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GAS-LIQUID CHROMATOGRAPHIC SEPARATION OF STEROIDS AND THEIR DERIVATIVES ON A DUAL COMPONENT COLUMN OF HIGH THERMAL STABILITY*

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SUMMARY

This study describes the gas chromatographic behavior of a large number of steroids on a highly reproducible gas chromatographic column, consisting of a binary mixture of a relatively non-polar phase, SE-30 (methylpolysiloxane) and a polar phase, TMCBA (tetramethylcyclobutanediol adipate). Standard retention time data are presented for the parent unaltered compounds as well as for a series of their derivatives, the trimethylsilyl ethers, chloromethyldimethylsilyl ethers, heptafluorobutyrates and chlorodifluoroacetates. The column described in this report has the distinct attribute of being more thermostable than most other columns described in the literature and can resist prolonged use over a period of even up to a year without alterations in its retention characteristics.

INTRODUCTION

The application of gas–liquid chromatography to the separation of closely related isomeric steroids constitutes a major advance in the analytical methodology of the steroid hormones[1,2]. This technique has been adapted by several workers for the assay of urinary steroids, using single component phase columns[1–3,11]. In a previous report we described a highly reproducible column consisting of a binary mixture of a relatively non-polar phase, SE-30 (methylpolysiloxane) and a polar phase, NGS (neopentylglycolsuccinate) for the simultaneous separation of 17-ketosteroids and estrogens in the form of their trimethylsilyl ethers[7,10]. However, this column could not be operated at temperatures above 210° without seriously affecting the life of the column. The purpose of this report is to describe an improved column which is not only highly reproducible, but which is also suitable for analysis at temperatures higher than 230°.

We have used a combination of tetramethylcyclobutanediol adipate (TMCBA), a highly thermostable polyester, and SE-30, a relatively non-polar silicone rubber in

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formulating this column. The characteristics of this column including its resolving power, reproducibility, and thermostability are described in this paper. A precise gas chromatographic procedure has been developed for the separation of a large number of biologically important steroids on a single column as their trimethylsilyl ethers, chloromethyldimethylsilyl ethers, heptafluorobutyrate, and chlorodifluoroacetate derivatives using an argon ionization detector. The advantages of using the present column in combination with the halogenated derivatives for detection of nanogram amounts of steroids by using a ^{63}Ni high temperature electron capture detector are indicated.

MATERIALS

Standard steroids were obtained from commercial sources (Steraloids, Inc., Rawling, N.Y.; Mann Research Laboratories, New York, N.Y., and Sigma Chemical Co., St. Louis, Mo.). All solvents (Fisher certified reagents) were distilled before use. The stationary phase, SE-30, was obtained from Analabs, Hamden, Conn. Tetramethylcyclobutanediol adipate (TMCBA) and column support, 100–120 mesh Gas-Chrom P were purchased from Applied Science Laboratories, Inc., State College, Pa. Regisil (bistrimethylsilyl trifluoroacetamide + 1% trimethylchlorosilane) was obtained from Regis Chemical Co., Chicago, Ill. Chloromethyldimethyl chlorosilane, 1,3-bis-(chloromethyl)tetramethyldisilazane, heptafluorobutyryl chloride and chlorodifluoroacetyl chloride were purchased from Pennisular Chemresearch Inc., Gainesville, Fla.

A modified Barber–Colman Model 15 gas chromatograph equipped with a high gain electrometer–high voltage unit and an argon ionization detector with a ^{90}Sr source was used in this study. Some of the halogenated derivatives were examined in a MicroTek Model GC 2000 MF gas chromatograph equipped with a modified high-temperature electron capture detector (the original ceramic insulators were replaced with identical ones made out of Teflon) containing a 10 mC source of ^{63}Ni. Dry nitrogen served as the carrier gas, and the detector was operated with a polarizing voltage of 55.

METHODS

Gas chromatography

Acid washed, 100–120 mesh Gas-Chrom P was deactivated with 4% dimethyldichlorosilane in toluene (200 ml for 1 h), washed with methanol, and dried overnight at 90°. The support was then coated with a mixture of phases: SE-30 (2 g in 150 ml of toluene) and TMCBA (1 g in 50 ml of chloroform) in a side arm flask under partial vacuum to remove trapped air bubbles. The resulting slurry was left for 1 h and filtered under suction. The coated support was dried at 90° overnight as described in a previous report[7]. U-shaped borosilicate glass columns, 6 ft. × 5 mm I.D. were used. The operating conditions of the gas chromatograph (Barber–Colman) were generally as follows: column temperatures: 195° for heptafluorobutyrates, 205° for trimethylsilyl ethers, 210° for chlorodifluoroacetates, and 228° for chloromethyldimethylsilyl ethers. The detector temperature was maintained at 240°, the inlet temperature at 265°, and the outlet flow rate at about 100 ml/min. The argon ionization detector was operated at an ionizing voltage of 1000.

