prepare apolar chemically bonded glass capillary columns following the aforementioned method (12,13). Typical chromatographic parameters observed with such capillary systems are illustrated in Table I. Study of the retention indices of a number of steroid metabolites showed that the resulting stationary phases behave quite similarly to commercially available methylpolysiloxanes such as OV 101, SE 30 and OV 1. Figure 1 gives an example of the separations obtained for a mixture of the major human urinary steroid metabolites as methyl oxime-trimethylsilylether derivatives.

The stability of the glass-polymer bonding was examined by determination of the chromatographic properties of these columns before and after extensive treatment with dichloromethane for 18 hr. Since no significant changes in chromatographic performance could be found, it was concluded that the stationary phase was most probably unextractable because it was covalently bonded to the glass surface. This was confirmed by the fact that no detectable change in the silicium content (which is directly related to the amount of coated polysiloxane phase) was detected after dichloromethane extraction (13).

Chemically Bonded Phases of Varying Polarity

Obtained after homologous polymerization. Polysiloxanes were obtained after hydrolysis of a single substituted dichlorosilane with various alkyl or aryl substituents. Diethyldichlorosilane yielded ethylpolysiloxane which was successfully used to prepare largely apolar chromatographic systems which exhibit some selectivity in their sensitivity to steric factors as compared to the aforementioned apolar methylpolysiloxane. This is shown in Table II in the case of several hydroxylated steroids as methyloxime trimethyl silylethers.

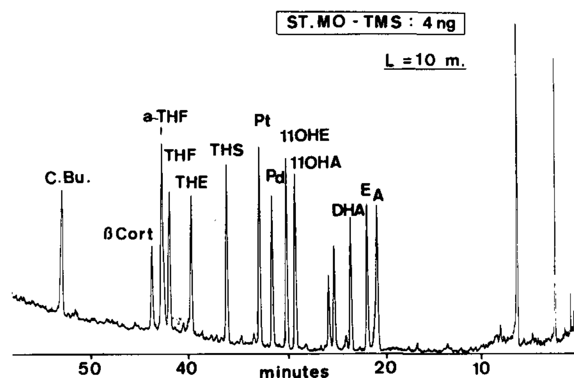


FIG. 1. Separation of a model mixture of urinary steroid metabolites (4 ng each) as methyl oxime-trimethylsilylethers, on a 10-m methyl polysiloxane capillary column. A, androsterone; E, etiocholanolone; 11 OH E, 11β hydroxy etiocholanolone; 11 OH A, 11β-hydroxy androsterone; Pd, pregnanediol; Pt, pregnanetriol; THS, tetrahydrodeoxycortisol; THE, tetrahydrocortisone; THF, tetrahydrocortisol; α THF, allotetrahydrocortisol; β Cort, β cortolone; C.Bu., internal standard (cholesterol butyrate). Temperature programming from 180 to 300 C (2 C/min).

A moderately polar phase was obtained after hydrolysis of methyl phenyl dichlorosilane. The theoretical 50% phenyl substitution in the monomer was, as expected, found in the resulting methyl phenyl polysiloxane (MPPS) as confirmed by infrared (IR) spectroscopy study. As shown in Table II, this MPPS capillary column exhibits

TABLE II

Retention Indices (Methylene Units) of Several Steroid Metabolites (As Methyloxime-Trimethylsilylderivatives) on Glass Capillary Columns with Methyl Polysiloxane (MPS), Ethyl Polysiloxane (EPS) and Methyl-Phenyl (50% Phenyl) Polysiloxane Irreversibly Bonded Stationary Phases

