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AN EVALUATION OF "OV" LIQUID PHASES FOR THE GAS–LIQUID CHROMATOGRAPHIC SEPARATION OF BIOLOGICALLY ACTIVE STEROIDS

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SUMMARY

Selected steroids with androgenic, estrogenic or progestational activity were chromatographed either as the native steroid alcohols or as steroid esters under varying conditions on six different "OV" liquid phases coated on Chromosorb W HP. The results, based on optimal retention times and molar responses, indicate that OV-225 is preferred for chromatography of the native steroid alcohols while OV-101 is favored for chromatography of steroid heptafluorobutyrates and perfluorooctanoates.

INTRODUCTION

SEVERAL gas–liquid chromatographic (GLC) procedures capable of separating native steroids possessing biologic activity have been described[1, 2]. Other reports have depicted the synthesis, characterization and absolute quantification of highly fluorinated steroid esters by GLC[3–6]. To date, most steroid separation procedures employ either the dimethylsiloxane polymer, SE-30[7], the cyanoethyl methyl silicone, XE-60[8], or cyanopropylmethyl-phenylmethyl silicone, OV-225 [6, 9], as the liquid phases in such analyses. We have previously reported the usefulness of the OV-225 liquid phase for routine analysis of the plasma levels of testosterone, androstenedione, dehydroepiandrosterone, progesterone[9], and 17β-estradiol diheptafluorobutyrate[6] in man. Now, in an effort to aid in the selection of an "ideal" liquid phase for the GLC analyses of these and additional steroids, we have evaluated the six "OV" liquid phases currently available using eight selected steroids of human biologic importance. This appraisal has furnished us with sufficient information to significantly improve our standard GLC procedures for these steroids, offering not only improved retention times (which is most important due to the presence of contaminating impurities in many biologic samples), but increased molar responses for the steroids tested as well.

EXPERIMENTAL

Materials

Solvents. All solvents were of reagent grade and were distilled prior to use.

Steroids. Steroids were obtained from Mann Research Laboratories, N.Y., and their purity established by thin layer chromatography. Standard solutions of

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steroids were prepared in 95% ethanol and stored at 5°C to prevent concentration change due to solvent evaporation.

Heptafluorobutyric anhydride (HFBA) and perfluorooctanoyl chloride (PFO) were purchased from PCR, Inc., Gainesville, Florida, and stored in a desiccator at 5°C. Great care must be taken to prevent contamination of such substances with atmospheric moisture, as immediate decomposition will result.

Liquid phases. All OV gas–liquid chromatographic phases were purchased from the Ohio Valley Specialty Chemical Co., Inc., Marietta, Ohio, and were used without further purification. Gas Chrom CLH (60–80 mesh), coated with 1% XE-60, was purchased from Applied Science Laboratories, Inc., State College, Pennsylvania.

Steroid ester preparation

Standard solutions of the steroid esters were prepared by dissolving known quantities of hydroxysteroid in 0·5 ml of benzene, adding 0·1 ml of either HFBA or PFO (molar excesses), and allowing the reaction to proceed for 30 min at 60°C. The residual HFBA or PFO and benzene were removed by evaporation under reduced pressure at 110°C. Sufficient heptane was then added to the residue to effect the desired concentration of steroid ester. Proof of the quantitative conversion of a steroid to its respective ester was obtained by employing tritiated steroid in the reaction mixture and partitioning the product(s) between heptane and 70% aqueous methanol. In all cases, over 99% of the radioactivity was contained in the heptane phase.

Instrumentation

Analyses of steroid alcohols were carried out on a Packard Analytical Gas–Liquid Chromatograph, Model 7621, while an F & M (Hewlett–Packard) Model 402 Gas–Liquid Chromatograph equipped with a ^{63}Ni electron capture detector was used for steroid ester quantifications. It should be emphasized that since two different instruments equipped with different types of detectors were used in these analyses, no attempt to compare their respective relative sensitivities should be made.

GLC operating conditions: (column and detector temperatures for each phase tested are noted separately in the tables)

	Packard Model 7621	F & M Model 402*
Column length (meters)	0·61	1·83
Column type	Coiled glass	U-tube, glass
Column internal dia. (mm)	2	2
Flash heater temp. °C	285	270
Carrier gas	Helium	Ar:CH_4 (9:1, v/v)
Flow rate carrier (ml/min)	90	100
Flow rate oxygen (ml/min)	250	–
Flow rate hydrogen (ml/min)	30	–
Purge gas	none	none
Recorder-chart speed (cm/min)	1·02	1·25
Electrometer sensitivity setting	3×10^{-11} A 150 V	Range 1 Attenuation 64

*The electron capture detector is operated in a pulsed mode, (+ 30 V/50 μs).

Column packings (noted individually in tables) are 3% of the respective OV phase coated on Chromosorb W HP, 100–120 mesh. All columns were conditioned at a temperature 20° above operating temperature for one week prior to use.

