BAYKULOV A.K., AKRAMOV D.KH., BOZOROV KH.A.

PHARMACEUTICAL ANALYSIS METHODS

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The first part

"BILIG-ILMIY FAOLIYAT" NASHRIYOTI
SAMARQAND – 2024

UDC 811.111 -057.875 (076.5)

Pharmaceutical analysis methods. Recommended by Samarkand State Medical University. Samarqand: "Bilig-ilmiy –faoliyat" nashriyoti, –2024.– 136 b.

Authors: BAYKULOV A.K., AKRAMOV D.KH., BOZOROV KH.A.

Reviewers: **A.O. Nasrullaev**
Samarkand State UniversityInstitute of Biochemistry. associate professor of the department of "Organic Synthesisand bioorganic chemistry", PhD.

D.R. Siddikov
Institute of The Chemistry of Plant Substances of the Republic of Uzbekistan. laboratory of "Terpenoids and phenol compoundschemistry", PhD.

The use of analytical sciences in the discovery, development and manufacture of pharmaceuticals is wide ranging. From the analysis of minute amounts of complex biological materials to the quality control of the final dosage form. the use of analytical technology covers an immense range of techniques and disciplines. This study guide concentrates on the analytical aspects of drug development and manufacture. focusing on the analysis of the active ingredient or drug substance.

This study guide can be used in the course of teaching the subjects "Pharmaceutical analysis", "Pharmaceutical chemistry", "Toxicological chemistry" and "Organic synthesis" in the curriculum of the third-course Pharmacy- 60900700. senior. undergraduatePharmacy- 5510500 and master's degree programs of the Pharmaceutical Chemistry and Pharmacognosy- 70910701.

ISBN: 978-9910-9184-4-5

Preface

The pharmaceutical industry is one of the most active areas for the application and development of new methods in the analytical sciences. This volume provides those joining the industry or other areas of pharmaceutical research with a source of reference to a broad range of techniques and their applications, allowing them to choose the most appropriate analytical technique for a particular purpose.

No book on the analysis of pharmaceutical materials should ignore the important area of quality and regulation. The first chapter provides an up-todate overview of the philosophy and practicalities of working in a regulated environment, with reference to current regulations and guidance.

Subsequent chapters cover the major disciplines of separation sciences and spectroscopy. Recognising the importance and breadth of the area of separation sciences, our authors concentrate on method development in high performance liquid chromatography (HPLC or LC), capillary electrophoresis (CE), gas chromatography (GC) and thin layer chromatography (TLC), discussing traditional approaches in addition to the newer computational and chemometric methods.

One of the most important and challenging areas in the analysis of pharmaceuticals is the determination of chiral purity. It is therefore highly appropriate that a chapter is devoted to this area. The importance of chiral analysis is described, together with the development of techniques across the separation sciences and beyond.

In considering the spectroscopies, the development and widespread use of coupled techniques forms a major part of the volume in the chapters covering nuclear magnetic resonance (NMR) and mass spectrometry (MS). In the NMRchapter, extensive coverage is given to state-of-the-art coupled LC/NMR. The chapter also covers multi-nuclear NMR, computer-aided spectral interpretation, quantitative NMR and solid-state NMR - all important techniques applied in the pharmaceutical development laboratory.

Recent years have seen many important developments in MS. This book devotes a chapter to the technique, focusing on the varied instrumental capabilities, their basic principles of operation and their

applicability to pharmaceutical analysis. The applications of mass spectrometry, both in structure elucidation and quantitative analysis, are considered. Quantitative analysis MS is covered in detail, to allow the reader to gain background knowledge of a technique that is becoming important in drug substance analysis.

Vibrational spectrosopy can be used to support structural elucidation by NMR and MS, but more typically it is used for identity testing, because IR and Raman spectra act as a fingerprint for molecular structure. However, both IR and Raman find their principal application in the investigation of polymorphism. Examples are described in this chapter, together with the benefits of coupling these techniques to microscopy. Additional solid-state techniques are covered in the chapter on solid-state analysis and polymorphism. The determination and control of the solid-state form, in respect of both crystal structure and particle characteristics, are important. The physical properties of the drug substance will influence its behaviour during handling processes and formulation, and can have a dramatic effect on dissolution, solubility and therefore bioavailability.

Although microscopy and imaging are used in a number of the above disciplines, a separate chapter is devoted to the use of optical and electron imaging techniques and image analysis, which play an increasingly important role in contaminant analysis and drug-excipient distribution.

The final chapter deals with the increasingly important area of process analytical science. There is increasing interest in in-process measurements from the FDA and other regulatory bodies, and this seems certain to be a rapidly expanding area of the analytical sciences in the pharmaceutical industry.

The regulatory, environmental, technological and commercial drivers in the pharmaceutical industry have profound implications for the analytical chemist. We hope that this volume, contributed by specialists from both the industrial and the academic sectors, will prove to be a useful source of reference for all those interested in this rapidly changing field of science.

1 QUALITY CONTROL AND REGULATION

1.1 Introduction

Any person working within the pharmaceutical industry or allied/support industries in a scientific capacity will be well aware that a good deal of their everyday work requires conformance to quality standards dictated by various regulatory bodies. For a scientist coming directly from university into the industry, there may well be some culture shock and bewilderment caused by the plethora of standards and procedures that require to be followed as part of their job. Additionally, it may be disturbing to discover that non-compliance with these quality standards and regulatory requirements could result in severe penalties for their employer and loss of their own job.

One of the major disciplines impacted by these requirements is the analytical sciences. The issue could arise in a variety of departments that either directly or indirectly provide data for the assessment of pharmaceutical materials or the support of various regulatory filings. These pharmaceutical materials could be drug products (the formulated dosage form), active pharmaceutical ingredients (API, also referred to as drug substance or bulk pharmaceutical chemical), raw materials, starting materials and intermediates used for the production of these pharmaceutical materials, or even materials derived from toxicology experiments. The filings could, for example, be for an investigational new drug application (IND), a new drug application (NDA) or a marketing authorisation application (MAA). This list is by no means exhaustive, but is meant to illustrate the variety of roles governed by regulatory bodies that may be encountered by an analyst within the pharmaceutical industry, whether in an R&D or a commercial environment. These terms will be explained more fully later when it will also become clear that every aspect of the work that analysts perform is influenced by the requirements of these regulatory bodies and the need for analysts to be able to defend their work.

This chapter provides an overview of the quality systems and regulations an analyst may encounter in the pharmaceutical industry and the reasons for these systems, as well as a description of the various

regulatory bodies that they may have to interact with. The overview may serve as refresher training for those working in the industry at present, and as a tool to aid the understanding of the extent of regulation for those scientists thinking of entering the industry or who work outside of the pharmaceutical industry or in allied industries. The most current detail of requirements may be found in the various references quoted, which will normally direct the reader to an appropriate website.