	MPS	EPS	MPPS
Androsterone	2501	2556	2792
Etiocholanolone	2520	2554	2806
Dehydroepiandrosterone	2563	2623	2908
11-Keto-androsterone	2603	2656	2983
11-Keto-etiocholanolone	2614	2643	2976
11-OH androsterone	2697	2715	2816
11-OH etiocholanolone	2714	2710	2928
Pregnanediol	2757	2778	2887
Pregnanetriol	2789	2783	2860
Tetrahydrodeoxycortisol	2863	2838	2975
Tetrahydrocortisone	2960	2947	3104
Tetrahydrocortisol	3021	2980	3073
Allo-tetrahydrocortisol	3034	3000	3086
Cortolone	3045	3010	3141
β Cortolone	3074	3033	3148

TABLE III

Chromatographic Characteristics of Glass Capillary Columns with Irreversibly Bonded Methyl-Phenyl-Polysiloxane Phases of Increasing Phenyl Substitution

% Phenyl substitution	5	10	20	25	50	75
Column length (m)	19	20.5	20	22	26	18.5
No theoretical plates/m	3145	2630	2691	2550	2500	2080
HETP (mm)	0.32	0.36	0.37	0.39	0.40	0.46
TZ	28	24	27	28	27	20

Experimental conditions as in Table I.

a selective retention of steroid structures with oxygenated functions or unsaturated rings.

Mixed polysiloxanes substitution after heterologous polymerization. Starting from a mixture of dimethyldichlorosilane (DMCS) and diphenyldichlorosilane (DPCS) it was thought that polysiloxanes of controlled phenyl content may be obtained and used as chemically bonded stationary phases of controlled polarity. A series of polysiloxanes was prepared from starting chlorosilane mixtures containing increasing ratios of DPCS to DMCS (from 5:95 to 75:25). After washing as described under Methods, the resulting polymers were characterized by IR spectroscopy. In all cases, a 1441 cm^{-1} absorption band was observed, indicating the presence of a phenyl-Si bond (18), while 1025 and 1080 cm^{-1} bands attributable to Si-O-Si bonds characteristic of linear polysiloxanes (19) were also present. In addition, the IR Phe-Si signal in the various polymers progressively increased in intensity as the content of DPCS in the corresponding starting mixture was raised (20). In order to check whether the amount of phenyl substitution in the final methyl phenyl polysiloxane (MPPS) could be accurately related to the DPCS/DMCS starting ratio, the amount of phenyl substituents was evaluated by IR spectroscopy, using commercially available MPPS of known phenyl content as calibration standards. A linear relationship was obtained between the percentage of DPCS in the starting DPCS-DMCS mixture and the amount of phenyl substituents in the resulting MPPS polymer (21). It may be pointed out that the content of phenyl substituent represents an average value and that it gives no evidence whether the Me and Phe substituents are evenly distributed along a

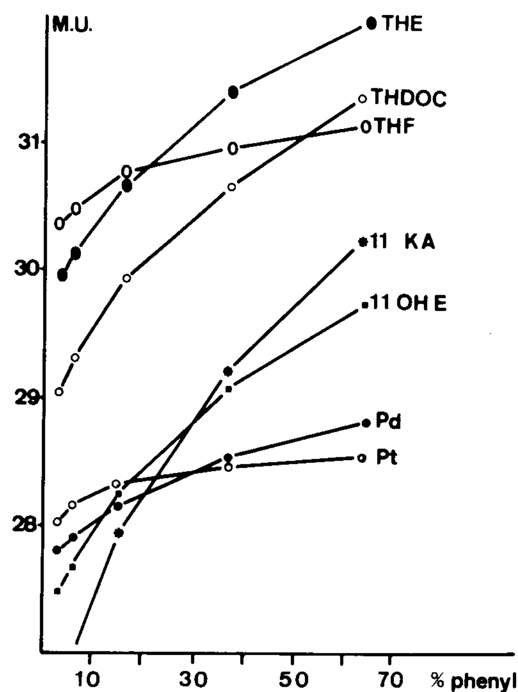


FIG. 2. Variation of the retention indices (methylene units [MU]) of several steroids (as MO-TMS derivatives) on methyl phenyl capillary columns with increasing phenyl substitution (0-70%). THE, tetrahydrocortisone; TH DOC, tetrahydrodeoxycorticosterone; THF, tetrahydrocortisol; 11 K A, 11 oxo-androsterone; Pd, pregnanediol; Pt, pregnanetriol.

