CRC

HANDBOOK of CHROMATOGRAPHY

Analysis and Characterization of Steroids

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CRC Handbook of Chromatography

Analysis and Characterization of Steroids

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CRC Press
Boca Raton Ann Arbor London Tokyo

Library of Congress Cataloging-in-Publication Data

Lamparczyk, Henryk
Analysis and characterization of steroids / author, Henryk Lamparczyk ; editor-in chief, Joseph Sherma.
p. cm. — (CRC handbook of chromatography) (CRC series in chromatography)
Includes bibliographical references and index.
ISBN 0-8493-3008-4
1. Steroids—Analysis. 2. Chromatographic analysis. I. Sherma, Joseph. II. Title. III. Series. IV. Series: CRC series in chromatography. V. Series: CRC series in chromatography.
[DNLM: 1. Chromatography, High Pressure Liquid—methods. 2. Steroids—analysis. QU 85 L237a]
QP752.S7L36 1992
574.19'243—dc20
DNLM/DLC
for Library of Congress 92-6838
CIP

Direct all inquiries to CRC Press, Inc., 2000 Corporate Blvd., N.W., Boca Raton, Florida, 33431.

International Standard Book Number 0-8493-3008-4

Library of Congress Card Number 92-6838

Printed in the United States of America 1 2 3 4 5 6 7 8 9 0

Printed on acid-free paper

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SERIES PREFACE

This volume is the 26th volume in the CRC Handbook of Chromatography Series, which began in 1972 with two general handbooks on chromatography edited by the late Dr. Gunter Zweig and me. Starting with the third volume in the series in 1982, each handbook has been devoted to a particular class of compounds. The first volume on steroids was written by Dr. Joseph C. Touchstone and was published in 1986. The amount of information published on steroid chromatography since that time suggested that a new volume on this topic was in order, and I am very pleased that Dr. Henryk Lamparczyk, a recognized authority on steroid chromatographic analysis, agreed to update the field from the point at which the coverage of the first volume ended.

Future volumes of the Handbook of Chromatography Series are now being written or planned. These will cover a particular compound type or chromatographic method and will include additional volumes on pesticides, polymers, lipids, and hydrocarbons, and coverage for the first time of industrial pollutants, toxins, vitamins, chiral separations, and supercritical fluid extraction and chromatography.

I would appreciate hearing from readers who have suggestions for topics or authors for subsequent volumes in the series. I would also be interested in receiving corrections or comments on the present volume or any others that were published earlier.

Joseph Sherma, Ph.D.
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CRC Handbook of Chromatography Series
January, 1992

THE EDITOR-IN-CHIEF

Joseph Sherma, Ph.D., received a B.S. in chemistry degree from Upsala College, East Orange, NJ in 1955 and a Ph.D. in analytical chemistry from Rutgers University, New Brunswick, NJ in 1958. His thesis research at Rutgers was in the area of ion exchange chromatography under the direction of the late William Rieman III. Dr. Sherma joined the faculty of Lafayette College, Easton, PA in September, 1958, and is presently John D. and Frances H. Larkin Professor and Head of the Chemistry Department. At Lafayette, he teaches three courses in analytical chemistry and directs an active undergraduate research program in chromatography.

Dr. Sherma, independently and with others, has written or edited more than 350 research papers, books, book chapters, reviews, and manuals involving chromatography and other analytical methodology. In addition to being Editor-in-Chief of the CRC Series in Chromatography, he co-edits the series *Modern Methods of Pesticide Analysis* with Dr. Thomas Cairns of the FDA for CRC Press. Earlier, he co-edited and edited 17 volumes of the series *Analytical Methods for Pesticides and Plant Growth Regulators* for Academic Press. He is editor for residues and trace elements of the *Journal of AOAC International* and is a member of the editorial board of the *Journal of Planar Chromatography.* He is consultant on chromatography and trace analysis methodology for industrial companies and federal agencies.

