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HIGH-PRESSURE LIQUID CHROMATOGRAPHY OF STEROIDS

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CONTENTS

1. INTRODUCTION

As recently as 1975, when the chromatography of steroids was last reviewed[1], there were only a few attempts to introduce high-pressure liquid chromatography (HPLC) into the steroid field. The prediction "that these accomplishments will be considerably improved upon by the time this book has been published" (ref. 1, p. 100) has come true, and the time is now ripe to evaluate the progress which has been made and to point out directions which future research might profitably take. As far as we know, the HPLC of steroids has not been surveyed previously, except for a review of HPLC limited to the steroid hormones[2].

The steroids are quite narrowly defined chemically as substances containing the cyclopentanoperhydrophenanthrene nucleus, but their physical characteristics and chromatographic properties run the gamut from the lipophilic sterols to the hydrophilic cardiac glycosides. Fortunately, most analyses are confined to only one of the many classes of steroids, but even so, there may be a large difference in polarity between various members, *e.g.*, in the pregnane series between progesterone and aldosterone.

Chromatographic systems must be designed not only to perform well in the polarity range of the class of steroids one wishes to fractionate, but also to provide the capacity and sensitivity called for by biological extracts. One of the difficulties inherent in the analysis of steroids in biological extracts is that they are usually present not only in a large excess of other lipids, but also in mixtures of closely

related analogs. Some of these are present in very low concentration, *e.g.*, using the same example, aldosterone occurs in urine in a 1000-fold excess of other pregnane derivatives. A chromatographic system capable of detecting 1 μg of aldosterone would therefore have to have enough capacity to handle 1 mg of analogous metabolites or several milligrams (depending on the extent of prefractionation) of crude extract.

One of the major attractions of liquid column chromatography (LC) is the relatively large capacity. This means that it can handle biological extracts with a minimum of preliminary purification. For instance, the application of gas–liquid chromatography (GLC) to the analysis of pharmaceutical preparations of vitamin D requires preliminary purification by thin-layer chromatography (TLC), whereas HPLC accomplishes this analysis directly and more accurately in less time[3].

Large capacity also allows HPLC to be used in the preparation of adequate amounts of material for further testing. Preparative HPLC columns can typically handle sample weights up to one-thousandth of the sorbent weight[4–6]. Such columns are conveniently prepared by axial compression[7]. The efficiency of preparative columns depends on the sample volume, as demonstrated with plant extracts containing cardiac glycosides[8]. Chromatographic systems suitable for preparative HPLC may be derived from preliminary tests by TLC[9–11]. Sometimes, a preparative column may be coupled with an analytical column, as, *e.g.*, in the analysis of corticosteroids in serum, where a pre-column packed with porous styrene–divinylbenzene copolymer particles was used for concentrating and purifying the steroids[12].

Another attractive feature of HPLC is that compounds, such as aldosterone, which are not very stable can be handled without derivatization or exposure to heat, light, and air. The effluent from the chromatographic column may be continuously monitored and assayed, or fractions may be collected and either subjected to any suitable physical, chemical, or biological test or battery of tests, or they may be accumulated for future uses.

LC has a greater potential for accomplishing difficult separations than GLC, because it can take advantage of a larger array of sorption mechanisms[13], *viz.* adsorption, partition, reversed-phase partition, chemisorption, gel permeation, ion exchange, and ion-pair formation. The sorbents enlisted for HPLC of steroids include the adsorbents silica and alumina and the permanently bonded octadecylsilane, which usually supports the less polar phase of so-called reversed-phase partition systems.

Silica is supplied either in the form of porous particles of a narrow size range down to 5 μm or in pellicular form, coating inert support beads. The activity of alumina[14] or silica[15] changes with the water content of the eluent, and the character of the chromatographic system gradually changes from adsorption to partition as the water content is increased[16]. Silica columns may be prepared for partition chromatography by loading them either before[17] or after [18] packing with a stationary phase, *e.g.* formamide, which has been diluted with a less viscous solvent. An interesting way to apply argentation chromatography to steroids is the incorporation of silver nitrate in the eluent[19].

A large array of proprietary packing materials for reversed-phase partition chromatography is available. They may be minute beads of polymers[20] or, frequently, pellicular coats of hydrophobic material, which is permanently bonded to beads of silica or other inert materials. Products of different manufacturers may

exhibit considerable differences in selectivity towards steroids[21]. Hydrophobic supports coated with a non-polar stationary phase usually give combined lipophilic adsorption and reversed-phase partition effects[22].

The rational design of two-phase solvent partition systems for HPLC from ternary mixtures was demonstrated by Hesse and Hövermann[23]. Jandera *et al.*[24] have developed a theoretical approach to the selection of the optimal composition of the mobile phase and have applied it to sterols. Following a discussion of solvent strength and solvent selectivity, as applied to the liquid–solid chromatography of steroids[25], Hara and Hayashi[26] have made an extensive study of the retention behavior of 43 steroids in adsorption (Corasil II*) and reversed-phase partition (Bondapak C_{18}) HPLC. From these data they have derived a series of retention parameters for the functional groups of steroids in binary solvent systems. Hara and co-workers[27,28] later based a systematic design of such solvent systems on the retention behavior of mono- and difunctional steroids on silica gel columns. O'Hare *et al.*[29] have devised gradient-elution schemes for the separation of a large array of steroids by reversed-phase partition chromatography. In addition to composition and flow-rate, the temperature is a variable which can be exploited to improve the resolution[30].