A PMS

1 : cholestérol TMS
2 : brassicastérol TMS
3 : campestérol TMS
4 : stigmastérol TMS
5 : β sitostérol TMS

B MPPS

1 : cholestérol TMS
2 : brassicastérol TMS
3 : campestérol TMS
4 : stigmastérol TMS
5 : β sitostérol

FIG. 3. Analysis of a sterol fraction isolated from gentian root, as trimethylsilylether derivatives. Temperature programming 200-250 C (2 C/min). (A) on a methylpolysiloxane (MPS) capillary column. (B) on a methyl phenyl polysiloxane (MPPS) column (20% phenyl substitution).

unique type of polymer or distributed preferentially in different types of polymeric chains. However, for practical purposes, the average phenyl content in the final polymeric preparation could indeed be accurately predicted on the basis of the starting DPCS-DMCS ratio in the polymerization mixture. The Phe/Me + Phe substituent ratio in the monomeric mixture remained the same in the resulting corresponding polymeric preparation (21).

These Me-Phe polysiloxanes (MPPS) of increasing Phe content were used to prepare glass capillary columns following the aforementioned procedure developed with apolar polysiloxanes. Treatment with dichloromethane confirmed that the MPPS stationary phase was mostly irreversibly bonded to the glass surface (21). As illustrated in Table III, the resulting systems exhibited quite good chromatographic performances. As indicated by the number of theoretical plates (in all cases ⩾ 2000/m) the resolution appears satisfactory, although it progressively declines as the polarity (Phe content) increases, as could have been expected. The stability of these MPPS columns appeared as remarkable as that of the apolar systems, both with time and under repetitive temperature programming up to 300 C.

One unique feature of the procedure is that it opens the possibility of making a high resolution chromatographic system of controlled polarity which may be "tailor-made" for a given analytical problem. This is illustrated in Figure 2 in the case of some human urinary steroid metabolites analyzed as methyloxime-trimethylsilylethers (MO-TMS) derivatives. It can be observed that the best overall separations in this case are obtained for the extreme values in Ph Phe content (i.e., 0 and 70%), whereas intermediate polarity (i.e., from 10 to 35% phenyl substituent) have no special interest in this case. It may be pointed out that relative retention indices of some metabolites (e.g., 11 oxo androsterone and pregnanediol) are inverted when the polarity (Phe content) of the stationary phase is increased. These results show that it is possible to determine the best separation conditions to be used for a given analytical problem possibly considering additional factors such as elimination of interfering contaminants present in biological extracts.

Application to Biochemical Analysis

Plant triterpenes and sterols. Both apolar (MPS) and polar (MPPS) irreversibly bonded phases were used in a study of the components of lipophilic extracts from gentian roots. The final characterization and identification of the major structures were carried out by capillary gas chromatography and combined mass spectrometry (22) after preliminary separation of the crude extract by lipophilic sephadex gel chromatography. In the case of the sterol fraction analyzed after trimethyl silylation, the separation was very similar whatever the type of capillary column used (MPS or MPPS of various polarity). In all cases, five main components, including cholesterol (minor) and β sitosterol (major), were nicely separated, as shown in Figure 3. In the case of a triterpene fraction containing α and β amyrin, lupeol and friedelin, an MPPS column with 20% phenyl substitution yielded a satisfactory profile (Fig. 4). The same column resolved satisfactorily the erythrodiol, uvaol and betulin mixture as TMS derivatives (Fig. 5). During these studies these columns were used in a gas chromatography-mass spectrometry (GC-MS) combination (Riber Mag) and the