We shall concentrate on the area of new chemical entities (NCEs) rather that new biological entities or biopharmaceuticals. These latter materials are governed by very similar regulations and quality requirements, but are somewhat outside of the experience of the author. The focus will be on regulatory requirements in the USA and Europe.

1.2 The quality of medicines

1.2.1 The meaning of quality

I could not continue to discuss quality systems, regulation and regulatory bodies that have jurisdiction over the pharmaceutical industry, without first discussing the meaning of quality in the context of medicines and why quality and hence regulation, are so important both in the commercial and R&D environments.

Most people have their own subjective view of the meaning of *quality*in everyday life. When asked to define exactly what they understand by the term, then their definitions tend to be rather woolly and it is obvious that there are different meanings depending on the environment that the term is used in. The traditional view of *quality*derives from the inspection/measurement approach used in quality control when *quality*consists of conformity with a pre-determined specification. For example, 'quality is the degree to which a specific product conforms to a design or specification'.

The view of *quality*, especially within the context of the pharmaceutical industry and quality assurance, has now moved away from this rather narrow view, and the definition more or less accepted at present tends to be *fitness for purpose*. The International Organisation for Standardisation (ISO) has further refined this definition in the application of the ISO 9000 quality standards to mean *fitness for purpose with*

customer satisfaction. This is to take into account the fact that the ISO 9000 standard covers an extremely wide range of products and services where customer satisfaction is the main determinant of quality.

When this definition of *fitness for purpose*is applied to the pharmaceutical industry, it can be seen to fit well with the concepts of quality. Personnel have to be shown to be *fit for purpose* for the job they are employed to do (training, education and experience). Equipment needs to be demonstrated as *fit for purpose*based on qualification/validation, maintenance and calibration. Manufacturing processes are deemed *fit for purpose*based on in-process testing, process validation, etc. Process materials are shown to be *fit for purpose* based on testing appropriate to their intended use in the process. This use will vary depending on the stage of processing and whether it is a primary (chemical) process or a secondary (formulation) process.

1.2.2 Medicines are special

Why are medicines different from other consumer products? Why is there so much regulation of the pharmaceutical industry? Why, in the light of modern analytical techniques, can't we rely on thorough testing of the final product (end-product testing) and do away with all of these bureaucratic systems? These are just some of the questions that are always asked of quality professionals in the pharmaceutical industry and I will try and answer them.

Medicines are special because virtually no other product is consumed by the public on such utter trust – trust that the medicine will not do them more harm than the illness it is meant to cure. Their doctor prescribes the medicine; it is dispensed by a pharmacist and is taken by the patient in the belief that it will cure their ill health and that it will not make it worse! This trust can only be assured if the medicine has been adequately tested during development. This testing should assure that side effects have been established and that the medicine is efficacious. When the medicine is given to patients it must have been appropriately manufactured, tested and packaged to assure that:

- It is the correct product.
- It is the correct strength.
- It has not degraded.

- It is free from harmful impurities and micro-organisms.
- It has not been contaminated.
- It is correctly labelled.
- It is properly sealed in a suitable container.

1.2.3 End-product testing

End-product testing (quality control) is the reliance only on appropriate analytical tests to demonstrate the quality of a medicinal material. End-producttesting alone is considered as *testing quality into the product*. You only acceptthose materials that pass specification. This is not adequate to ensure that themedicine is free from all manufacturing faults, that test methods employed areadequate to establish its purity and that a small proportion of defective materialswould be detected. I will give several examples to illustrate this point.

Example 1.1. All pharmaceutical industry regulators prohibit the manufacture
of penicillins in the same facility as other medicinal materials. The reason for this is the very high potential of extremely low levels of penicillin to cause serious side effects in individuals who are sensitised to this class of compounds. For this reason, cross-contamination levels are set at zero and the only way to ensure compliance is to not have the penicillin there in the first place. Firstly, the penicillin may contaminate at such a low level that existing analytical methods would not detect the presence of it, even if it were suspected of being present. Secondly, the penicillin contamination may not be distributed evenly in the medicinal material, making detection very difficult.

Example 1.2. (This example is taken from a real incident.) A solution of a chemical intermediate used for the production of an API was stored temporarily whilst waiting further processing. The storage containers used had previously contained a potent pesticide that had not been completely removed. These pesticide residues continued through the whole process to the final drug product, without detection. Apparently, the chromophore of the pesticide was very different from that of the material under test at each stage of manufacture and the analytical methods being used could not detect these low levels of contamination.

In conclusion, end-product testing is not adequate for the following reasons:

- You only test for what you expect to find.
- You only test a small portion of the bulk of the test material. (There couldbe a small proportion of defective material in a batch, which only 100%testing would detect, e.g. microbiological contamination of sterile vials.)
- It is doubtful whether, in the case of medicines, the consumer wouldddetect defects.

To assure the quality of medicinal products, quality must be built in at each stage of the manufacturing process and not merely tested in. Any factor that could have an effect on the quality of the final medicinal product must be controlled. These factors could be anything from the design of the production facility used or the environment the material is isolated in, to the analytical test methods employed at each stage of production. The philosophy of quality assurance is that batch to batch consistency should be maintained by reducing variability of all supporting processes, sub-processes and procedures. Hence, if written procedures control all of these factors and trained personnel follow these procedures, then a product consistently meeting its predetermined specification should be produced. End-product testing then becomes just a final check of the quality of the product. This testing is then used in conjunction with the written records, which demonstrate that all critical factors have been controlled, as the supporting documentation to allow the material to be released for use. This is what is meant by a quality system.

1.3 General quality system requirements

There are a number of quality systems that may be encountered by the pharmaceutical analyst but by and large the major one will be good manufacturing practice (GMP). Compliance to GMP is a requirement for the manufacture and testing of a drug product or API destined for human use, whether in the context of commercial manufacture or a clinical trial. Similarly, data supporting non-clinical safety assessment of chemicals requires to be generated to good laboratory practice (GLP). The majority of other quality systems are voluntary and are commercially driven.

Before discussing the various quality systems encountered by the pharmaceutical analyst, I would like to more clearly define some of the quality terms that are used to ensure some consistency, as these definitions may vary depending on the quality system. These definitions are taken from the GMP guide for API, ICH Q7A [3] as there is a good chance that more than one interested group has input into the definition.

Quality control (QC): Checking or testing that specifications are met.

Quality assurance (QA): The sum total of the organised arrangements made with the objective of ensuring that all materials are of the quality required for their intended use and that quality systems are maintained.

Quality unit: An organisational unit, independent of production, which fulfils both QA and QC responsibilities. This can be in the form of separate QA and QC units or a single individual or group, depending on the size and structure of the organisation.

Quality: Fitness for purpose.

Good manufacturing practice (GMP): GMP is that part of QA which ensures products are consistently produced and controlled to the quality standards appropriate to their intended use and as required by the marketing authorisation or product specification. GMP is concerned with both production and QA.