HPLC differs from other methods of LC mainly in the use of ultrafine particles of sorbents. This accounts for the high efficiency of this separation method, but this also necessitates the application of pressure to force solvents through packed columns. Finely powdered sorbents will yield highly efficient separations only if they are properly packed, and because packing them properly is so difficult, most researchers either buy packed columns from supply houses or decide to pack their columns with somewhat coarser particles. The limited selection and expense of ready-made columns is one of the obstacles to the development and acceptance of new methods of separating steroids by HPLC.

Other obstacles are the capital investment in equipment, which may be considerable when complete liquid chromatographs with luxury features are purchased, and the inadequacy of commercially available detectors. Practically the only detectors suitable for steroids are the ultraviolet (UV) and the refractive index (RI) detectors, which may be used either separately or in tandem[31]. Until the variable-wavelength detector[32] made the far UV accessible, it was common practice to make those steroids which do not exhibit appreciable absorption at 254 or 280 nm, where the fixed-wavelengths detectors operate, UV-absorbing by derivatization. Thus, *e.g.*, hydroxy-steroids have been esterified to form benzoates or *p*-nitrobenzoates[33], and keto-steroids have been converted to 2,4-dinitrophenylhydrazones[34,35].

By post-column reaction with isonicotinylhydrazine in methanolic aluminum salt solution Δ^4-3-ketones may be converted to hydrazones, which fluoresce owing to complex formation with aluminum ions[36]. This permits the detection of picomole quantities of steroids with greater specificity than by UV spectrometry. Similarly, cardiac glycosides react with concentrated hydrochloric acid to yield fluorescent products which may be exploited for post-column derivatization and an automated, highly sensitive fluorometric assay[37].

* For chemical composition and manufacturers of commercial products the reader is referred to p. X, ref. 1. Reference to a company and/or product named by the Department is only for the purpose of information and does not imply approval or recommendation of the product named to the exclusion of others which may also be suitable.

Moving-wire flame-ionization detectors (FID), although quite expensive and temperamental, are beginning to find acceptance in lipid research[38,39]. A heated thin-layer plate, moving slowly past the column outlet, has been advocated as an alternate transport detector[40]. Although off-line monitoring by mass spectrometry[41] is now common, field desorption[42] and atmospheric pressure ionization[43] mass spectrometry have rarely been coupled with HPLC in steroid analysis. More use of the infrared and light-scattering[44] detectors could be made in the future.

HPLC, like all new methods, must overcome the competition of highly developed and generally accepted methods of steroid chromatography: GLC and TLC. For many applications, GLC will remain the method of choice, because it is extremely efficient, sensitive, rapid, and convenient. However, these are advantages which are gradually becoming the attributes of HPLC, and the gap between the two competitive methods is narrowing. For instance, completely automatic HPLC systems for pharmaceutical analysis are now available[45,46]. The two main advantages of TLC are that several samples or reference standards can be chromatographed simultaneously and that the relatively unreactive steroids can be subjected to a large array of detection methods, most of which require corrosive reagents. Adding the modest cost and portability to its attractive features, we may venture to predict that in steroid research TLC will probably survive GLC and HPLC in the long run[47].

2. STEROLS

This section deals with C_{27}, C_{28}, and C_{29} steroids with one or two oxygen functions. The *seco*-steroids are discussed in section 4 on vitamins D and the more highly oxygenated sterols with insect-molting hormone activity are treated in section 3 on ecdysteroids. No reports on the behavior of bile alcohols in HPLC have come to our attention. The natural sterols have an oxygen function, usually a hydroxyl group, at C-3 and a hydrocarbon chain at C-17. They differ from each other in the way rings A and B fused, in the presence and location of double bonds, and in the presence, nature, and orientation of the alkyl group at C-24. Additional oxygen functions may occur in the nucleus as well as in the side chain.

Smith and Hogle[48] separated the benzoates of C_{27} sterols in the order of increasing number of double bonds by the use of a column of Corasil II, containing 5% silver nitrate. Gradient elution with increasing concentrations of dichloromethane in light petroleum produced the sequence cholestanol, cholesterol, 7-dehydrocholesterol, and ergosterol. At the same time, Rees *et al.*[49] chromatographed steryl acetates and benzoates on a μBondapak C_{18} column and monitored the effluent with a RI detector. When mixtures of methanol, chloroform, and water were used as eluents, the esters emerged in the order of decreasing polarity, *e.g.*, cholesteryl benzoate, followed by a mixture of stigmasteryl and campesteryl benzoates, and then sitosteryl benzoate. In another chromatogram, ergosteryl acetate was eluted before brassicasteryl acetate, which was followed by the acetates of campesterol and then sitosterol. In a third example, desmosteryl acetate was followed by 7-dehydrocholesteryl and lathosteryl acetates.