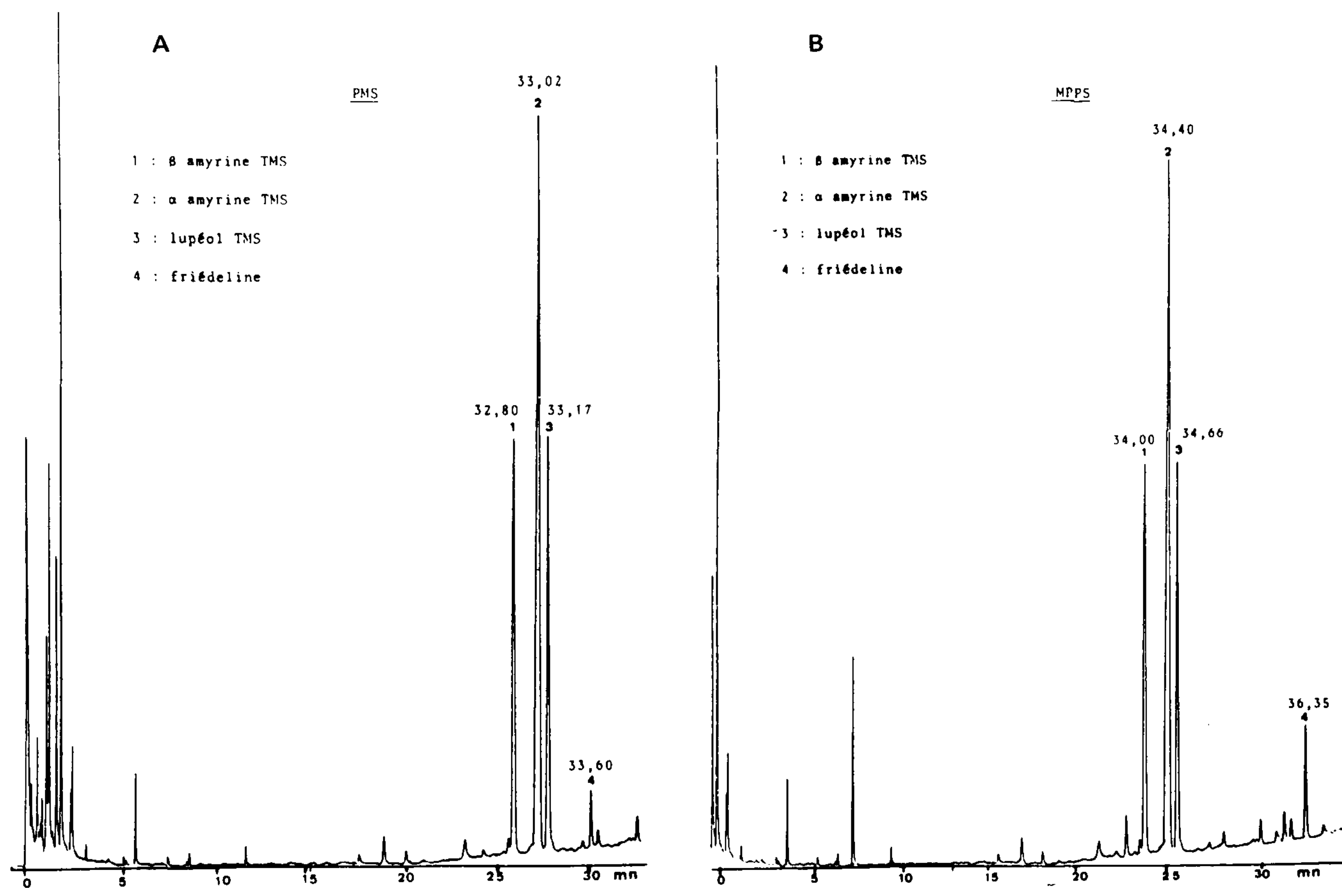


FIG. 4. Separation of a triterpene fraction extracted from gentian flower (A) on an apolar column PMS and (B) on a methyl phenyl polysiloxane (MPPS) column (20% phenyl substitution). Analytical conditions as in Figure 3.

stability of the stationary phase bonding appeared of interest in this context since the "column bleeding" and subsequent MS background remained at a very low level.