Dr. Sherma has received three awards for superior teaching and scholarship at Lafayette College and the E. Emmet Reid Award for excellence in teaching presented by the Middle Atlantic Region of the ACS. He is a member of ACS, AIC, AOAC, Phi Lambda Upsilon, and Sigma Xi. Dr. Sherma's current research interests are in modern quantitative high performance TLC, mainly applied to clinical analysis and the determination of drugs, pesticides, lipids, and food additives.

THE AUTHOR

Henryk Lamparczyk, Ph.D., is an associate professor at the Medical Academy of Gdańsk and a professor at the Medical Center of Postgraduate Education, Pharmaceutical Branch, in Bydgoszcz, Poland, where he is head of the Biopharmacy Department.

Dr. Lamparczyk received his higher education at the Medical Academy of Gdańsk. After graduation, he joined the Department of Physical Chemistry of the Medical Academy, working in the beginning on the synthesis of organosilicon compounds. In 1976 he started his research on analysis and metabolism of polycyclic aromatic hydrocarbons. In 1978 he obtained his Ph.D., with a thesis on analysis and structure-retention relationships in gas chromatography. During 1982 to 1983 he joined professor P. Sims' Laboratory at the Chester Beatty Research Institute (London) as a fellow on postdoctoral studies, granted by the World Health Organization, where he performed research on the metabolism of anthracene derivatives.

He next focused his research interests on intermolecular interactions involved in chromatography. In this field he has studied the effect of electrostatic interactions on retention and proposed an electrostatic index system common to GLC, HPLC, and TLC. In 1985, Dr. Lamparczyk worked as a Visiting Scientist at the Alcala Henares University, Spain.

In 1986 he defended his thesis to qualify as associate professor. After that, he won a competition sponsored by the Japan Society for the Promotion of Sciences and spent one year in Japan as a visiting scientist.

Since 1974 Dr. Lamparczyk has published over 60 scientific papers, mainly relating to environmental analysis, structure-retention relationships, and chromatography.

At present, his research is mainly concerned with the analysis of steroids and the application of physicochemical methods for the standardization of commercial products. With the scientific team at the Medical Center of Postgraduate Education in Bydgoszcz he is also performing bioavailability studies on human beings in cooperation with domestic and foreign pharmaceutical enterprises.

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Chapter 1

INTRODUCTION

Within the last several years, the use of chromatographic techniques and especially high performance liquid chromatography has become increasingly an important tool for the separation and the determination of steroids. Although many steroids can be determined using radioimmunoassay, fluorescence polarization immunoassay, and even colorimetric or fluorimetric measurements, the identification and determination of the individual steroids requires an efficient separation that can be performed only by extraction and chromatography.

Steroids play a part in many biological processes and have many therapeutic applications. In most biological and medical applications, the ratio of analyte to the biological matrix is very poor; hence, samples usually must be prepared for analysis. The sample preparation is intended to improve the specifity of the assay by removing the majority of the matrix while concentrating the analyte. The specifity of any assay is derived partly from the analysis but also from the initial purification process. The first step in a sample preparation has been for many years liquid-liquid extraction. While solvent extraction is still in use, liquid-solid extraction has become the method of choice for recovery of steroids, particularly from aqueous solution. Over the past decade, great advances can be observed in the development of analytical equipment. Until recently, these advances were not matched by improved sample cleanup procedures. Under these circumstances, sample purification methods could become the rate-limiting step for a laboratory.

The great diversity of steroid structures and their wide range of polarities present special problems for the simultaneous analysis of different classes of steroids. Therefore, analysis of steroids requires a well-equipped laboratory in which such techniques as gas chromatography, high performance liquid chromatography, thin layer chromatography, and even supercritical liquid chromatography are readily available.

This book is a continuation of the *Handbook of Chromatography: Steroids* by J. C. Touchstone. Therefore, many important methods, still in use, were not included in this volume. In order to make the two volumes compatible, the organization of chapters devoted to the particular steroid class (i.e., Chapters 5 through 12) is similar, while the contents are completely new, covering the years 1985 through 1991. Chapters 1 through 4 are intended to provide the user with general information on steroid nomenclature, chromatographic methods employed in steroids analysis, and sample preparation.