In previous sections, I have given a number of reasons why the whole aspect of medicine development, manufacture and testing requires to be performed in a different manner to that of any other product. There are many documented examples in the history of the pharmaceutical industry that demonstrate the necessity for regulations and regulatory bodies to oversee compliance to these regulations. There are cases of people being harmed by inadequate toxicity testing during development (e.g. thalidomide). There have been examples of the inadequacy of systems to ensure the integrity of tests performed in support of drug registration (resulting in the introduction of GLP). There have been not a few examples of the lack of assessment of the effect on the quality of drugs resulting from changes to the manufacturing environment and manufacturing route (resulting in the establishment of Good Manufacturing Practices Regulations). The establishment of these

regulations has been independently mirrored outside of the pharmaceutical industry by the introduction of quality systems, such as ISO 9000, in an attempt to bring consistency of quality to other industries and services.

There is a common theme to all quality systems whether used for pharmaceutical production or not.

- The quality system must be described in written documents approved by

management (policies, quality manual, etc).

- The quality system must be regularly reviewed.
- There must be senior management involvement in quality.
- All operations that can effect quality must be described in written andapproved procedures (standard operating procedures, SOPs).
- Materials must be appropriately approved prior to use.
- Output (product) must be appropriately inspected prior to release.
- Equipment used must be fit for purpose (qualified/validated, calibratedand maintained).
- Personnel must be trained in the quality system and in operations theyperform.
- Written records must be kept to demonstrate quality procedures havebeen followed.
- There must be regular internal quality audits to ensure quality is maintained.

In conclusion, all quality systems are to do with people, materials, equipment, records and procedures.

1.3.1 ISO 9000

This quality standard is a voluntary standard operated by many industries worldwide. Compliance to this standard demonstrates to customers that defined systems have been followed for the design, manufacture and/or testing of products.

The ISO 9000 quality standard was developed from the original BS 5750 quality standards and was issued in 1987. The standard was adopted as a European standard, EN29000, in 1988, and is one set of a whole set of ISO standards. The main part of the ISO 9000 standard is made up of three separate standards:

ISO 9001 Covers design, development, production, installation and servicing. This is the most comprehensive of the three ISO 9000 systems and is applicable to a supplier involved in original design. It is also commonly applicable to service activities where the service is being designed to meet specific requirement. This is typically found in contractors to the pharmaceutical industry where the development of a manufacturing process is performed. Similarly, it will be relevant to developers of computer software, although the ISO 9001-TickIT standard (specific to the development of software) would be more appropriate in this case.

ISO 9002 Covers production, installation and servicing.

This system is identical to ISO 9001 except it does not include the design part. This is the most common system used worldwide and can be applied to either manufacturing or service industries providing a standard product or service. A number of pharmaceutical manufacturers are certified to this standard.

ISO 9003 Covers final inspection and test.

This system has limited value and application as it encourages quality to be inspected in rather than designed in. It would be rare to find this standard applied within the pharmaceutical industry as the output of data is normally considered to be the provision of a service under ISO 9002.

These standards are operated as follows:

A national Accreditation Body, such as the United Kingdom Accreditation Service (UKAS), accredits a certification body (e.g. Lloyds, BSI, etc.) who in turn certifies individual companies that make a voluntary application to them. This certification is based on successful audits by trained ISO 9000 auditors. The company maintains its ISO standards by means of internal auditors and by annual or biannual audits and regular follow-up audits from the certification body.

These standards are a voluntary set of worldwide standards that a whole range of industries and services have adopted. Their relevance to the pharmaceutical industry has been questioned in the past, particularly by the USA Food and Drug Administration (FDA), on the basis that the system ensures consistency of development, manufacture and testing, but does not address the key issue of product quality, this being left to the

customer and supplier to agree. However, these standards have been adopted by a number of pharmaceutical companies as a means of laying a quality foundation with respect to quality management and on which to base and support further quality systems such as GMP. The ISO 9000 quality systems will also be followed by the majority of material suppliers and contractors to the pharmaceutical industry where the use of these materials and services does not require compliance to a higher standard such as GLP or GMP. In fact, it would be unusual for most pharmaceutical industries to accept any of these materials or services from a company that did not follow the appropriate ISO 9000 standard. For these reasons it is important for those working in the pharmaceutical industry to have some understanding of these standards.

The latest up date of the ISO 9001 standard is ISO 9001–2000 and may be obtained via the ISO's Website.

1.3.2 UKAS

Analytical scientists may encounter UKAS in a number of work environments. UKAS is the sole national accreditation body recognised by government to assess, against internationally agreed standards, organisations that provide certification, testing, inspection and calibration services. UKAS is a non-profit-distributing company, limited by guarantee, and operates under a memorandum of understanding with the government through the Secretary of State for Trade and Industry.

The knowledge that conformity assessment organisations are accredited by UKAS gives the confidence that they have been independently evaluated for their impartiality, competence and performance capability.

One of the responsibilities of UKAS is the accreditation of laboratories to issue NAMAS (National Accreditation of Measurement and Sampling) certificates and reports.

1.3.3 NAMAS

The NAMAS designation on a report or calibration gives the assurance that the work has been performed to the highest standards and that the laboratory has been stringently assessed by independent experts. There is further assurance that the work has been performed according to

agreed methods and specifications and that all measurements are traceable to national and international standards.

A laboratory may apply to UKAS for accreditation with respect to specific tests or calibrations. The laboratory is assessed by UKAS for that specific work and if it meets NAMAS requirements, then the laboratory will be accredited for those areas of work and can then issue NAMAS reports and certificates. UKAS publishes the NAMAS Concise Directory that lists all accredited laboratories and services.

As part of the accreditation process, UKAS assesses all technical aspects of the laboratory's practices and organisation and not just the quality system. Typical areas of assessment would be:

- Organisation
- Quality system
- Quality audits and review
- Personnel
- Equipment
- Measurement traceability
- Methods and procedures
- Environment
- Sample handling
- Records
- Complaints
- Sub-contractors and purchasing.

This assessment is very similar to the assessment performed for ISO 9000 certification and laboratories meeting NAMAS requirements for calibration and testing, where they are the supplier of these services, comply with the requirements of ISO 9002.

NAMAS accreditation is similar to the ISO 9000 certification process, in that it requires a thorough assessment by independently appointed industry experts. Six months following accreditation, a full follow-up visit is made by UKAS, and annual audits are made thereafter. Four years after accreditation a full re-accreditation assessment is made, although UKAS can make unannounced visits at any time. Laboratories found to be unsatisfactory on inspection will lose NAMAS accreditation until such time as that laboratory again meets the required standard.

NAMAS accreditation is an acceptable quality standard in a large number of countries both within the European Union and outside, and there exist a number of memoranda of understanding with these countries for mutual acceptance of standards.

Standards for the operation and accreditation of laboratories were originally set and published by the European Committee for Standardisation (CEN) in EN45001 and EN45002, (equating to ISO Guides 25 and 54) which were equivalent to the British standards, BS7500 series. The International Standard ISO/IEC 17025:1999 entitled *General requirements for the competence of testing and calibration laboratories* now replaces ISO/IEC Guide 25 and EN45001.