Urinary steroid metabolites and physiopathological investigations in humans. As illustrated in Figure 1, the major individual neutral urinary steroid metabolites can be separated as MO-TMS derivatives, using our glass capillary chromatographic systems. In this case, the best overall result is obtained with an apolar system (MPS phase), especially for individual separation of corticosteroid metabolites. Due to the resolution of these systems, this type of analysis can be satisfactorily carried out on rather short capillary columns (e.g., 10 m, Fig. 1). Apolar MPS irreversibly bonded phases have been in routine use for this kind of analysis in our laboratory over the past four years under rather harsh conditions (repeated temperature programming, injection of crude extracts). Their stability has proven to be one of the major advantages of these systems, the chromatographic performances being retained for years under these conditions, thus making these column remarkably inexpensive to use.

Study of steroid production in humans using the aforementioned "metabolic profile" approach by glass capillary gas chromatographic analysis has been widely applied in

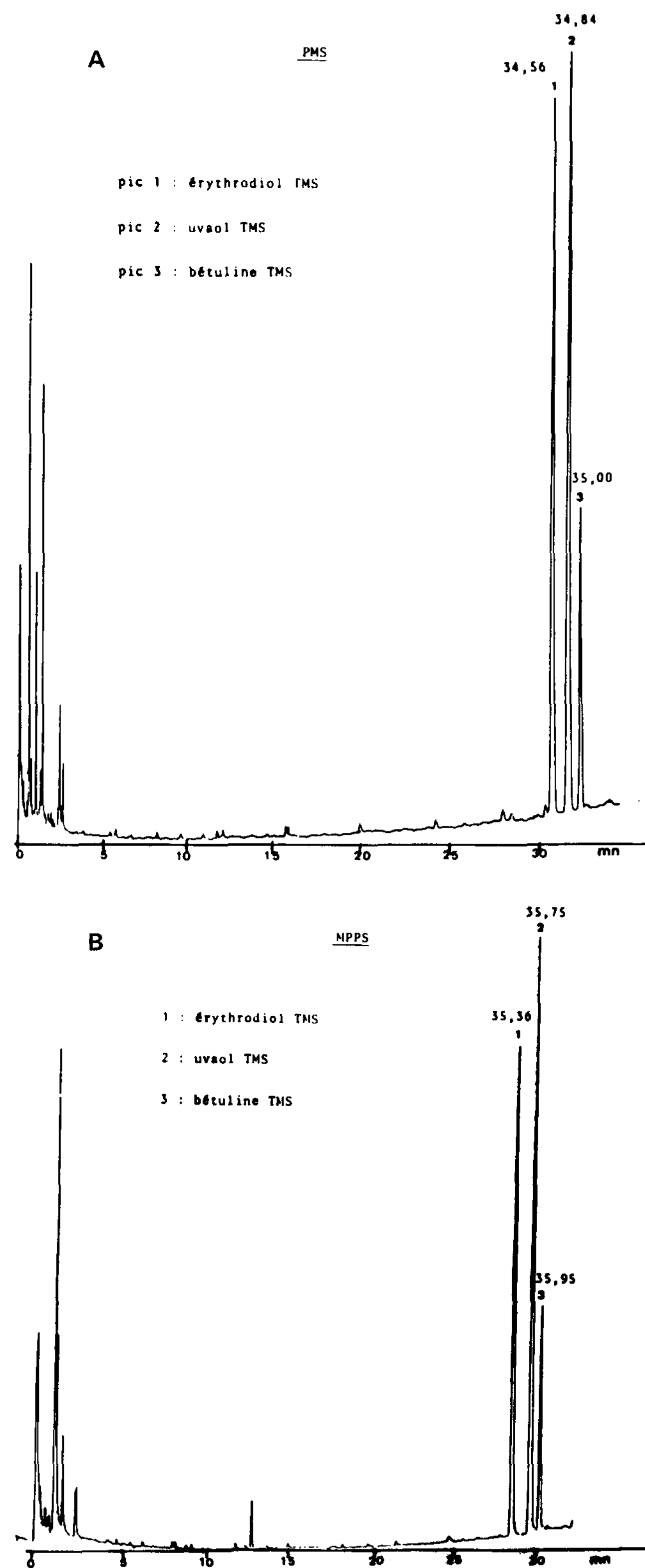


FIG. 5. Analysis of a triterpene fraction isolated from gentian root. Experimental conditions as in Figure 4: (A) apolar MPS column, (B) phenyl methyl column MPPS.

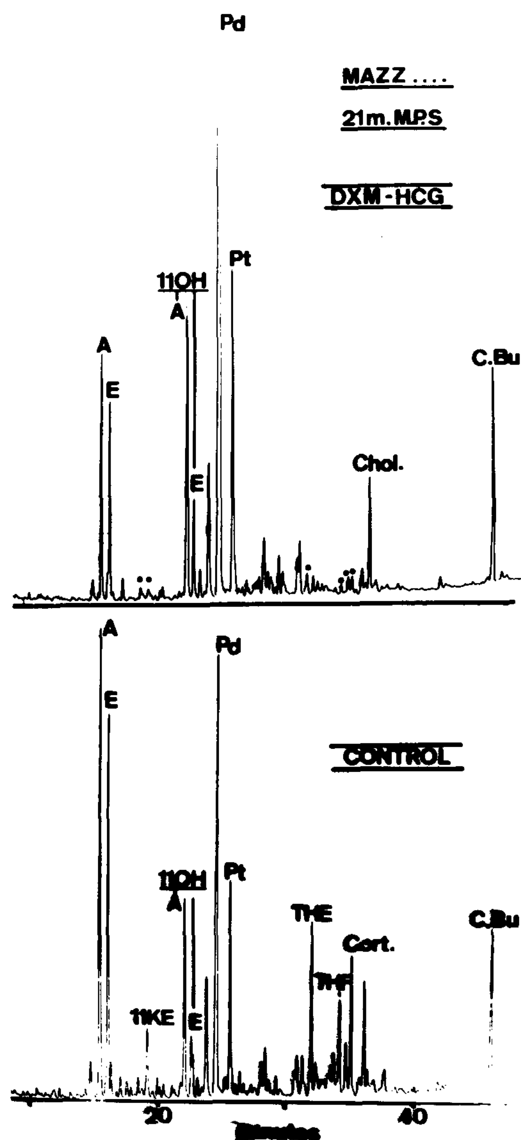


FIG. 6. Analysis of a neutral urinary steroid extract (as MO-TMS derivatives) before (control) and after dexamethasone (DXM) and chorionic gonadotropin (HCG) administration in a normal woman. Capillary column with methylpolysiloxane (MPS) bonded phase; abbreviations and experimental conditions as in Figure 1. Stars point to metabolites which disappeared after DXM administration.