Chapter 2

STEROIDS AS A CLASS OF COMPOUNDS

NOMENCLATURE

Steroids are compounds possessing the skeleton of cyclopenta[*a*]phenanthrene or a skeleton derived therefrom by one or more bond scissions or ring expansions or contractions. An alkyl side chain may be present at C-17, and methyl groups are often present at C-10 and C-13. Sterols are steroids carrying a hydroxyl group at C-3 and most of the skeleton of cholestane.[1] The four-ring steroid structure, designated with rings A, B, C, and D, is numbered as in Figure 1.

Saturated steroid nucleuses have seven asymmetric carbon atoms (atom numbers 5, 8, 9, 10, 13, 14, 17) and, therefore, 128 possible stereoisomers can exist. In fact, the number of naturally occurring isomers of the ring systems is found to be very few. When the rings of a steroid are denoted as projections onto the plane of the paper, the formula is normally to be oriented as in Figure 1. An atom or group is termed α if it lies below the plane of the paper or β if it lies above the plane of the paper. When the configuration is not known, this is indicated by the letter ζ. The A/B ring junction may be either *cis* or *trans;* i.e., the substituents or hydrogen atoms on C-5 and C-10 may be on the same *(cis)* or opposite *(trans)* sides of the C-5–C-10 bond to give 5β or 5α compounds, respectively. The B/C ring junction is *trans* in steroids and bile acids and *cis* in cardioactive steroids. Unless implied or stated to the contrary, use of a steroid name implies that atoms or groups attached at the bridgehead positions 8, 9, 10, 13, and 14 possessed 8β, 9α, 10β, 13β, 14α orientations. A side chain attached at position 17 is assumed to be β-oriented.

The numbering system and the systematic names of steroids are described in the IUPAC IUB definitive rules for the nomenclature of steroids.[2] This system was revised and modified in light of the current practice.[1] Some trivial names are generally accepted and may be used without reference to their systematic names.[3] These include etiocholanone, adolsterone, androsterone, cholesterol, cholic acid, corticosterone, cortisol, cortisone, dehydroepiandrosterone, deoxycorticosterone, ergosterol, 17β-estradiol, estriol, estrone, progesterone, and testosterone. Trivial names may be prefixed to denote their derivatives or sterioisomers. The following prefixes are frequently used: hydroxy, oxo, epi (inversion of substituent), dehydro (removal of two hydrogen atoms), deoxy (replacement of a hydroxy group by a hydrogen atom), and dihydro (addition of hydrogen in double bonds). Use of the prefix "allo" (change from 5β to 5α configuration) and the symbol Δ^x (saturation at position x) are not recommended. International nonproprietary names (INN) have been given to many steroids of pharmaceutical importance (e.g., betamethasone, ethynylestradiol, hydrocortisone, mestranol, norgestrel).[4,5] These names should not be used as a basis for the names of modified compounds.

The main classes of steroid hormones, each defined by their physiological function, are the androgens, the estrogens, the progestogens, the glucocorticoids, the mineralocorticoids, and vitamin D. The estrogens and androgens are female and male sex hormones, respectively. The progestogens are involved in the preparation and maintenance of pregnancy. The glucocorticoids have a distinct effect on carbohydrate metabolism and also have anti-inflammatory properties. Mineralocorticoids affect ion transport so that sodium is conserved in the cell while potassium is lost. Vitamin D is involved in the regulation of calcium transport. Apart from mammalian hormones, steroid structure is found in many naturally occurring compounds of biological interest such as cardiac glycosides, bile acids, sterols, ecdysterols, and steroidal sapogenins.

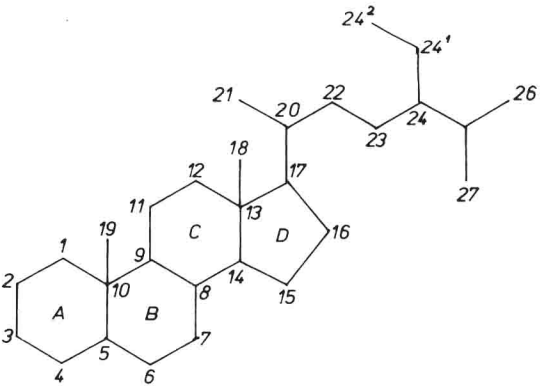

FIGURE 1. Numbering and ring lettering system for steroids.