Preparation of derivatives

Trimethylsilyl ethers. The standard steroid (1.0 mg) was dissolved in 1.0 ml of

acetonitrile and allowed to react with 0.1 ml of Regisil at 100° for 1 h in a Teflon-lined screw-capped tube. The solvent and the reagent were removed under a stream of nitrogen, and the derivative was redissolved in hexane.

Chloromethyldimethylsilyl ethers. The standard steroid (1.0 mg), dissolved in a mixture of 0.5 ml of dichloroethane and 0.5 ml of acetonitrile, was allowed to react overnight at room temperature with 0.3 ml of 1.3-bis(chloromethyl)tetramethyldisilazane and 0.1 ml of chloromethyldimethylchlorosilane in a glass-stoppered centrifuge tube. The supernatant was transferred to another centrifuge tube. The precipitate was extracted twice with about 1.0 ml of dichloroethane, and the extracts were combined with the original supernatant. The sample was evaporated under a stream of nitrogen and the residue reconstituted to a standard volume with hexane.

Heptafluorobutyrates. Heptafluorobutyryl chloride (0.1 ml) was added to a solution of 1.0 mg of the steroid in a mixture of 0.2 ml of pyridine, 0.5 ml of benzene and 0.5 ml of hexane, and allowed to react at room temperature for 30 min in a glass-stoppered centrifuge tube, after which 2.0 ml of hexane were added. The precipitated pyridine hydrochloride was centrifuged and the supernatant was transferred to a second centrifuge tube. The precipitate was washed twice with 1.0 ml of hexane and combined with the original supernatant before being evaporated under a stream of nitrogen. The heptafluorobutyrates were dissolved in a standard volume of hexane.

Chlorodifluoroacetates. This derivative was prepared in a manner similar to that of the heptafluorobutyrates, except that chlorodifluoroacetyl chloride was used in place of heptafluorobutyryl chloride.

RESULTS AND DISCUSSION

Table I presents relative retention data for a series of biologically important steroids and their trimethylsilyl ethers, chloromethyldimethylsilyl ethers, heptafluorobutyrates and chlorodifluoroacetates. The trimethylsilyl ethers and the heptafluorobutyrates exhibited retention times shorter than those of their parent compounds in contrast to the relatively longer retention times of chloromethyldimethylsilyl ethers and the chlorodifluoroacetates. Among the chlorodifluoroacetates, there were a few exceptions in which these derivatives were eluted earlier than their parent compounds. However, no common structural features could be recognized which would contribute to this behavior.

In this column, steroids possessing axial substituents are usually eluted before the corresponding equatorial forms. In addition, the column exhibits selectivity for unsaturation in that the Δ^5 and Δ^4 compounds are retained on the column longer than the corresponding saturated ones.

Since the trimethylsilyl ethers, heptafluorobutyrates, chloromethyldimethylsilyl ethers and chlorodifluoroacetates have retention times which are widely separated from one another, closely related steroids which would overlap in the unaltered form could be readily resolved by conversion to one of these derivatives. Moreover, these halogenated derivatives have afforded us sensitivities several-fold more than conventional derivatives, through the use of the highly sensitive ^{63}Ni electron capture detector, as would be expected[9].

In the preparation of heptafluorobutyrates and the chlorodifluoroacetates, the acylchloride, if it is used alone or in the presence of pyridine or benzene, results in the

TABLE I

RELATIVE RETENTION TIMES OF STEROIDS AND THEIR CORRESPONDING TRIMETHYLSILYL ETHERS, CHLOROMETHYLDIMETHYLSILYL ETHERS, HEPTAFLUOROBUTYRATES, AND CHLORODIFLUOROACETATES ON SE30/TMCBA[a]

PC: parent compound, TMSi: trimethylsilyl ethers, CMDSi: chloromethyldimethylsilyl ethers, HFB: heptafluorobutyrates, CDFA: chlorodifluoroacetates.

