

Steroid Analysis

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Preface to the Second Edition

The second edition of this book has drawn heavily on the first edition but a huge amount of research on steroid analysis has been published over the last 15 years. As a result, the Editors decided to let the first edition of this book stand on its own and direct readers interested in pre-1995 steroid analysis to it, simply because the post-1995 research can on its own fill the second edition. We have tried to keep a balance but equally have allowed authors of each chapter a significant degree of freedom to approach their particular topics as they thought fit – they are after all the experts in their field. We hope that readers will agree that we have got the balance right.

In re-writing or updating these chapters, we have been greatly assisted by the developments in the availability of research publications electronically. Huge strides have been made in this area since 1995 and the ability to read a paper on one's computer rather than trekking to the British Library is a tremendous advantage. The editors wish to express their gratitude to their respective institutions (St. Bartholomew's and the Royal London School of Medicine, Queen Mary University of London and Kings College London) for providing us with electronic access to research journals from our home computers. Without such access, it would have been impossible to complete this book. Increasingly it is being recognised that while the purpose of research is to discover new scientific facts, discoveries must be disseminated to the scientific community. Unfettered and easy access to research publications is therefore vitally important. In the main, we have experienced very little difficulty in accessing research papers published in the last 20 years, though difficulties in accessing pre-1980 publications still persist, which is sad because the 'old methodology' may still offer solutions to today's problems. Regrettably, there still are journals which we have been unable to access – articles published in these journals are therefore not cited, unless, as has occurred on numerous occasions, authors of research papers in these journals have kindly provided us with copies of their publications. Where this has been done, we are very grateful. We recognise the difficulties which publishers, often learned societies which rely on income from their journals, have in allowing unrestricted access to their journals, but some solution must be found; otherwise those journals which do not allow access will not be cited and low citation rates will discourage authors from submitting their work to them.

At a late stage in the production of this book, we realised that enzyme nomenclature had moved on since the ‘dehydrogenase’ and ‘hydroxylase’ days and that cytochrome P450s have now been codified (Nelson et al. (2004); Nelson (2006)) and attempts are being made to re-name steroid dehydrogenase/reductase enzymes (Kavanagh et al. (2008); Persson et al. (2008)). Some chapter authors have been strict and have used the proper CYP names but others have not. We have not requested any author to change the steroid enzyme names which they have used, as in all cases, their terminology is clear and understandable to other colleagues in this area. We have, however, asked authors to ensure that there is no ambiguity in their text when referring to the enzyme or when referring to the gene which codes for the protein – mutations of course take place in genes and may be reflected in altered amino acid sequence and enzyme activity of the expressed protein.

We are immensely grateful to our colleagues in this endeavour, who have produced their chapters and put up with all the problems inherent in any multi-authored book such as this. We thank all our colleagues throughout the world who have willingly given their permission for us to reproduce their work. We hope that our cumulative endeavours satisfy our readers but we encourage anyone who finds errors or disagrees with opinions expressed herein to write to the editors or authors to let us know their views. Good opinions are nice but to avoid complacency send us the bad opinions as well.

We would like to thank our publisher (Springer) for being so patient with us and for their gentle encouragement during the course of chapter delivery. It is 18 years since David Kirk died in the course of production of the first edition – we miss him now as much as we did then.

Finally and most importantly we thank our wives, Margaret and Dorothea, for putting up with us and not complaining too much when things needed to be done, finding that we were ‘too busy with the book’ to help. Their contribution has been considerable and without them it would have been a more onerous and lonely task.

Hugh L.J. Makin
D.B. Gower

References

- Kavanagh KL, Jörnvall H, Persson B, Oppermann U (2008) The SDR superfamily: functional and structural diversity within a family of metabolic and regulatory enzymes. *Cell Mol Life Sci.* **65**; 3895–3906.
- Nelson DR (2006) Cytochrome P450 nomenclature, 2004. *Methods Mol Biol.* **320**; 1–10.
- Nelson DR, Zeldin DC, Hoffman SM, Maltais LJ, Wain HM, Nebert DW (2004) Comparison of cytochrome P450 (CYP) genes from the mouse and human genomes, including nomenclature recommendations for genes, pseudogenes and alternative-splice variants. *Pharmacogenetics.* **14**; 1–18.
- Persson B, Kallberg Y, Bray JE, Bruford E, Dellaporta SL, Favia AD, Duarte RG, Jörnvall H, Kavanagh KL, Kedishvili N, Kisiela M, Maser E, Mindnich R, Orchard S, Penning TM, Thornton JM, Adamski J, Oppermann U (2008) The SDR (short-chain dehydrogenase/reductase and related enzymes) nomenclature initiative. *Chem Biol Interact.* **178**; 94–98.

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