This method has been applied to marine invertebrates[50] and yeast mutants[51]. In a study of the *in vitro* biosynthesis of cholesterol, Thowsen[52] chromatographed the acetates of various C_{27} sterols on a μPorasil column and monitored the

effluent with a RI detector. Using hexane–benzene (9:1) as the eluent, she obtained the following order of elution: Δ^0, Δ^5, $\Delta^{8(14)}$, Δ^8, Δ^7, $\Delta^{5,7}$, $\Delta^{8,14}$ and $\Delta^{7,14}$, *i.e.* the acetates were in the order of increasing polarity.

Rees *et al.*[49] have also chromatographed the free sterols in their reversed-phase system, which was later modified by Hansbury and Scallen[53]. Similarly, Kikuchi and Miki[54] have employed a Zorbax ODS column with acetonitrile–water–0.1 *M* sodium acetate (90:10:1) as the eluent to separate some of the free sterols obtained from dates. In this case, the absorbance at 210 nm was used for monitoring the effluent, which yielded, in sequence, cholesterol, stigmasterol, campesterol, and sitosterol. For the specific analysis of ergosterol in fungus-infested grains, Seitz *et al.*[55] have chosen to set their detector to 282 nm, where other sterols do not interfere.

In a systematic study of the behavior of the ketonic C_{27} sterols in HPLC, we took advantage of their UV absorption at 254 and 280 nm[56]. Using two UV detectors in series, we followed the separation of eleven C_{27} ketones, separated on a 50-cm column of LiChrosorb Si 60–10, with dichloromethane–*n*-hexane–ethyl acetate (94:5:1) as the eluent. Under these conditions, the elution sequence was: 5β-cholestan-3-one, 5α-cholestan-3-one, 5-cholesten-3-one, and 4-cholesten-3-one. With dichloromethane–ethyl acetate (99:1) as the eluent, it was: 3,5-cholestadien-7-one, 5α-cholest-7-en-3-one, 4-cholestene-3,6-dione, 4,6-cholestadien-3-one, 1,4-cholestadien-3-one, 3β-hydroxy-5α-cholestan-7-one, and 3β-hydroxy-5α-cholestan-6-one. Generally, the A/B-*trans* steroids are more strongly adsorbed than A/B-*cis* steroids[57]. Thus, 5α-cholestan-3-one follows 5β-cholestan-3-one. The saturated steroids are less adsorbed than unsaturated steroids, and among the unsaturated steroids the ones containing conjugated double bonds are more strongly adsorbed than the ones with isolated double bonds. Thus, 4-cholesten-3-one follows 5-cholesten-3-one.

For the separation of various sterols in the free form, we[58] chose to use Bondapak C_{18}–Porasil B as an adsorbent rather than as the carrier of the less polar phase in a reversed-phase partition system. When 0.5% 2-propanol in *n*-hexane was used as the eluent, the sterols, detected by their UV absorption at 205 nm, emerged in the order of increasing polarity: ergocalciferol, sitosterol, stigmasterol, campesterol, cholesterol and finally, ergosterol. In another chromatogram, cholecalciferol was followed by cholesterol, then lathosterol and, finally, 7-dehydrocholesterol. The glass columns with an I.D. of 1/8 in. were suitable for preparative chromatography, allowing the isolation of 18 μg cholesterol from a 10-mg sample of commercial sitosterol. In the same year, Cortesi *et al.*[59] separated the sterols in vegetable oils by HPLC on Micropak Si-10, using hexane–diethyl ether (4:1) as a developer and the UV absorption at 210 nm for detection.

An outstanding example of the power of HPLC is the resolution of the epimeric 26-hydroxycholesterols[60]. By recycling 2.5% ether in *n*-hexane through a 60-cm Microporasil column, Redel and Capillon[61] succeeded in separating 25β(*S*)-26-hydroxycholesterol from the 25α(*R*)-epimer, which is slightly more polar.

3. ECDYSTEROIDS

Owing to the presence of many hydroxyl groups, the molting hormones are quite polar. They are easily detected by their UV absorption at 254 nm, which is due to their α,β-unsaturated carbonyl group. Although ecdysteroids are easily

separated by HPLC, surprisingly little work has been reported on the use of modern sorbents, perhaps because reference compounds are not easily obtainable.

Nigg *et al.*[62] have demonstrated the ability of Corasil II, in combination with mixtures of chloroform and 95% ethanol, to separate various analogs of insect-molting hormones. Gilgan[63] used this method to isolate, in the order of emergence: ecdysone, makisterone A, and 20-hydroxyecdysone plus inokosterone, which were unresolved. By reversed-phase partition with Bondapak Phenyl-Corasil as the column material and a linear gradient from water to ethanol as the eluent, he obtained, in sequence: 20-hydroxyecdysone, inokosterone plus makisterone A, ecdysone, and ponasterone A. More recently, Ogawa *et al.*[64] determined 20-hydroxyecdysone and inokosterone separately by HPLC on a Permaphase ETH column, which was developed with *n*-hexane–ethanol (9:1) at 50°.

4. VITAMINS D

HPLC is an ideal method of analysis for the vitamin D analogs, because they are light- and heat-sensitive and it is difficult to convert them quantitatively to derivatives. These UV-absorbing lipids are readily handled by LC, but the separation of closely related analogs is sometimes difficult. Adsorption chromatography on silica[65,66] or alumina[67] does not adequately separate vitamin D_2 (D_2) from vitamin D_3 (D_3), but it is very effective for the fractionation of their metabolites and photoisomers, which are usually detected at 254 nm.