The pharmaceutical industry accepts a number of UKAS/NAMAS standards for a large variety of calibration and tests, although most companies would normally audit to confirm the acceptability of these standards in the case of very critical calibrations or tests.

1.4 Good laboratory practice (GLP)

1.4.1 Organisation for economic co-operation and development (OECD) GLP guide

The principles of GLP were originally developed under the auspices of the OECD and were first published in 1981 with later updates and guidance documents [7]. These principles are not legally binding but all OECD member countries have agreed to abide by them. However, the European Directive 87/18/EEC (amended by Commission Directive 1999/11/EC) requires that all EU member states must incorporate the OECD Principles of Good Laboratory Practice and Monitoring into national legislation. This has been accomplished in the UK as what is commonly known as The GLP Regulations. In the USA, the GLP regulations have been incorporated into national law under Code of Federal Regulations (CFR) 21 part 58 and may be found on the FDA's web site.

1.4.2 Principles of GLP

The principles of GLP define a set of rules and criteria for a quality system concerned with the organisational process and the condition under

which ***non-clinical*** health and environmental safety studies are planned, monitored, recorded, archived and reported. These principles have been developed to promote the quality and validity of data generated in the testing of chemicals. Apart from assuring the quality of data obtained from these studies that may have implication for human health, the other main advantage is the recognition by regulatory authorities of one country, of the data generated in other countries which hence avoids duplicative testing.

Any facility that produces data in support of these non-clinical studies is required to comply with the principles of GLP and will be audited for compliance by the appropriate regulatory authority. In the case of the UK this would be the United Kingdom GLP Compliance Monitoring Authority, which is part of the Department of Health. In the USA this would be the appropriate FDA department.

GLP must not be confused with that part of GMP that is concerned with operations in QC laboratories that assure the quality of medicines for human use (sometimes referred to as *good quality control laboratory practice*). Although there are a number of similarities in the practical aspects, GLP is in place to assure the integrity and quality of data; GMP is there to assure the quality of the product, i.e. its conformance to specification. There have been a number of statements recently from FDA sources on this subject.

- '(GLP)...is not the same as lab. work that tests finished drugs and activepharmaceutical ingredients. The GMP regulations have specific requirements for drug lab controls.'
- '. . . . manufacturers should not confuse good manufacturing practices(GMPs) with good laboratory practices (GLPs). The issue occasionallyarises, as some firms confuse the terms.'
- 'It's a semantic issue . . . . but you can be sure there is no confusion atFDA. FDA does not care how a company refers to its practices. It caresabout what those practices are.'
- 'While drug makers must account for many of the same issues in GMPsand GLPs, experts agreed, assessing quality and conformation to product specification is not the same as proving safety.'

One could of course argue that assurance of the integrity of data is also a requirement under any other quality system such as GMP. This

assurance requires that analysts are trained, that procedures are written and approved, that analytical equipment is calibrated and maintained, that reagents and test materials are controlled and that accurate records and original raw data are kept.

One could of course argue that assurance of the integrity of data is also a requirement under any other quality system such as GMP. This assurance requires that analysts are trained, that procedures are written and approved, that analytical equipment is calibrated and maintained, that reagents and test materials are controlled and that accurate records and original raw data are kept.

1.5 Good manufacturing practice (GMP)

GMP is probably the most widespread quality system followed across the pharmaceutical industry as a whole. GMP compliance is a requirement within the R&D environment for the manufacture and testing of clinical trial materials (both drug product and API) and for commercial manufacture and testing of these materials for human and animal consumption. R&D facilities performing these operations may be subject to audit for compliance to GMP; commercial facilities *will* be audited by the appropriate regulatory authority, possibly without prior warning.

1.5.1 USA GMP regulations

The USA Food, Drugs and Cosmetics Act (FD&C Act) states that 'All drugs shall be manufactured, processed and packaged in accordance with current good manufacturing practice' [Section 501 (a)(2)(B)]. No distinction is drawn between the manufacture of drug products (secondary manufacture) and the manufacture of APIs (primary manufacture). It is also noted in the preamble to the FD&C Act that the act applies to all drugs for human use, and this therefore includes the requirement for both APIs and drug products manufactured for clinical trials, to be manufactured according to cGMP.

The requirements for compliance to cGMP are lain down in the following Code of Federal Regulations (21CFR):

Part 210 Current Good Manufacturing Practice in manufacturing, processing, packing or holding of drugs.

Part 211 Current Good Manufacturing Practice for finished pharmaceuticals.

It must be noted that the US regulations refer to *current* GMP. The regulations as detailed in 21CFR parts 210 and 211, give the pharmaceutical manufacturer plenty of scope to interpret the requirements appropriately for his specific facility and process, but in doing this the regulations require the manufacturer to adopt best *current* practice. The onus is placed upon the manufacturer to keep current with what the industry is doing (best practice), with what the current interpretation of the regulations are, and what the US FDA's expectations are.

Although the FD&C Act requires all drugs (products and APIs) to be manufactured to cGMP, the regulations 21CFR parts 210 and 211 are only mandatory for the manufacture of drug products and not APIs. In the past the onus has been on the pharmaceutical industry to interpret these requirements with respect to the manufacture of APIs. FDA has published guidelines in the form of guides for FDA investigators, to assist industry to meet compliance to cGMP and to place their interpretation on cGMP requirements for APIs and a number of other key areas such as impurities in new drugs, allowable solvent residues and stability testing. Guides issued by ICH have now supplemented most of these guidelines and these, along with other FDA guidelines, will be discussed in more detail later in this chapter.

These regulations and guidelines may not always be appropriate for the manufacture of clinical trial materials. Although most of the regulations are reasonably applicable in an R&D drug product environment they may become inappropriate where attempts are made to apply them to the early manufacture of clinical APIs within an R&D environment. It is only with the issue of the International Conference on Harmonisation (ICH) Harmonised Tripartite Guideline ICH Q7A – *Good Manufacturing Practice Guide for Active Pharmaceutical Ingredients* in November 2000, that the worldwide pharmaceutical industry finally received detailed guidance for manufacture of APIs for both commercial and R&D purposes. (The scope and more detail of this guide will be discussed later.)

If one looks at the major headings of 21CFR part 211, the similarity with other

quality systems becomes apparent. It is mandatory to have controls in the following areas:

Subpart A – General provisions

Subpart B – Organisation and personnel

Subpart C – Buildings and facilities

SubpartD – Equipment

Subpart E – Control of components and drug product containers and closures

Subpart F – Production and process controls

Subpart G – Packaging and labelling control

Subpart H – Holding and distribution

SubpartI – Laboratory controls

Subpart J – Records and reports

Subpart K – Returned and salvaged drug products.