PHYSICOCHEMICAL PROPERTIES OF STEROIDS WHICH INFLUENCE THE CHROMATOGRAPHIC SEPARATION

It is believed that the majority of the retention properties of a solute in chromatography can be explained on the basis of solute-phase electrostatic interactions. These include interactions between dipoles, inductive, dispersive (London), and hydrogen bonding.[6-9] The potential energy of electrostatic interactions is given by Equation 1

$$E_{int} = -\frac{1}{r^6}\frac{2}{3kT}\mu_a^2\mu_b^2 + \alpha_a\mu_b^2 + \alpha_b\mu_a^2 + \frac{3}{2}\frac{I_aI_b}{I_a + I_b}\alpha_a\alpha_b \quad (1)$$

where E_{int} is the interaction energy between molecules a and b, μ is the dipole moment, k is Boltzmann's constant, T is the absolute temperature, r is the distance between interacting molecules, α is the molecular polarizability, and I is the first ionization potential. The first term within the brackets corresponds to the electrostatic energy, the second and the third terms to the inductive energy, and the fourth term to the dispersive energy.

Assuming that the electrostatic interactions are substantial for most of the chromatographic techniques, and considering that other specific interactions might also influence the retention,[10] it follows from Equation 1 that

$$\log X = A\mu^2 + B_{ph,x}\alpha_x + C\eta_x + D\gamma_x + \cdots \quad (2)$$

where X is a retention parameter. Coefficients A, B, C, D, etc. denote constants for a given stationary phase, and $B_{ph,x}$ is a constant depending on both the phase and the solute. The Greek letters denote solute properties. In particular, μ is the dipole moment, α denotes molecular polarizability, η is the molecular shape parameter, and γ refers to any other parameter characterizing the specific interactions of the solute. When the solutes are nonpolar and their ionization potentials are nearly identical, the operating interactions are inductive and dispersive. Under these circumstances the molecular polarizability (α) of the solute appears to be the most important factor governing the retention order of the sample solutes.

The molecular polarizability can be easily calculated with good approximation using the equation derived by Miller and Savchik[11] (Equation 3)

$$\alpha = \frac{4}{N}\left(\sum_A \tau_A\right)^2 \quad (3)$$

Table 1
ATOMIC HYBRID COMPONENTS FOR SELECTED ATOMS[11]

Atom (A)	Hybridization of A	τ_A	Example
H	σ	0.314	—
C	tetetete	1.294	Methane
C	trtrtr$\pi\pi$	1.428	Ethylene
C (aromatic)	trtrtr$\pi\pi$	1.800	Naphthalene
C	didi$\pi\pi\pi$	1.393	Acetylene
O	te^{2}te^{2}tete	1.290	Methanol
O	tr^{2}tr^{2}tr$\pi\pi$	1.216	Acetone
O	tr^{2}trtrπ^2	1.099	Furan
F	σ	1.046	—

where N is number of electrons in the molecule, and τ_A refers to the atomic hybrid components. The summation proceeds over all atoms A in the molecule. The atomic hybrid components useful for calculation of the α values for steroids are given in Table 1.

When the solutes are polar (they possess a permanent dipole moment) but their ionization potentials are identical, according to Equation 2, both μ and α values have an important role in governing the retention order. Examples of experimentally determined dipole moments for steroids found in the reference literature[12,13] are compiled in Table 2.