Jones and DeLuca[68] used a Zorbax SIL column and 2.5% 2-propanol in Skellysolve B to obtain the following elution sequence: D_2 plus D_3, 24-hydroxy-D_2, 24-hydroxy-D_3, 25-hydroxy-D_2 and 25-hydroxy-D_3. For the mono- and dihydroxy-vitamins D, they used a 10% instead of a 2.5% 2-propanol solution to elute, in order: D_3, 25-hydroxy-D_3, 24,25-dihydroxy-D_3, 1α-hydroxy-D_3, 25,26-dihydroxy-D_2, 25,26-dihydroxy-D_3, 1,25-dihydroxy-D_2 and 1,25-dihydroxy-D_3.

Similarly, Ikekawa and Koizumi[69] used a Zorbax SIL column and either 2% methanol in dichloromethane or a gradient from 0.02 to 6% methanol in dichloromethane for the separation of D_3 metabolites. The polarity, which depends on the number and position of the hydroxyl groups, increased in the following order 24<25<1α<24,25<1,24<1,25<1,24,25. For the most difficult separations, they converted the metabolites to trimethylsilyl derivatives and eluted with 2% dichloromethane in *n*-hexane: first 24β(*S*),25-dihydroxy-D_3 and then 24α(*R*),25-dihydroxy-D_3, but with 3.5% methanol in dichloromethane: first 1α,24α,25-trihydroxy-D_3 and then 1α,24β,25-trihydroxy-D_3.

For the determination of the photoisomers of D_2, Tsukida *et al.*[70] developed a Zorbax SIL column with pentane–diethyl ether–methanol (2000:40:3) and obtained, first, a mixture of 5,6-*trans*-D_2 and iso-D_2, then pre-D_2, lumisterol$_2$, isotachysterol$_2$, D_2, tachysterol$_2$, and ergosterol. With chloroform–pentane (11:9), iso-D_2 was eluted before 5,6-*trans*-D_2. Similarly Tartivita *et al.*[71] eluted a column of μPorasil with chloroform–*n*-hexane–tetrahydrofuran (70:30:1) to determine, individually and in sequence, *trans*-D_3, pre-D_3, lumisterol$_3$, isotachysterol$_3$, tachysterol$_3$, D_3, and 9,7-dehydrocholesterol. Analogous results were obtained by the Vanhaelens[72]. An earlier method employed a Vydac column for the separation of precalciferol from D[73].

The determination of D in biological specimens, where the concentration

is in the ppb (ng/g) range, by UV absorption requires some prefractionation. Columns of Sephadex LH-20[74,75] and hydroxyalkoxypropyl Sephadex[76] have been used for the analysis of blood and milk, respectively. Kosky and VanDerSlik[77] used a partition column of Celite 545 for this purpose. The mobile phase was *n*-pentane and the stationary phase was 80% aq. methanol. Low-pressure[78] and high-pressure[79] silica chromatography have also been utilized as prefractionation procedures.

The earliest HPLC method for D employed a Permaphase ODS column and an eluent of 78% aq. methanol, which produced a partial separation of D_2 and D_3[80]. Later, a linear elution gradient from 30 to 80% aq. methanol was used in this method[81]. When a μBondapak C_{18} column was developed with a solvent composed of 865 ml methanol, 135 ml water, and 2.4 g silver nitrate, D_2 was eluted ahead of D_3[19]. Complete separation of the two vitamins was also accomplished by developing a Vydac 201TP column with 90% aq. methanol[82]. Other octadecylsilane columns used for D analogs were developed with methanol–1% aq. ammonium carbonate (19:1)[83] or methanol–acetonitrile (1:1)[84]. For the preliminary isolation of D from blood, Koshy and VanDerSlik first used low-pressure chromatography on silica gel alone[85], but later they combined it with partition chromatography on diatomaceous earth[86]. The purified material was then analyzed for D by reversed-phase chromatography on Zorbax ODS with acetonitrile–methanol–water (94:3:3), but adsorption chromatography on Zorbax SIL with *n*-hexane–2-propanol (97:3) was used for the separation of 25-hydroxy-D_2 from 25-hydroxy-D_3[87].

The plasma analysis scheme of Lambert *et al.*[88] involves a preliminary chromatography on Sephadex LH-20 with *n*-hexane–chloroform–methanol (9:1:1), yielding fractions of D, monohydroxy-D, and dihydroxy-D, which are separately chromatographed on μBondapak C_{18} with 90, 87 and 80% aq. methanol, respectively, but a fraction containing 1α,25-dihydroxy-D was resolved on μPorasil with *n*-hexane–2-propanol (22:3). Jones[89] first obtained two fractions by low-pressure silica gel chromatography, using hexane with stepwise increasing 2-propanol concentrations for elution. He later accomplished this preliminary purification by HPLC on Zorbax SIL, which yielded a D fraction and a 25-hydroxy-D fraction[90]. Both fractions were chromatographed on a Zorbax ODS column, the former with 98.5% aq. methanol, the latter with 91% aq. methanol.