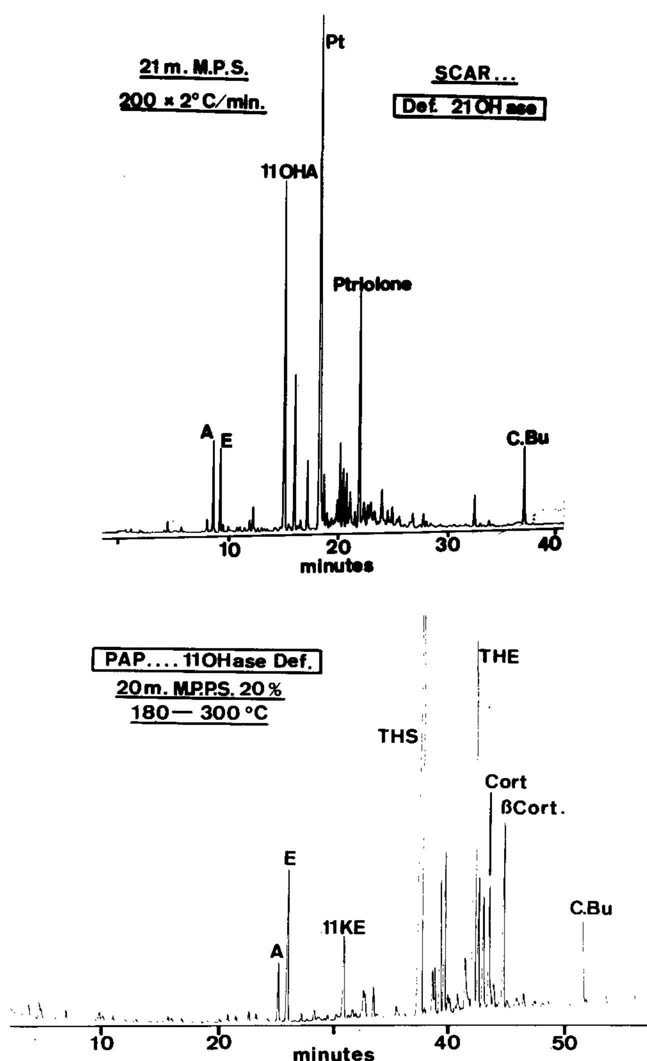


FIG. 7. Neutral urinary steroid metabolic profile (as MO-TMS) in cases of congenital adrenal hydroxylase defects in human newborn. (A) 21 hydroxylase defect; methylpolysiloxane column (21 m). (B) 11β hydroxylase defect; methyl phenyl polysiloxane column (20% phenyl substitution). Abbreviations and experimental conditions as in Figure 1. Ptriolone = pregnanetriolone.

physiopathological investigations (23). Figure 6 illustrates a typical example of an ovarian test where the neutral urinary steroid metabolites are examined under administration of dexamethasone (which suppresses the adrenocortical secretion) and chorionic gonadotropin, which stimulates ovarian activity. Simple inspection of the gas chromatography recording in the case of a normal response indicates that the adrenal cortex activity is indeed blocked (11 oxo metabolites have almost totally disappeared), whereas C 19 and C21 (e.g., pregnanediol) steroid metabolites of ovarian origin are considerably elevated. As seen in Figure 6, the peak with the retention time of 11β-hydroxyandrosterone is not diminished after dexamethasone administration. This somewhat surprising finding can be explained by the fact that in addition to this C 19 metabolite, the peak obtained with an apolar phase (MPS, Fig. 6) also contains a pregnanediolone of ovarian origin. This was shown by using a more polar system (MPPS with 50% Phe substitution), which yielded a complete separation between the two components.

Another application has been the search for adrenocortical enzymatic defects in human newborn and subsequent follow-up of the therapy in these cases. Figure 7 shows that a very typical "fingerprint" is obtained using capillary gas chromatography profiles of a neutral steroid metabolite extract: 21 hydroxylase or 11β hydroxylase defects may be recognized upon simple inspection of the metabolic profiles.

Quantitative analysis and adsorption problem. Our glass capillary systems with irreversibly bonded polysiloxane phases have been found highly satisfactory for quantitative analysis of biochemical compounds in the submicrogram range. Particular attention was focused on possible adsorption processes, occurring at low sample concentrations, especially in the case of polyfunctional steroids. The inherent needs of the preparation procedure (reactive polymer and glass surface for covalent condensation) is not compatible with classical deactivation procedures such as silanization of the capillary wall (24) or use of additional coating (25). However, it was found that the adsorption property of our systems could be greatly minimized when the chemical bonding step at high temperatures was carefully controlled (21). By slowly increasing the temperature at this stage (0.4 C/min), one may favor a smooth glass-polymer reaction and limit intramolecular polymer condensation which takes place at high temperatures (>300 C). Using these conditions, our MPS and MPPS capillary systems show no detectable loss of oxygenated steroidal compounds (e.g., free progesterone) in the range 1-40 ng. The adsorption phenomenon was detectable below 600 pg of injected individual compounds. However, this was obviated when hydroxy-steroids were analyzed after derivatization (e.g., trimethylsilylethers), which indeed represents the usual analytical conditions. For instance, no loss could be detected for androsterone methyl oxime-trimethylsilylether, down to 400 pg injected (limit of our flame ionization detection sensitivity).