The areas of these regulations that will be most important for a pharmaceutical analyst will be:

Organisation and personnel – this includes the requirement to have a QC unit having . . . responsibility and authority to approve and reject all components, drug product containers, closures, in-process materials, packaging materials, labelling and drug products, and the authority to review production records to assure that no errors have occurred or, if errors have occurred, that they have been fully investigated. Further requirements cover laboratory facilities and the responsibility of the quality unit for approving or rejecting all materials, specifications and procedures. The responsibilities of the quality unit must be described in written procedures.

Laboratory controls– this part covers mainly calibration of equipment, testing and release procedures, stability testing, reserve samples, laboratory animals and penicillin contamination.

Records and reports– this part describes the key records that require to be retained. These include drug product component and container/closure records, labelling records, production records, production record review, laboratory records, distribution records and complaint files.

These requirements can be further compared with the ICH guidelines for API manufacture later in this chapter when I discuss worldwide harmonisation.

In conclusion, the USA cGMP regulations apply to interstate commerce within the USA and to any facility worldwide, that exports pharmaceutical materials (drug products, APIs, or components of these products) to the USA or, wishes to perform clinical trials in the USA. These facilities are open to inspection for cGMP compliance by US FDA inspectors and for those facilities found to be in non-compliance with these requirements the material will be deemed *adulterated with respect to identity, strength, quality and purity*. Products from these facilities will be refused entry for sell or use within the USA. Data from these facilities may not be accepted in support of regulatory filings.

1.5.2 EU/UK GMP requirements

Two European directives lay down the principles and guidelines for GMP in the EU, one for medicinal products for human use and the other for veterinary products. These directives have been incorporated in the national law of member states. The European Commission has issued nine volumes of *the rules governing medicinal products in the EU*. The latest edition was issued in 1998. Volume four covers GMP for medicinal products for human and veterinary use. These are now used as a basis for inspection by the various national regulatory authorities (e.g. Medicines Control Agency (MCA) in the UK).

If one looks at the requirement of the EU GMP rule, the similarity with 21CFR part 211 is clear, as is the consistency with other quality systems. The basic requirements are detailed under the following chapter headings:

Chapter 1: Quality assurance
Chapter 2: Personnel
Chapter 3: Premises and equipment
Chapter 4: Documentation
Chapter 5: Production
Chapter 6: Quality control
Chapter 7: Contract manufacture and analysis
Chapter 8: Complaints and recall

Chapter 9: Self-inspection

There are a further 14 Annexes:

Annex 1: Manufacture of sterile medicinal products

Annex 2: Manufacture of biological medicinal products for human use

Annex 3: Manufacture of radiopharmaceuticals

Annex 4: Manufacture of veterinary medicinal products other thanimmunologicals

Annex 5: Manufacture of immunological veterinary medicinal products

Annex 6: Manufacture of medicinal gases

Annex 7: Manufacture of herbal medicinal products

Annex 8: Sampling of starting and packaging materials

Annex 9: Manufacture of liquids, creams and ointments

Annex 10: Manufacture of pressurised metered dose aerosol preparations for inhalation

Annex 11: Computerised systems

Annex 12: Use of ionising radiation in the manufacture of medicinal products

Annex 13: Manufacture of investigational medicinal products

Annex 14: Manufacture of products derived from human blood or human plasma

1.5.3 USA/EU GMP differences

Historically there have been distinct and fundamental differences between USA regulation and EU/UK requirements for GMP. As discussed previously, the US required all *drugs*to be made to GMP requirements and performed inspections throughout the world in support of these requirements. In the UK, only drug products and biological manufacturers (not APIs, except some specified antibiotics) were inspected by the regulatory authority for compliance to GMP. Other EU countries, such as France and Italy, did require audits of API manufacturers, but the requirements and standards varied widely throughout the EU.

Although drug product manufacturers have always been audited by the UK authorities, the UK GMP guideline (The Orange Guide) was not

mandatory and did not have the force of law. (Although non-compliance with GMP would result in non-approval of the facility to manufacture drug products for selling.) The original European Directive defined a medicinal product as 'Any substance or combination of substances presented for treating or preventing disease in human beings or animals.' This applied to finished pharmaceutical dosage forms (drug products) only.

There are fundamental differences between a drug product and a starting material (API) that makes the application of many GMP drug product requirements difficult or inappropriate. An API is normally prepared by chemical processes that involve purification at each stage of manufacture, and early raw materials and processing stages may not have much influence over the quality of the final API. Impurities that are present in the final API will not be removed and will still be present in the manufacturer drug product. Similarly,if the morphic form of the API is changed through unassessed changes in the API manufacture, this could have a considerable effect on the bioavailability of the drug product. To use the API based on end-product testing, as previously discussed, is not in keeping with the principles of QA.

Historically, in the UK and Europe, there has been no legal requirement to manufacture drug products or APIs to GMP for use in clinical trials (investigational medicinal products, IMPs). This has always been a requirement under the USA FD&C Act.

The situation in the EU with respect to APIs and IMPs is now changing with the requirement for consistent standards throughout the EU and the wish to harmonise inspection standards and other regulatory requirements with other countries. The lack of GMP controls for APIs and IMPs has been seen as a major barrier to harmonisation with the USA. Harmonisation with the US through a Mutual Recognition Agreement (MRA) is seen as a big saving of inspection resources to both the EU and the USA, through mutual acceptance of facility inspection reports.

The EU *Starting Materials Directive* has now been resurrected and is expected to be included as an amendment to the EU GMP legislation. The Starting Materials Directive was originally drafted in September 1997, with a second draft in 1998 after a large number of amendments. The original definition of a starting material included the API and also

inactives such as excipients and the container/closure, but it is now expected to only include APIs. Because of the changes in the European Commission in 1999, the Directive was no longer seen as a priority and was never accepted by the European Parliament. With the advent of ICH Q7A, to which the EU is a signatory, the Starting Material Directive is now expected to be included as an amendment to the EU GMP legislation, with ICH Q7A used as a basis for defining GMP requirements for APIs within the EU.

The Clinical Trials Directive requires clinical materials (IMPs) to be manufactured to GMP and must be incorporated into each member state's national law by 2004. The European Commission has issued the detailed requirements for IMPs as Annex 13 of volume four of the GMPs rules.

The Clinical Trials Directive:

• Mandates that all EU member states incorporate GMP for IMPs intotheir national legislation.

• It should provide a standard set of GMP requirements across all memberstates.

This Directive, through Annex 13, will require for all IMP manufacturing facilities:

• compliance of all facilities to (GMP) Directive 91/356;

• inspection by competent authority (e.g. UK MCA);

• registration of manufacturer/importer;

• licensing of site of manufacture;

• qualified person (QP) to certify/release products. (Duties of QP defined in Directive 75/319/EEC);

• all clinical trials to be conducted to good clinical practice (GCP).

1.5.4 International GMPs

Once again I must apologise for concentrating on quality systems in the USA and the EU, although I mean no disrespect to the rest of the world. There is a very good reason for this. A large number of those countries that have pharmaceutical industries, especially generic pharmaceutical manufacturers, have set up GMP QS based on USA requirements for the simple reason that the USA has the largest pharmaceutical market in the world. A large number of API

manufacturers export to the USA and hence have required to comply with USA cGMP for pharmaceutical manufacture.