5. STEROIDAL SAPOGENINS AND ALKALOIDS

So far, only one publication on HPLC of steroidal sapogenins has come to our attention. Higgins[91] has applied reversed-phase chromatography on LiChrosorb RP-8 to the quantitative determination of sapogenins isolated from *Agave*. The sapogenins, in the form of benzoates, were eluted with 80% aq. acetonitrile and detected at 235 nm. The elution sequence was 9(11)-dehydrohecogenin, hecogenin, 9(11)-dehydrotigogenin and tigogenin in one chromatogram, and diosgenin followed by sarsasapogenin in another chromatogram.

The separation of steroidal alkaloids by HPLC has apparently also been reported only once. We[92] observed the following order of elution from a Porasil A column: tomatidine, solanidine, solasodine, rubijervine, veratramine, and jervine. The eluent was changed from acetone–*n*-hexane (2:1) to 97% aq. acetone between solasodine and rubijervine. In the absence of a detector, the effluent was analyzed by

TLC. The relatively large column (3/8 in. O.D. × 8 ft.) was suitable for fractionating 2 g of a crude alkaloid extract.

6. WITHANOLIDES

The withanolides are a class of plant steroids characterized by an α,β-unsaturated carbonyl and an α,β-unsaturated δ-lactone group, both of which give rise to appreciable UV absorption. Because the withanolides have been discovered rather recently and reference material is not generally available, little is known about their chromatographic properties.

Gustafson *et al.*[93] have separated five synthetic derivatives and two microbial metabolites of withaferin A on a μPorasil column with ethyl acetate or ethyl acetate–hexane (5:1), monitoring with a RI detector. Using the UV absorption at 225 nm for detection, we[94] have chromatographed 12 withanolides on a 12-ft. coiled column of Porasil A. Elution with *n*-hexane–2-propanol (9:1) yielded, in sequence, withanolides G, J, 27-deoxwithaferin A, withanolide D, 20-hydroxywithanone and withanolide E. Elution with *n*-hexane–2-propanol (3:2) separated, in sequence, 4β,7β-dihydroxy-8,14-dihydrowithanolide G, withanone, 4β-hydroxywithanolide E, and withaferin A. Thus, the effect of hydroxyl groups on adsorption is in the order primary>secondary>tertiary.

7. PREGNANE DERIVATIVES

Most of the published applications of HPLC to pregnane derivatives deal with the adrenocortical hormones. This is partly due to their medical importance and partly to the fact that the natural and synthetic corticosteroids (as well as some other hormones) are characterized by a Δ^4-3-keto group, which absorbs UV light and is easily detected at 254 nm.

In one of the earliest methods, silica was used as an adsorbent[95], but soon investigators began to take advantage of its ability to act as a carrier of the more polar phase in partition systems, either consciously, by presaturating the silica column[96] and using the organic phase of biphasic solvent systems, such as dichloromethane–ethanol–water (948:35:17)[97–99], as eluent, or by simply developing the silica column with water-containing solvents, such as dichloromethane–95% ethanol (19:1)[100], chloroform–methanol–water (983:150:2)[101], chloroform–methanol (197:3)[102], or dichloromethane–ethanol–water–methanol (963:20:12:5)[103]. Excellent separations can be obtained in this way, the corticosteroids emerging from the columns in increasing order of polarity and aldosterone being eluted between cortisone and cortisol. Gradient elution may be used for this application[104,105].

Hydrophobic packing materials can be used as adsorbents with non-aqueous eluents[105,106], but more commonly, such nonpolar groups as octadecylsilane[107–109], cyanoethylsilane[110], cyanopropylsilane[111], nitro[112], or phenyl[113–115] and others[116,117], bonded to silica, selectively hold an organic solvent stationary. The mobile phase, which is more polar, is a water-containing organic solvent, such as methanol[110,113,118], 2-propanol[107], acetonitrile[113,114], or tetrahydrofuran[109]. The corticosteroids emerge from such reversed-phase columns in the order of decreasing polarity, giving very sharp and symmetrical elution peaks. Burgess[119] has shown that the optimum flow-

rate for such columns may be doubled when the temperature is raised from ambient to 60°.

HPLC is now widely used for the analysis of synthetic corticoid drugs. Silica columns have been recommended for prednisone and prednisolone[120,121], dexamethasone[121,122], and triamcinolone acetonide[123], but non-polar packing materials, especially those containing octadecylsilane groups, are more popular. The latter have found use in the analysis of fludrocortisone acetate[124], triamcinolone acetonide[125], prednisolone[126,127], methylprednisolone[101,128], and other steroid drugs[129]. For the corticosteroid phosphates, buffers are suitable eluents, because they control ionization[130,131]. The resolution of epimeric corticoid drugs by reversed-phase HPLC on Bondapak C_{18} deserves special mention. The epimers of ethynodiol diacetate[132] and of budesonide[133,134] could not have been separated as efficiently by any other method.

8. ANDROSTANE DERIVATIVES

In contrast to the HPLC of the pregnane derivatives, the HPLC of androstane derivatives has received surprisingly little attention. In order to make androstane derivatives detectable at 254 nm, the pioneers of this application, Fitzpatrick and co-workers, converted them to benzoates or *p*-nitrobenzoates[33] or 2,4-dinitrophenylhydrazones[35]. From a Permaphase ODS column, the esters were eluted by 66% aq. methanol in the order androsterone, dehydroepiandrosterone and epiandrosterone. The 2,4-dinitrophenylhydrazones were eluted with isooctane from a column of Zipax, which had been coated with β,β'-oxydipropionitrile, in the following sequence: epietiocholanolone, androsterone, epiandrosterone, etiocholanolone and dehydroepiandrosterone.