Nevertheless, from the point of view of electrostatic interactions, steroids are difficult to investigate because they possess different molecular polarizabilities, dipole moments, and ionization potentials. Moreover, most of these parameters are unknown and are difficult to determine experimentally for a large number of steroids and their metabolites. On the other hand, it is evident that only certain types of interactions are possible between steroids and other molecules. Except for the phenolic OH group in the estrogens, there are no possibilities for true ionic bonds. Even with the estrogens, it is most unlikely that ionic interactions are important, since the phenolic hydroxyl group has a pK of 10 to 10.5. Covalent attachment to macromolecules is also rare. Hence, it can be suggested that a significant part of the unspecific interactions of steroids in biology, their adsorption and desorption from drug systems, and their chromatographic properties may be explained by the electrostatic forces.[14]

Because, as was mentioned above, prediction of the retention for steroids using the basic electrostatic interaction concept might be difficult, the idea of so-called hydrophobic interactions is employed, particularly in reversed-phase HPLC and TLC. The partition coefficient of a solute between *n*-octanol and water is commonly assumed to be the numerical measure of hydrophobicity. Numerous papers have been published dealing with the correlation of the HPLC data with the logarithm of partition coefficient (log P) determined in a standard *n*-octanol–water-partitioning system. The relevant literature has been thoroughly reviewed in various periodicals.[15–17] The partition coefficient data were used for estimation of the chromatographic retention, and the chromatographic data were also used for calculation of the hydrophobicity parameters. However, in general, the correlations of log P and HPLC capacity factors are significant only as long as the solutes analyzed are more or less closely mutually related.[17] The decrease in correlation coefficient between log P and log k′ with increasing structural diversity of solutes results mainly from specific interactions of the compounds chromatographed. Moreover, it should be emphasized that the chromatographic separation is a dynamic process, while partition between two liquid phases is an equilibrium phenomenon.

Three principal sources of the partition data were found, namely, the steroid partition coefficients measured in an ether-water system by Flynn,[18] the coefficients measured in an

Table 2
EXAMPLES OF EXPERIMENTALLY DETERMINED DIPOLE (μ) MOMENTS FOR STEROIDS

Steroid	μ (D)	Ref.
Cholesterol	1.99	12
Dihydrocholesterol	1.81	12
Cholestane-3β,7α-diol	2.31	13
Cholestane-3β,7β-diol	2.55	13
Cholestane-3β-ol-7-one	2.98	13
Cholest-5-ene-3β-ol-7-one	3.79	13
Androsterone	3.70	13
3β-Hydroxy-5α-androstane-17-one	2.95	13
Androst-5-en-3β,17α-diol	2.89	13
Androst-5-en-3β,17β-diol	2.69	13
Testosterone	4.32	13
17α-Hydroxyandrost-4-en-3-one	5.17	13
Androst-5-en-3β-ol-17-one	2.46	13
Androst-4-en-3,17-dione	3.32	13
5α-Androstane-3α,17α-diol	2.29	12
5α-Androstane-3β,17α-diol	2.99	12
5α-Androstane-3,17-dione	3.25	12
17-Methyl-5α-androstane-3β,17α-diol	2.78	12
17-Methyltestosterone	4.17	12
3,17-Dimethylandrostane-17α-ol	2.06	12
3,17-Dimethylandrost-4,6-diene-17α-ol	1.81	12
Gitogenin	2.64	12
Chlorogenin	2.67	12

octanol-water system by Leo et al.,[19] and octanol-water partition coefficients compiled by Craig.[20] The data of Flynn[18] were translated into an octanol-water system by Ebling et al.[21] Experimentally determined log P values for a number of steroids are listed in Table 3.

The general equation (Equation 2) describing the distribution coefficient of a solute between chromatographic phases includes the parameter related to solute molecular shape. To describe this parameter in quantitative terms is a difficult task, even for relatively rigid molecules[22–24] and is particularly difficult for steroids. Moreover, the importance of shaped differences in determining retention is generally minor in comparison to differences resulting from electrostatic interactions. On the other hand, molecular shape plays a decisive role in chromatographic separations of positional, geometrical, and optical isomers.

The average values of interatomic distances of steroid molecules are summarized in Table 4.