Particularly stable glass capillary gas chromatographic columns can be produced by a reproducible and inexpensive procedure making use of covalent bonding between a reactive polymeric stationary phase and the glass surface. Several questions remain to be answered with regard to the precise structure of the polymers, especially in the case of heterologous polymerization and with regard to the glass-polymer coupling reaction mechanism. These points are currently under study in order to consider the development of a much wider range of analytical systems with fully controlled and "tailor-made" chromatographic properties.

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REFERENCES

1. Alexander, G., G. Garzo and G. Palyi, J. Chromatogr. 91:25 (1974).
2. Novotny, M., and K.D. Bartle, Chromatographia 3:272 (1970).
3. Simon, J., and L. Szepezy, J. Chromatogr. 119:495 (1976).
4. Schieke, J.D., and V. Pretorius, Ibid., 132:223 (1977).
5. McKeag, R.G., and F.W. Hougen, Ibid., 136:308 (1977).
6. Blumer, M., Anal. Chem. 45:980 (1973).
7. Cronin, D.A., J. Chromatogr. 97:263 (1974).
8. Van-Hout, P., J. Szafranek, C.D. Pfaffenberger, and E.C. Horning, Ibid., 99:103 (1974).
9. Marshall, J.L., and D.A. Parker, Ibid., 122:425 (1976).
10. Blakesley, C.N., and P.A. Torline, Ibid., 105:385 (1975).
11. Lin, S.N., C.D. Pfaffenberger and E.C. Horning, Ibid., 104:319 (1975).
12. Rigaud, M., P. Chebroux, J. Durand, J. Maclouf, and C. Madani, Tetrahedron Lett. 44:3935 (1976).

13. Madani, C., E.M. Chambaz, M. Rigaud, P. Chebroux, J.C. Breton, and F. Berthou, Chromatographia 10:466 (1977).
14. Madani, C., E.M. Chambaz, M. Rigaud, J. Durand, and P. Chebroux, J. Chromatogr. 126:161 (1976).
15. Digiorgio, P.A., L.H. Sommer and F.C. Whitmore, J. Amer. Chem. Soc. 68:344 (1946).
16. Patenode, W., and D.F. Willock, Ibid., 68:358 (1946).
17. Chambaz, E.M., C. Madani, and A.J. Hadjian in "Chromatography," edited by S.G. Perry, 72, Vol. 1, Bartholomew Press, Dorking, 1973, p. 89.
18. Lady, J.H., G.M. Bower, R.E. Adams, and F.P. Byrne, Anal. Chem. 31:1100 (1959).
19. Coleman, A.E., J. Chromatogr. Sci. 11:198 (1973).
20. Noll, W., Acad. Press (1968).
21. Madani, C., and E.M. Chambaz, Chromatographia, 11-725 (1978).
22. Mariotte, A.M., Thèse de Doctorat Pharmacie, Grenoble, 1978, 2, USMG Grenoble.
23. Madani, C., and Chambaz, in "Symposium International sur les Hormones Stéroïdes", B. Kekhia Edit., Tunis, 1979, p. 273.
24. Novotny, N., L. Blomberg and K.D. Bartle, J. Chromatogr. Sci., 8:390 (1970).
25. Rutten, G.A.F.M., and J.A. Luyten, Ibid. 74, 177 (1972).

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