Using a RI detector, Lafosse *et al.*[135] separated these steroids, as well as their sulfates and glucuronides, by reversed-phase partition chromatography on Micropak CH with methanol–water mixtures. The order of elution for the free and conjugated 17-ketosteroids was dehydroepiandrosterone, epiandrosterone, etiocholanolone, and androsterone. Thus, the equatorial hydroxyl group makes the first three steroids more polar than the axial hydroxyl of androsterone. Among the steroids with equatorial hydroxyl groups, the most polar one is dehydroepiandrosterone with a double bond at C-5, followed by the A/B *trans*-compound epiandrosterone, and then the A/B *cis*-compound etiocholanolone (*cf.* ref. 57). A μBondapak C_{18} column was used to isolate labeled 19-hydroxyandrostenedione from sow ovaries[136] and to follow the enzymatic dehydrogenation of Δ^4-3-ketosteroids at C-1[137].

Synthetic androgens have been analyzed by reversed-phase partition chromatography on columns of ODS Permaphase[138], Nucleosil CN, Nucleosil C_{18}, Bondapak CN, and Hibar RP-8[139–141], using a concave gradient of methanol in water for elution. The drugs emerged in the following order: methandione, testosterone, methyltestosterone, Embadol, Ultandren, nortestosterone, and ethisterone. As little as 1 ng could be detected by the UV absorption at 254 nm. Higgins[32] recently chromatographed the acetates of some 19-norsteroids on LiChrosorb RP-8 with 50% aq. acetonitrile as the eluent and a detector, made from a Beckman DB spectrophotometer, which was set at 260 nm. Adsorption chromatography on silica gel columns with *n*-hexane–2-propanol–1, 2-dichloroethane (82:15:3) as the eluent gave the elution

sequence methyltestosterone, methandrostenolone, 6β-hydroxymethandrostenolone, and 6α-hydroxymethandrostenolone[142].

9. ESTROGENS

Being phenolic steroids, the estrogens are easily detected by their strong UV absorption. Therefore, work on HPLC of estrogens started early and has been extensive. In 1971, Huber *et al.*[143] reported the quantitative analysis of urinary estrogens, based on partition chromatography with a combination of a diatomaceous earth column and solvent systems of 2,2,4-trimethylpentane–ethanol–water. In 1973, Butterfield *et al.*[144] succeeded in separating the equine estrogens by hydrophobic adsorption chromatography with a column of Permaphase ETH. Elution with *n*-hexane–tetrahydrofuran (49:1) produced the following sequence: estrone, equilin, equilenin, 17α-estradiol, 17β-estradiol, 17α-dihydroequilin, 17β-dihydroequilin, 17α-dihydroequilenin and 17β-dihydroequilenin. Thus, the polarity increases with the number of double bonds and hydroxyl groups, and the 17β-hydroxysteroids are more polar than the 17α-epimers. Elution with *n*-heptane–2-propanol (99:1) gave similar results[145].

For the separation of iodinated estradiols, a column of μPorasil was developed with chloroform[146]. The 2,4-diiododerivative was followed by 4-iodo-, then 2-iodo-estradiol, and finally estradiol. Dolphin[147] analyzed the urinary estrogens by adsorption chromatography on a Corasil I column, eluted with ethanol hexane mixtures. He later used the Partisil 5/*n*-heptane–ethanol (19:1) adsorption system as well as the Partisil 10-ODS/methanol–0.1 % aq. ammonium carbonate (11:9) reversed-phase system[148]. Aqueous methanol in combination with the reversed-phase packings Zorbax ODS[149] and μBondapak C_{18}[150] have also been used for estrogen chromatography. The resolution may be increased by adding silver nitrate, which forms complexes with the estrogens, to the mobile phase[19].

Van der Wal and Huber[151] have made a detailed study of the separation of estrogen conjugates by HPLC on ion-exchange cellulose columns. The anion exchangers, Cellex E, B 300, and ET 41, developed with perchlorate–phosphate buffers, separated the glucuronides (G) in the following sequence: estriol 3-G, estrone G, estradiol 3-G, estriol 17-G, estriol 16-G, and estradiol 17-G. For the sulfates (S) the following sequence was obtained: estriol 3-S, estrone S, equilin S, 17α-dihydroequilin 3-S, equilenin S, and 17α-dihydroequilenin 3-S[152].

Experiments with other stationary phases showed that cellulose and polystyrene anion exchangers have the greatest selectivity for the site of conjugation, but hydrophobic adsorption chromatography on LiChrosorb RP-18 or RPZ and on RP-18, coated with a liquid anion exchanger, are also suitable for this fractionation[153]. Musey *et al.*[154] devised a similar scheme, with μPartisil 10-SAX as the strong anion exchanger and 0.1 *M* NaCl at pH 4.8 or 0.01 *M* KH_2PO_4 at pH 4.2 as the developer, but the order of elution was different: estriol 17-G, estriol 3-G, estradiol 17-G, estrone G, estradiol 3-S and estrone S.