There is structural strain in steroids. In the regular structure of diamonds, every carbon atom is surrounded by four other carbon atoms, so that the bond angles are all 109.5° and the torsion angles are all 60°. Two angles in steroids are particularly distorted from 109.5°. These are C-8–C-14–C-15, which has an average value of 119.3°, and C-14–C-13–C-17, which has an average value of 99.2°.

There are several conformations of six- and five-membered rings. The most important conformations of six-membered rings are the chair, the half-chair (four atoms in a plane, and two adjacent atoms above and below the plane, respectively), and the sofa (five atoms in a plane). The most important conformations of five-membered rings are the half-chair (three atoms in a plane and two adjacent atoms above and below the plane, respectively) and the envelope (four of the five atoms in a plane). Steroid hormone molecules are flat in the sense that a ball-and-stick model of fused cyclohexane rings in a chair conformation

Table 3
LOGARITHMIC FORM OF *n*-OCTANOL/WATER PARTITION COEFFICIENTS FOR SELECTED STEROIDS

Steroid	log P	Ref.	Steroid	log P	Ref.
Triamcinolone	1.03	21	Triamcinolone acetonide	2.53	20
Aldosterone	1.08	19	Halometasone	2.58	20
Digoxin	1.26	20	Canrenone	2.68	20
Cortisone	1.42	21	Estrone	2.76	20
Prednisone	1.46	19	Dicirenone	2.82	20
Cortisol	1.55	21	Deoxycorticosterone	2.90	20
Hydrocortisone	1.61	20	Betamethasone acetate	2.91	20
Prednisolone	1.62	21	Dexamethasone acetate	2.91	20
Dexamethasone	1.83	21	Desoxycorticosterone acetate	3.05	20
6α-Methylprednisolone	1.85	21	Hydrocortisone butyrate	3.18	20
Triamcinolone diacetate	1.92	20	Testosterone	3.29	20
Corticosterone	1.94	21	17-Methyltestosterone	3.36	20
Betamethasone	1.94	20	Betamethasone valerate	3.60	20
Flumethasone	1.94	21	Clobetasone butyrate	3.76	20
Fluorometholone	2.00	20	Hydrocortisone valerate	3.79	20
Cortisone acetate	2.10	20	Clobetasol propionate	3.83	20
Hydrocortisone acetate	2.19	20	Diflucortolone pivalate	3.86	20
Doxibetasol	2.35	20	Flumethasone pivalate	3.86	20
Prednisolone acetate	2.40	20	Progesterone	3.87	19
Cortexolone	2.46	20	Estradiol	4.01	20
Cortodoxone	2.52	20	Chenodiol	4.14	20

Table 4
AVERAGE VALUES OF INTERATOMIC DISTANCES IN STEROID MOLECULES

Bond/atom	Distance (Å)
C–C	1.54
C–C	1.34
C–C (aromatic)	1.39
C–O	1.20
C–OH	1.42
van der Waals radii	
H	1.0
O	1.4
C	1.7

Note: Compiled using data from Reference 25.

may be laid flat on a surface. Thermodynamically, this is the most stable situation with all ring fusions *trans*. However, those steroids that have two bulky axial methyl groups (C-18 and C-19) are slightly bowed. For example, in androsterone, the axial bonds C-10–C-19 and C-13–C-18 are not parallel, but lie at an angle of approximately 13° to each other.[26]

If all six-membered rings of a steroid molecule were fully saturated, the molecule would be fairly rigid, and each ring would be shaped like a chair. However, some flexibility is introduced when the molecule contains double bonds in any of its six-membered rings. This

is contrary to the usual notion that a double bond is associated with decreased flexibility. In addition, a saturated five-membered ring is flexible and can assume many conformations. This ability is called pseudorotation. For example, in androgens the ring A is flexible, while the ring B is flexible in estrogens. This flexibility of naturally occurring hormones may have very important biological consequences because a steroid may assume different conformations for optimal binding to metabolizing enzymes, steroid receptors, or transport proteins.[27] It can be also assumed that the unusual retention behavior of steroids in HPLC and TLC can be explained as a result of changes in molecular shape due to flexibility of steroid molecules.

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