The World Health Organisation (WHO) for a number of years has been very active in setting global GMP standards for both drug products and APIs. They have issued two chapters of guidelines for GMP. Chapter 1 concerns general considerations for GMP, more detailed guidance under the heading of Good Practices in Production and Quality Control, an international pharmacopoeia and a very useful glossary and definition of terms. Chapter 2 gives guidance on GMPs for APIs and excipients. It will come as no surprise that the headings of the guidance covers those already given earlier when discussing USA and EU requirements.

GMPs represent a technical standard upon which is based the 'WHO Certification scheme on the quality of pharmaceutical products moving in international commerce'.

1.6 International harmonisation of quality standards

Many countries have seen the advantages of harmonisation of standards and have formed MRA. The European Community (EC) has negotiated MRAs between the community and third countries, which include mutual recognition of standards of GMP and arrangements to ensure compliance by pharmaceutical manufacturers. At present such agreements, operating with New Zealand and Australia, are expected to be in operations soon with Canada and Switzerland, and are in a transitional evaluation phase with the USA. Negotiations are also underway with Japan. Under an MRA, the regulatory authorities accept each other's inspection reports, and routine inspection by one authority of manufacturers in the other's territory is not required. In addition, the re-testing of imported products is normally not required.

The main instrument for the harmonisation of standards between the EU, USA and Japan is through the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH). Detailed information on the guidelines issued to date can be found through their Website.

The following is a summary of the quality topics issued by ICH as draft or full guidelines:

Q1 Stability Q1A Stability testing of new drugs

Q1B Photostability testing

Q1C Stability testing of new dosage forms

Q1D Bracketing and matrixing designs for stability testingof drugsubstance and drug products.

Q2 Analytical validation

Q3 Impurities

Q4 Pharmacopoeias

Q5 Biotechnological quality

Q6 Specifications Q6A Chemical substances

Q6B Biotechnological substances

Q7 GMP Q7A GMP for APIs.

1.7 Quality control, quality assurance and regulatory filings

No medicinal product may be sold without being licensed by an appropriate regulatory authority. Normally clinical trials can only be carried out under the control of the appropriate regulatory authority and a filing of some form is required in all countries.

It is very important to realise that information obtained during both the preclinical and clinical phases of drug development are pivotal to the success of the registration of the product. The toxicology testing is performed on early supplies of material. The health and welfare of patients on clinical trials is determined by the quality of the test material and its equivalence to the material tested in the pre-clinical programme. Eventually, all batches of the commercial material must be shown to be equivalent in quality to that used in clinical trials and the toxicology programme. No further clinical or toxicology studies are normally performed before the material is commercially manufactured.

A regulatory inspection usually concentrates on what the facility has done, rather than what it will do. Even in the latter case, as with compliance to a product licence, what the facility will do in future must be based on its past record, the systems that have been put in place and its compliance with them. At present in an R&D facility, possibility of inspection does not normally arise until an application is made for a product licence, which could be a number of years after the work has been

done. For these reasons it is vital that everything that is done is accurately recorded. An FDA inspector was once quoted as stating 'If it's not written down it's only rumour.' For the pharmaceutical analyst this may mean they have to defend their work possibly five or more years after it was completed. Even worse, the analyst may have to defend the work of a peer or a subordinate. Without good records this will not be possible and failure to do so could adversely affect a regulatory submission.

There is a considerable difference in the standard and type of information required by the various national regulatory authorities throughout the world. For example, until recently it was possible to perform clinical trials within some European countries without submission of any manufacturing information; in other European countries Phase I clinical trials could be performed solely on the permission of a local ethics committee or just a doctor or dentist's approval. Requirements have now been harmonised across the EU with the Clinical Trials Directive discussed previously. Further attempts at harmonisation continue through ICH or are being driven by various MRAs. In the USA, a considerable amount of information has always been required for all phases of clinical trial from Phase I to Phase III.

In the space of this review, I cannot discuss all of the different national requirements for the various stages of development although I will give some guidance on general expectations for the pharmaceutical analyst.

1.7.1 Pre-clinical development

Materials do not have to be manufactured under GMP conditions, but it is recommended that GMP controls or controls approaching those of GMP should be in place for the following reasons:

- It is not a good practice to have dual systems (GMP and non-GMP) operating in the same facility, otherwise the standards will always drift to the lowest standard.
- It is a business risk in that undetected impurities can give a false result in a toxicology study, resulting in premature termination of the project.

• Frequently the same batch of material that is used in the pivotal toxicology study is needed for Phase I clinical trials. There is then pressure to
retrospectively assign GMP status to that batch of material based on the toxicology study. This is not in accordance with QA principles where quality should be built in rather than tested in.

Having made these suggestions, *it is* a GLP requirement that the testing of these materials is performed according to GLP or an equivalent standard (see discussion previously). Facilities directly responsible for the toxicology studies or supporting those studies *will* be audited by the national or other interested GLP monitoring authority. Some national authorities will audit the test facility for the test article analysis.

It is not expected that analytical methods will be fully validated and that a specification will be in place, but the other quality requirements previously discussed such as equipment calibration, reagent control, training, etc. must be in place in the analytical facility. Some best estimate of the stability and storage conditions for the test article is required under GLP.

1.7.2 Early phase development (Phases I/II)

Clinical supplies (API and drug product) must be made and tested to an appropriate level of GMP to assure patient safety. In an analytical facility the level of GMP does not significantly change throughout development, excepting that initially analytical methods may not be fully validated, and specifications or other acceptance criteria are likely to change, as more information becomes available.

It is recognised that during early development of a manufacturing process and of the analytical methodology, there will be changes. What is vital is that all changes made to the process are assessed for their impact on the quality of the final product. Major changes to the route of manufacture normally require updating of the regulatory filing and an analytical investigation to determine if any new impurities are formed as a consequence of the change. Additionally changes at the final stage, especially for APIs, should be carefully assessed for their impact on the morphic form of the material, as changes in the form can have serious effects on bioavailability. Similarly, major changes to analytical methods

must be assessed for equivalence and normally require updating in the regulatory filing. Analytical methods for the API and drug product should be stability indicating. Special attention should be paid to changes of process solvents, where the allowable levels of solvent residues may change. New stability studies on materials obtained via major route changes would normally be initiated.

At the present time, it would be unusual for a regulatory authority to audit an R&D facility that manufactures clinical supplies for Phase I and II studies, for compliance to GMP or for compliance to a clinical trials *chemistry, manufacturing, controls* (CMC) part of a submission. This is however, likely to change in Europe with the implementation of the Clinical Trials Directive, when facilities (possibly including API facilities) will require to be certified. It is still always possible that a facility named in a submission will be audited for cause. For example, if something goes wrong during a clinical trial and a patient or volunteer becomes seriously injured or dies, the appropriate regulatory authority may include in its investigation, the R&D facility that made the test material. At this time it is highly likely that all records and quality systems will be subject to close scrutiny and all personnel may have to justify decisions they have made, and defend their work. Personnel should be reminded that fraud and negligence could be severely punished by a regulatory authority.