A different principle was applied to this problem by a Swedish group. In liquid–liquid ion-pair chromatography, the glucuronides or sulfates are selectively transferred from an aqueous phase to an organic phase by addition of a cation. Fransson *et al.*[155] chose tetraethylammonium ion as this counter ion, which was held stationary in the

form of its bromide on a support of LiChrospher Si-100. The ion pairs, extracted by a mobile phase of dichloromethane–1-pentanol (9:1), emerged in the order estradiol 3-S, estradiol 17-S, estriol 17-S, and estriol 3-S. Hermansson[156] used reversed-phase ion-pair chromatography with 1-pentanol, held stationary on a column of LiChrosorb RP-2 or RP-18, and tetrapropylammonium as the counter ion in a mobile phase containing phosphate buffer at pH 6.4. The following elution sequence was obtained: estriol 17-G, estrone G, estradiol 3-G, and estradiol 17-G.

The following reversed-phase partition systems have been used for HPLC of estrogens analogs: LiChrosorb RP-8/methanol–0.01 *M* phosphate buffer at pH 7 (3:1) for mestranol and norethisterone[157], Spherisorb S5-ODS/aq. methanol for ethinylestradiol[158], and LiChrosorb RP-8-10A/acetonitrile–water (11:9) for miboleronе[159].

10. BILE ACIDS

The bile acids, which cannot be detected with fixed-wavelength detectors, have in many instances been monitored with the RI detector. Although the RI detector is not very sensitive, the concentrations of bile acids in most biological fluids are sufficiently large for this instrument. Free bile acids are best chromatographed by reversed-phase partition, but after their conversion to esters, both partition and adsorption chromatography give satisfactory results. Certain esters also facilitate detection by UV absorption. One of the advantages of analyzing bile acids by HPLC rather than GLC is that their conjugates do not need to be hydrolyzed or derivatized.

Parris[160], who had earlier used a Zorbax SIL column, which was developed with pentane–2-propanol (7:3), for the chromatography of free bile acids, reported better results with a Zorbax ODS column, which he developed with methanol–phosphoric acid solution of pH 2 (4:1). However, cholic and chenodeoxycholic acids could not be separated. When Jefferson and Chang[161] applied adsorption chromatography on μPorasil with ethyl acetate–hexane (7:3) as the eluent to the separation of bile acid methyl esters, partial resolution of several epimeric pairs was achieved.

The phenacyl esters[162] of the bile acids were eluted from a Partisil ODS column by *n*-heptane–dioxane–2-propanol (14:5:1) in the following order: lithocholic, chenodeoxycholic, ursodeoxycholic, hyodeoxycholic, and cholic acid esters. Excess reagents and biological contaminants were removed by prior elution with *n*-heptane–dioxane (9:1). The phenacyl esters as well as the *p*-nitrobenzyl esters[163] are easily detected at 254 nm. The latter were chromatographed on Partisil 10 with isooctane–2-propanol (49:1)[163] or on MicroPak-NH_2 with an elution gradient from isooctane–dichloromethane (1:1) to dichloromethane–2-propanol (9:1)[164]. For the *p*-chlorobenzoyl esters of methylated bile acids, Porasil T and isooctane–diisopropyl ether (19:1) proved to be effective[163].

Okuyama *et al.*[164] chromatographed the glycine conjugates of bile acids in the form of *p*-nitrobenzyl esters, but for the taurine conjugates, which are detectable at 210 nm, they used μBondapak C_{18}, eluted with methanol–0.01 *M* KH_2PO_4 (3:1). Taurodeoxycholic and taurochenodeoxycholic acids were not separated from each other. Conjugated bile acids have also been analyzed by adsorption chromatography on Corasil II with 2-propanol–ethyl acetate–water–7 *N* ammonium hydroxide

(260:60:50:3) as the eluent[165] or on Vydac or Perisorb A columns by elution with a convex gradient from chloroform to ethyl acetate–ethanol (8:3)[166].

More commonly, however, reversed-phase partition chromatography and detection by refractometry have been applied to the conjugated bile acids[167–169]. One approach to the simplification of the complex mixture obtained by extraction of bile is the preliminary fractionation into free bile acids, glycoconjugates, and tauroconjugates by TLC[170]. Another approach is based on ion-exchange chromatography[171]. A column of piperidinohydroxypropyl Sephadex LH-20 is first washed with 90% aq. ethanol to remove lipid contaminants, then free bile acids are eluted with 0.1 *M* acetic acid in 90% ethanol. Later, the glycoconjugates are eluted with 0.2 *M* formic acid in 90% ethanol, and finally the tauroconjugates are eluted with 0.3 *M* acetic acid–potassium acetate buffer at pH 6.3 in 90% ethanol. Each group is then resolved on a column of μBondapak C_{18}. Elution with 0.3% ammonium carbonate–acetonitrile (9:4) gives a mixture of cholate and ursodeoxycholate, followed by chenodeoxycholate, deoxycholate, and finally lithocholate. A second chromatogram with an 11:4 mixture of 0.3% ammonium carbonate and acetonitrile separates ursodeoxycholate from cholate, which is eluted later.