Chemical name	*Trivial name*	*PC*[c]	*TMSi*[b]	*CMDSi*[c]	*HFB*[d]	*CDFA*[e]
Diketone compounds						
4-Androstene-3,17-dione		1.44				
1,4-Androstadiene-3,17-dione		1.72				
4-Pregnene-3,20-dione	Progesterone	2.17				
Monohydroxy compounds						
5α-Cholestan-3β-ol	Cholestanol	2.85	2.60	5.81	1.98	3.79
5-Cholesten-3β-ol	Cholesterol	2.85	2.55	5.18	1.87	3.62
5,7-Cholestadien-3β-ol	7-Dehydrocholesterol	D[f]	3.05	6.54	D	D
24β-Methyl-5,7,22(9β,10α)-cholestatrien-3β-ol	Lumisterol	2.54	1.67	3.72	D	D
24β-Methyl-5,7,22-cholestatrien-3β-ol	Ergosterol	3.80	3.38	7.27	D	D
24β-Ethyl-5-cholestaen-3β-ol	Sitosterol	4.72	4.56	9.27	3.30	6.21
Monohydroxymonoketone compounds						
5α-Androstan-3α-ol-17-one	Androsterone	0.97	0.46	1.43	0.36	0.84
5α-Androstan-3β-ol-17-one	Epiandrosterone	1.00	0.61	1.94	0.51	1.16
5α-Androstan-17β-ol-3-one	Androstanolone	1.19	0.60	2.22	D	1.28
5β-Androstan-3α-ol-17-one	Ethiocholanolone	0.88	0.53	1.61	0.41	0.98
5-Androsten-3β-ol-17-one	Dehydroepiandrosterone	1.01	0.61	1.89	0.47	1.05
4-Androsten-17α-ol-3-one	Epitestosterone	1.57	0.77	2.41	0.61	1.44
4-Androsten-17β-ol-3-one	Testosterone	1.64	0.96	3.01	0.83	1.78
5α-Pregnan-3β-ol-20-one	Allopregnanolone	1.49	1.03	2.82	0.80	1.74
5β-Pregnan-3α-ol-20-one	Epipregnanolone	1.33	0.88	2.40	0.65	1.51
4-Pregnen-20α-ol-3-one		2.65	1.95	5.33	1.61	3.22
4-Pregnen-20β-ol-3-one		2.33	1.75	4.79	1.37	2.85
5-Pregnen-3β-ol-20-one		1.54	1.02	2.84	0.78	1.67
1,3,5(10)-Estratrien-3-ol-17-one	Estrone	2.05	0.89	2.48	0.52	1.22

Monohydroxydiketone compounds						
5α-Androstan-3α-ol-11,17-dione	11-Ketoandrosterone	1.59	0.78	2.27	0.58	1.33
5β-Androstan-3α-ol-11,17-dione	11-Ketoethiocholanolone	1.51	0.93	2.40	0.69	1.70
4-Pregnen-17α-ol-3,20-dione	17-Hydroxyprogesterone		2.29	5.90	2.03	1.98
Dihydroxy compounds						
5α-Androstane-3α,17β-diol	Dihydroandrosterone	1.01	0.43	2.91	0.25	1.03
5-Androstene-5β,17α-diol		1.02	0.48	3.31	0.26	1.09
5-Androstene-3β,17β-diol		1.10	0.53	3.93	0.34	1.33
5α-Pregnane-3β,20β-diol		1.58	1.00	6.98	0.58	2.33
5β-Pregnane-3α,20α-diol	Pregnanediol	1.60	0.87	6.28	0.53	2.18
5-Pregnene-3β,20α-diol			1.09	7.22	0.65	2.46
1,3,5(10)-Estratriene-3,17β-diol	17β-Estradiol	2.34	0.70	4.90	0.36	2.58
Dihydroxymonoketone compounds						
5α-Androstane-3β,11β-diol-17-one		2.90	1.95	8.76	0.77	3.71
5β-Androstane-3α,11β-diol-17-one	11 Hydroxyethiocholanolone	2.42	1.48	6.84	0.57	2.95
1,3,5(10)-Estratriene-3,17β-diol-16-one		4.38	1.52	9.84	1.06	4.48
Trihydroxy compounds						
5-Androstene-3β,16α,17α-triol			1.06	15.22	0.42	3.06
5β-Pregnane-3α,17α,20α-triol	Pregnanetriol		1.52	12.10	D	D
1,3,5(10)-Estratriene-3,16α,17β-triol	Estriol		1.26	19.79	0.46	3.66

[a] Mean of three determinations. Data presented here are relative to cholestane = 1.00.
[b] Cholestane retention time: 14.6 min: column temperature: 205°.
[c] Cholestane retention time: 5.2 min: column temperature: 228°.
[d] Cholestane retention time: 21.5 min: column temperature: 195°.
[e] Cholestane retention time: 11.2 min: column temperature: 210°.
[f] D means that the compound decomposes.

formation of side products, probably due to enolization. The addition of hexane to the reaction mixture allowed the pyridine hydrochloride to precipitate, thus avoiding undesirable side reactions.

Under the experimental conditions described, the conversion of the parent compounds to the derivatives was complete and no extraneous peaks were observed except for androstanolone which gave two peaks. In the mixture of phases used, SE30 (a relatively non-polar silicone rubber) separated the steroids on the basis of molecular weight, and TMCBA (polar, high boiling polyester) provided the selectivity needed to distinguish positional isomerism and unsaturation. The combination of phases, in addition, possessed high thermal stability as evidenced by exposure of the column to 250° for 4 days and 240° for 7 days without any bleeding or change in its separation characteristics.

Several batches of coated support prepared under identical conditions are highly reproducible, in agreement with our earlier observations on mixed phase columns [4–7,10]. Columns, operated over a period of more than 7 months on a continuous basis with biological samples, retained their original characteristics. This type of column could be profitably used in applications such as in combined gas–liquid chromatography–mass spectrometry, where thermal stability and low column bleed are vital prerequisites to succesful operation.

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