1.7.3 Late phase development (Phase III)

Most of the GMP requirements mentioned for Phases I/II will still hold, although it is considered that the route and analytical methodology should be approaching the consistency expected of a commercial product. Specifications for the API and drug product must be in place, specifications for raw materials and intermediates should be nearing finalisation, and analytical methods should be fully validated to pharmacopoeial standards, etc.

Process validation would not be expected until commercial batches are produced but information on process boundaries and critical parameters should be obtained at this stage. It should also be pointed out that if the pivotal stability studies to be listed in the final registration (on the drug product or API) are performed on pilot batches, then this data

certainly could become subject to inspection. It is becoming increasingly common in Europe and the USA for R&D facilities to be audited as well as the commercial facility before approval of a manufacturing licence, even though the R&D facility has not manufactured any commercial supplies.

1.7.4 Commercial manufacture

Throughout the world the manufacture of pharmaceutical products for sale requires to be performed under the control of an appropriate regulatory authority. A manufacturing licence of some form must be obtained and compliance of the facility with GMP is a pre-requisite. Before a licence is approved the commercial facility will need to have been subject to a recent regulatory inspection and/or have demonstrated a good compliance history via previous, recent audit. This inspection will determine that the facility complies (or should comply) with the route of manufacture, the specification specified in the licence application, and is compliant with GMP requirements. Facilities that are not in compliance will be required to give an undertaking to improve, or could be refused a licence. In some countries (e.g. the USA) where cases of fraud, extreme negligence or non-compliance are discovered, such that there is a strong possibility of risk to public health, companies and individuals can be subjected to harsh penalties including heavy fines and/or imprisonment. For the USA, companies that are not in compliance, do not respond to inspection observations (FDA 483s) adequately or promptly, or do not complete corrective action plans as agreed with the FDA, risk a warning letter that could prohibit the sell of their products in the USA. Furthermore, a Consent Decree could be issued which effectively puts production and release of each batch of the affected product under close regulatory control, at the firm's expense, until such time as there is perceived to no longer be a risk to public health.

At the present moment the European regulatory authorities tend towards the situation where a company's compliance history and the fact that the specific facility is within a two-year satisfactory inspection window, to determine whether an audit is required. The USA authorities have in the past tended to rely on pre-approval inspections (PAI) directed specifically at the filing, along with a biennial inspection programme of

all facilities. They are at present moving more towards reliance on the biennial inspection to be consistent with European authorities leading up to the MRA (and to save inspection resources). These inspections, depending on the regulatory authority, can take anything from two days to a number of weeks and can be very resource intensive for the company under inspection. They can also be unannounced although foreign inspections tend to be pre-planned for logistic reasons. The US FDA issued a useful Foreign Inspection Guide [18] that gave guidance to inspectors visiting foreign companies. This type of inspection may now change following the FDA's acceptance of system's audits (discussed later).

1.8 Regulatory inspection key areas

Some examples of key areas of concern of some regulatory authorities are given
in the final part of this review, along with some actual examples taken from public files. Most of these examples are taken from the US FDA's observational findings, as these are available in the USA under the Freedom of Information Act. Many of the guidelines discussed below can be found on the web site of the appropriate regulatory authority.

1.8.1 Inspection of analytical test facilities

Problems with analytical facilities is regularly cited by the US FDA as one of the main reasons for the non-approval of a NDA or abbreviated new drug application (ANDA). The mid-Atlantic region of the US FDA issued *A Guide to Inspection of Pharmaceutical Quality Control Laboratories* [19] and although it is ten years old now, most of the information is still valid (except the more recent requirements on computerised systems which will be discussed next). In this guide to inspectors, the FDA discusses the various approaches to an inspection. The inspection might cover one or more of the following topics:

- The specific methodology which will be used to test a new product.
- The complete assessment of laboratory GMPs.
- A specific aspect of laboratory operations.

The following is a summary of some of the typical areas that an inspector would be required to scrutinise for both drug products and APIs.

Pre-approval inspections are designed to confirm that the data submitted in an application is authentic and accurate and that the procedures used in the application were actually used to produce the data contained in the application. Since not all data can be included in a submission, the inspection must determine if there is sufficient justification for certain data not being reported, especially that data pertaining to batches that fail to meet a predetermined specification. The review would include:

• A comparison of the results of analyses for those applications for whichmore than one batch has been produced.

• An evaluation of the methods and exceptions to the procedures or equipment
actually used from those listed in the application. Confirmation thatit is the same
method listed in the application.

• An evaluation of raw laboratory data for tests performed on biobatches andclinical batches and comparison with the data filed in the application.

Laboratory records and logs represent a vital source of information that allows a complete overview of the technical ability of the staff and of the overall QC procedures. Laboratory data review would include:

• An evaluation of raw laboratory data worksheets and personal notebooks, laboratory procedures and methods, laboratory equipment, andmethod validation data to determine the overall quality of the laboratoryoperation and the ability to comply with GMP regulation. This willinclude checking that data is not missing, results have not been changedwithout adequate explanation and traceability and calculations are accurate and have been checked by a second person.

• Ensuring that raw data is maintained in bound, not loose-leaf, books, or onanalytical sheets for which there is accountability (e.g. pre-numbered sheets).

• An examination of chromatograms and spectra for evidence of impurities, poor technique or lack of instrument calibration.

• An investigation of laboratory test failures. Evaluation of results ofanalyses of re-tested and failed batches. Review of decisions to releasebatches failing specification. Products cannot be *tested into compliance*.

• A check that data has not been transcribed without retention of the original records.

• Determining that the laboratory results are actually used to release lotsof the product and ensuring that data has not been selectively used.

The review of microbiological data regarding topical and parenteral manufacture would typically include:

• A review and evaluation of the preservative effectiveness.

• A review of the bioburden from both an endotoxin and sterility perspective.

• A review of environmental monitoring methods and data.

Laboratory standard solutions must be ascertained as being suitable for use in that theyare:

• stored correctly;

• have expiry dates appropriate to their stability;

• have adequate records of preparation.

*Methods validation*would be critically reviewed for completeness, accuracy and reliability. If an alternative to a compendial method is used, this in-house method must be compared to the compendial method to demonstrate it is equivalent or superior to the official procedure. Appropriate pharmacopoeial guidelines for methods validation should be followed.

*Laboratory equipment*usage, maintenance, calibration logs, repair records and maintenance procedures should be examined. Data would be suspect if it were generated from equipment known to be defective. Continuing to use and release products on the basis of such equipment would be a serious violation of GMP. The inspection would need to:

• Confirm the existence and condition of equipment specified in themethod.

• Verify that equipment was present and in good working order (maintained and calibrated to pre-defined procedures) at the time the batcheswere analysed.

• Determine that equipment was used properly.