Procedure

Appropriate quantities (0·005–0·15 μg) of the steroid alcohols were applied to a dry injector[10], the solvent allowed to evaporate and the residue injected directly onto the GLC column. In the case of the steroid diesters, on column injection of 1–2 μl of standard solutions, (0·5–3·0 ng steroid diester/μl heptane) was employed. Peak areas were determined by triangulation and the linearity of the detector response for each compound in the respective detector established.

The retention times were calculated from the injection point on the graph to mid-peak of the eluted steroid. Gaussian peaks were obtained for all steroids chromatographed except where indicated. Molar responses are presented as peak area (cm^2)/μmol of steroid or steroid diester in the injected sample. In all cases, operating temperatures were chosen such that the Gaussian nature of the recorded peaks was preserved.

RESULTS

The retention times and molar responses for eight native steroids tested on the six OV liquid phases are listed in Table 1. Also shown are the column and detector temperatures utilized in each instance.

Table 2 lists the molar responses and retention times along with column and detector temperatures for two highly fluorinated esters of 17β-estradiol. In addition to the six OV phases previously employed, XE-60 coated on Gas Chrom CLH was also included in this study. It should be noted that the reported retention times and molar responses are averages obtained from three different injections of each concentration of steroid or steroid diester on the respective columns. In the interest of brevity, the degree of variance (σ^2) and the standard deviations (σ) for the retention times and molar responses on any given phase are not reported as they were shown to be insignificant. Although dehydroepiandrosterone consistently yields rather short retention times on all OV phases examined, the values reported are very reproducible at the operating temperatures and conditions employed.

In addition, the retention times of the steroids or steroid diesters observed for the various liquid phases were not obtained at identical column and detector temperatures in each case. For example, the high polarity of estriol prevented its elution from the columns in a narrow, defined gaseous band below temperatures of 230°C for most OV phases and 250°C for OV-17 in particular. We noted that if chromatography was carried out at lower temperatures (below 230 or 250°C for OV-17) there was significant tailing of estriol upon elution and Gaussian peaks were not obtained.

DISCUSSION

The results show that steroids chromatographed on OV-225 consistently yield good molar responses, usually two to three times those observed with any other phase, and retention times which permit the elution of organic impurities frequently present in biologic extracts prior to the elution of the desired steroid.

Table 1. Retention times and molar responses of native steroids chromatographed at specific column and detector temperatures on various liquid phases

Steroid	Retention time (min)	Molar response ($cm^2/\mu mol) \times 10^{-4}$	Col. Temp. (°C)/ Det. Temp. (°C)
	Liquid phase = OV-1		
Progesterone	1·96	16·0	210/215
20α-Dihydroprogesterone	2·06	13·1	210/215
Testosterone	1·18	18·0	210/215
Androstenedione	1·08	14·8	210/215
Dehydroepiandrosterone	0·74	20·9	210/215
Estrone	1·18	9·8	210/215
Estradiol	1·18	6·5	210/215
Estriol	1·37	9·9	230/235
	Liquid phase = OV-17		
Progesterone	4·60	13·3	230/235
20α-Dihydroprogesterone	4·80	13·1	230/235
Testosterone	2·84	22·4	230/235
Andostenedione	2·94	23·7	230/235
Dehydroepiandrosterone	1·76	20·8	230/235
Estrone	2·94	16·0	230/235
Estradiol	2·94	13·3	230/235
Estriol	3·14	5·7	250/255
	Liquid phase = OV-25		
Progesterone	3·04	8·8	210/215
20α-Dihydroprogesterone	3·24	9·0	210/215
Testosterone	1·76	13·2	210/215
Androstenedione	1·96	16·2	210/215
Dehydroepiandrosterone	1·08	17·1	210/215
Estrone	2·25	13·1	210/215
Estradiol	2·16	8·4	210/215
Estriol	2·84	8·3	230/235
	Liquid phase = OV-101		
Progesterone	3·33	11·5	210/215
20α-Dihydroprogesterone	3·73	7·2	210/215
Testosterone	2·06	11·8	210/215
Andostenedione	2·06	15·1	210/215
Dehydroepiandrosterone	1·37	18·2	210/215
Estrone	2·06	9·1	210/215
Estradiol	2·16	7·7	210/215
Estriol	2·45	8·7	230/235
	Liquid phase = OV-210		
Progesterone	4·22	10·9	210/215
20α-Dihydroprogesterone	3·14	10·2	210/215
Testosterone	1·86	13·3	210/215
Androstenedione	2·94	18·0	210/215
Dehydroepiandrosterone	0·88	19·6	210/215
Estrone	1·47	11·0	210/215
Estradiol	1·08	8·3	210/215
Estriol	1·96	4·9	230/235

Table 1(*Cont.*)