Reversed-phase partition chromatography was also used by Shaw and coworkers[172,173], who separated conjugated bile acids on a Waters Assoc. fatty-acid analysis column by elution with 2-propanol–8.8 m*M* potassium phosphate buffer at pH 2.5 (8:17) in the following order: tauro-α-muricholate, taurocholate, taurochenodeoxycholate, glycocholate, taurolithocholate, glycochenodeoxycholate, and glycodeoxycholate. However, for the resolution of 5α/5β epimers, Shaw and Elliot[173] preferred adsorption chromatography on Corsail II or on μPorasil with recycling of acetonitrile–acetic acid (40:1).

Sulfated bile acids[174], which were detected by their absorption at 210 nm, emerged from an ODS SC-02 column, eluted with 0.5% ammonium carbonate–acetonitrile (13:4) in the sequence cholate, glycocholate, taurocholate, chenodeoxycholate, deoxycholate, glycochenodeoxycholate, glycodeoxycholate, taurochenodeoxycholate, and taurodeoxycholate. From the same column, eluted with 0.5% ammonium carbonate–acetonitrile (5:2), they emerged in the order taurodeoxycholate, lithocholate, glycolithocholate, and taurolithocholate. Thus, the polarity depends not only on the number of hydroxyl groups but also on the nature of the conjugate, the free bile acids being more polar and the tauroconjugates being less polar than the glycoconjugates.

11. CARDIAC GENINS AND GLYCOSIDES

One of the advantages of HPLC over GLC in the application to the cardiac glycosides is that they may be chromatographed without hydrolysis or derivatization. The unsaturated lactone ring makes the cardenolides and bufadienolides relatively unstable, but facilitates their detection by UV absorption.

Evans[175] in 1974 first reported the HPLC of cardiac glycosides on a SCX (strong cation-exchange) column at 45°. With 4% aq. amyl alcohol, he eluted, in sequence: digitoxigenin, its mono-, bis- and tris-digitoxoside (digitoxin), and lanatoside A. An analogous elution sequence was observed by Cobb[176] for the digoxigenin glycosides in adsorption chromatography on LiChrosorb Si 60-10 with cyclohexane–

ethanol–acetic acid (60:9:1) as eluent. This system separates the cardiac genins in the order digitoxigenin, gitoxigenin, and the cardiac glycosides in the order: digitoxin, gitaloxin, gitoxin, digoxin, and diginatin. A chromatographic method for the determination of gitoxin glycosides in *Digitalis* preparations is based on a silica–methanol adsorption system[177].

Using LiChrosorb Si 60-10 as a carrier for the stationary phase, Lindner and Frei[178] devised two partition systems for *Digitalis*. For the genins and less polar glycosides (digitoxigenin to diginatin, see above), they used isooctane–1-pentanol–acetonitrile–water (124:35:12:2). Heptane–*tert.*-butyl alcohol–acetonitrile–water (3560:1020:465:52) was used to elute the more polar glycosides in the following order: digitoxin, gitoxin, digoxin, diginatin, then lanatosides A, B, C, and D, followed by the desacetyl lanatosides A, B and C. The sensitivity of detection by UV absorption of 254 nm is greatly increased by converting cardiac aglycones or glycosides to the fully substituted 4-nitrobenzoates[179]. In experiments with Merckosorb Si 60-5 and either *n*-hexane–chloroform–methanol (20:2:1) or *n*-hexane–chloroform–acetonitrile (10:3:3) as eluents, as little as 20 ng/ml of the 4-nitrobenzoates could be detected[180]. The order of elution was the same as that observed for the underivatized compounds.

Comparison of partition and reversed-phase partition HPLC of *Digitalis* glycosides showed not only, as expected, that the order of elution was reversed, but also that the resolution is improved when the reversed-phase system Nucleosil C_{10}/37% aq. acetonitrile is used[181]. For the analysis of digoxin and digitoxin glycosides, Castle[182] applied an elution gradient from 25 to 40% aq. acetonitrile to a column of μBondapak C_{18}. As expected, the digoxigenin glycosides emerged before the digitoxigenin glycosides, but oddly within each group the genin was eluted first, followed by the mono-, then the bis-, and finally the trisglycoside. A similar chromatographic system has been used for the cardiac glycosides in milkweed plants and Monarch butterflies[183].

So far, only one group of investigators[184,185] has published data on the application of HPLC to bufadienolides. Best results were obtained with a column of μBondapak C_{18} and either 40% aq. tetrahydrofuran or 66.6% aq. methanol as the mobile phase. The order in which the functional group in bufadienolides and cardenolides retard the elution from that column is: 11α-OH<12β-OH<16β-OH <5β-OH=16β-OAc. The homologs of gamabufalitoxin emerged in the order gamabufotalin 3-succinoylarginine, 3-adipoylarginine and 3-pimeloylarginine esters, and, finally, gamabufalitoxin. The epimeric 14,15-epoxides were best resolved by adsorption chromatography on a Corasil I column with hexane–tetrahydrofuran (3:1) as the eluent.

12. SUMMARY

After a brief discussion of the merits and limitations of high-pressure liquid chromatography (HPLC) relative to other chromatographic methods, special problems in the application to steroids are discussed. Publications on HPLC of steroids are then discussed under the headings of individual classes, arranged generally in the order of increasing polarity.

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