Steroid	Retention time (min)	Molar response ($cm^2/\mu mol) \times 10^{-4}$	Col. Temp. (°C)/ Det. Temp. (°C)
Liquid phase = OV-225			
Progesterone	4·31	47·5	240/245
20α-Dihydroprogesterone	4·22	47·6	240/245
Testosterone	2·75	61·2	240/245
Andostenedione	3·13	46·5	240/245
Dehydroepiandrosterone	1·47	64·9	240/245
Estrone	3·63	40·4	240/245
Estradiol	3·33	34·9	240/245
Estriol	2·35	12·1	240/245

Table 2. Retention times and molar responses of 17β-estradiol-diheptafluorobutyrate and 17β-estradiol-diperfluorooctanoate chromatographed at specific column and dectector temperatures on various liquid phases

Liquid phase	Retention time (min)	Molar response ($cm^2/\mu mol) \times 10^{-4}$	Col. Temp. (°C)/ Det. Temp. (°C)
17β-Estradiol-diheptafluorobutyrate			
OV-1	0·94	0·93	240/255
OV-17	1·02	1·25	250/265
OV-25	0·27	*	240/255
OV-101	1·41	1·95	240/255
OV-210	1·65	*	240/255
OV-225	0·74	1·20	235/250
XE-60	0·37	1·10	235/250
17β-Estradiol-diperfluorooctanoate			
OV-1	2·36	2·00	240/255
OV-17	1·33	2·10	250/265
OV-25	0·35	*	240/255
OV-101	3·70	3·30	240/255
OV-210	4·33	*	240/255
OV-225	0·74	2·50	235/250
XE-60	0·47	2·10	235/250

*Molar response not calculated – noted in text.

This is in agreement with a previous report comparing the performance of OV-225 with that of XE-60 [9].

The molar responses of chromatographed steroids on OV-17 are one-third to one-half those obtained on OV-225, while retention times are notably longer for some of the compounds tested.

Under our experimental conditions, OV-1 yields reasonable molar responses for these compounds but consistently short retention times are also noted. OV-25, OV-101 and OV-210 tend to give more variable results. Although one or the other of these three phases occasionally yields an acceptable response for a particular steroid, the retention times and molar responses are generally not

as desirable as those obtained with OV-225 or OV-17. Molar responses for OV-25 and OV-210 were not calculated because the short retention time of the former and the broad peaks of the latter were not suitable for the accurate quantification of peak areas.

The highly fluorinated esters of estradiol, namely 17β-estradiol-diheptafluorobutyrate and 17β-estradiol-diperfluorooctanoate, yield improved molar responses and retention times when chromatographed on OV-101. Molar responses of these derivatives on OV-1, OV-17 and OV-225 are roughly equivalent, but less than with OV-101, with OV-225 showing shorter retention times than the other two phases. OV-25 and XE-60 yield very short retention times for both diesters. OV-210 demonstrated an acceptable retention time but elution of the compounds produced poor peaks with a considerable degree of tailing even at high column temperatures and optimal flow rates of the carrier gas. Of the two estradiol derivatives employed, the 17β-estradiol-diperfluorooctanoate gave slightly better retention times and molar responses on all liquid phases tested.

Thus we conclude from these data that OV-225 approaches an "ideal" liquid phase for the GLC separation and quantification of the eight native steroids tested. The retention times and molar responses observed under our chromatographic conditions have proven to be most adequate for the routine measurement of these steroid hormones in biologic samples. In addition, OV-101 has been shown to afford improved retention times and molar responses for the diheptafluorobutyrate and diperfluorooctanoate esters of 17β-estradiol.

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REFERENCES

1. Goldfien A., Jones J., Yannone M. E. and White B.: in *Gas Chromatography of Steroids in Biological Fluids* (Edited by Mortimer B. Lipsett). Plenum Press, N.Y. (1965) p. 35.
2. Yannone M. E., McComas D. B. and Goldfien A.: *J. Gas Chromatog.* **2** (1964) 30.
3. Attal J. and Eik-Nes K. B.: *Anal. Biochem.* **26** (1968) 398.
4. Exley D. and Chamberlain J.: *Steroids* **10** (1967) 509.
5. Wotiz H. H., Charransol G. and Smith I. N.: *Steroids* **10** (1967) 127.
6. Munson A. K., Mueller J. R. and Yannone M. E.: *Biochem. Med.* **3** (1969) 187.
7. Zmigrod A., Ladany S. and Lindner H. R.: *Steroids* **15** (1970) 635.
8. Kumari G. L., Collins W. P. and Sommerville I. F.: *J. Chrom.* **41** (1969) 22.
9. Mueller J. R., Killen M., Davis B. M. and Yannone M. E.: *Separation Science* **4** (1969) 507.
10. Yannone M. E.: *J. Gas Chromatog.* **6** (1968) 465.