• Evaluate completeness of equipment and maintenance logs.

Raw materials (*API*) quality and purity largely determines the safety and efficacy of the drug product. The same standard of laboratory control is required for testing of APIs as for the drug product. If the API is obtained externally, the drug product manufacturer must have a system in place for evaluating the supplier with respect to quality, and assuring the accuracy of the supplier's certificate of analysis (C of A). Normally this would be performed by an audit of the API facility for compliance to GMP and qualification of the C of A through duplicate testing of a number of batches.

*Stability indicating methods*must be used to test the samples of the batch. Evidence that the method is stability indicating must be presented, even for compendial methods. Manufacturers may be required to carry out accelerated or forced degradation of a product to demonstrate that the test is stability indicating. The inspection should:

• Evaluate the validation report for stability testing.

• Review the raw data and the results of testing at the various stabilitystations to determine if the data actually reported matches the data foundin site records.

• Evaluate the raw data used to generate the data filed documenting thatthe method is stability indicating and the level of impurities.

*Other areas of inspection focus*include sampling protocols, in process controls and specifications, further guidance on the quality of data, computerised systems (more details will be given in the next section), and laboratory management to determine the overall GMP compliance of the facility. This latter area would include supervisory control and review and personnel qualifications and training.

1.8.2 Computerised systems (21 CFR part 11)

Many regulatory agencies and consultants to the pharmaceutical industry throughout the world have put out some sort of guidance in the past concerning the use of computer systems. It is only within the last six years that this guidance has been given the force of law in the USA in the form of 21 CFR part 11 [20].

Guidance on the use of computerised systems within the pharmaceutical industry was given in the 1992 inspection guide discussed

in the previous section. This concentrated on computerised laboratory data acquisition systems and was concerned with system security, audit trails of changes/deletions and procedures to ensure validity of the data. Since the mid-1990s it has been a US FDA expectation that major computer systems would be validated to ensure these concerns would be addressed. Occasionally the pharmaceutical newssheets would be full of serious adverse findings with respect to the computer systems of major pharmaceutical companies.

In spite of this guidance and these adverse findings, the pharmaceutical industry was originally only pressing the US FDA to come up with some more guidance on the use of electronic signatures to be used in place of traditional hand-written signatures. Following industry comment, in 1997 21 CFR part 11, Electronic Records, Electronic Signature, Final Rule was issued by FDA. The rule covered nearly 40 pages in the Federal Register (although the actual rules only covered two pages, the rest being preamble, scope, etc.) and it became effective on 20 August 1997. From this date onwards all computerised equipment records and the use of electronic signatures in support of regulatory processes were required to comply with this legislation. The US FDA saw this rule as an attempt to encourage industry to modernise with respect to the use of electronic systems and electronic submissions. The FDA stated in the preamble 'These regulations, which apply to all FDA program areas, are intended to promote the widest possible use of electronic technology, compatible with FDA's responsibility to promote and protect public health.' In many cases it has had the opposite effect, with some companies trying to revert (erroneously in most cases) to hard copy records. This rule has had far ranging effects within the pharmaceutical industry and by some estimates is likely eventually to have cost the industry considerably more than the Year 2000 preparation. One of the main reasons is that the rule applies to *all records*generated after 20 August 1997, even though virtually no equipment, at that time, could comply with the requirements. Although the US FDA gave the industry time to comply with the rule and to even put temporary dual hard copy/electronic systems in place, the expectation is that all companies will now comply with 21 CFR part 11.

Some of the main requirements of 21 CFR part 11 are as follows:

• The rule applies to electronic records that are created, modified, maintained,archived, retrieved or transmitted, under any agency regulations. It alsoapplies to electronic records submitted to the agency under requirementsof the FD&C Act, even if such records are not specifically identified inagency regulations.

• Systems must be identified as *closed* (system access controlled by peopleresponsible for content) or *open* (system access not controlled by peoplewith the responsibility for content).

The following requirements will concentrate only on closed systems.

• The system must be validated to ensure accuracy, reliability, consistentintended performance, and the ability to discern invalid or altered records.

• The system must have the ability to generate accurate and completecopies of records in both human readable and electronic form.

• Records must be protected to enable their accurate and ready retrievalthrough the record's retention period.

• The system must limit access only to authorised persons.

• The system must have equivalent levels of controls expected of a papersystem such that there are computer-generated, time-stamped audit trailsof all modifications, deletions, additions, etc., the system identifies theperson making these changes, and the original entry is not obscured.

• Operational system checks should be in place to enforce permittedsequencing of steps and events, where appropriate.

• The system only allows authorised persons access and identifies thosewho are authorised to make changes to records.

• If appropriate, the system employs checks to determine the validity ofthe source of data input.

• All person involved with electronic records and systems have adequateeducation, training and experience.

• There should be adherence to written policies that hold individuals accountable and responsible for actions initiated under their electronic signature.

• There must be appropriate controls over system's documentation including distribution, revision and change control.

With respect to electronic signatures, the following are the key areas:

• Each company must certify to FDA that they intend to use electronicsignatures as being equivalent to hand-written ones.

• Each electronic signature must be unique to one individual and shall not be reused by, or reassigned to, anyone else.

• The electronic signature must contain information to indicate who signed, date and time, and the meaning of the signature.

• Non-biometric signatures must employ at least two distinct identification components such as an identification code and password.

• Electronic signatures must only be used by their genuine owner.

• Systems must require that use of an electronic signature by anyone otherthan its genuine owner require collaboration of two or more individuals.

• Systems must be in place to ensure that each combination of identification code and password is unique to the individual.

One of the biggest misunderstandings of 21 CFR 11, is that many companies have interpreted the rule as allowing the printing of hard copy of an electronic record and signing/dating of this hard copy, as a substitute for maintaining the electronic record in compliance with the rule. Although dual systems (maintaining both hard copy and electronic raw data records) were to some extent accepted by FDA in the early days following introduction of the rule, this is not now an acceptable practice. It is certainly not acceptable to delete original electronic raw data and only retain a hard copy. It has been made clear by FDA on numerous occasions that if equipment has the ability to store an electronic record, then the electronic record is the raw data, not the hard copy print out.

This rule has wide applicability across the pharmaceutical analysis area, in that if any computerised equipment generates data that may be used in support of regulatory filings or pharmaceutical manufacture, compliance with the rule is mandatory. This includes stand-alone equipment such as HPLCs, etc., although primary target for regulatory scrutiny would be laboratory information management systems (LIMS) and data acquisition systems.

1.8.3 Out-of-specification (OOS) test results

In a ruling dated 4 February 1993, Judge Alfred Wolin of the US District Court for the District of New Jersey handed down a ruling which

has had far reaching consequences as to how the pharmaceutical industry treats OOS and other suspect data. This ruling has become better known as the Barr Ruling, from the case USA vs Barr Laboratories. Although the ruling is not mandatory outside of the Court's jurisdiction, it has become the model by which the US FDA would treat laboratory failures. The US FDA considers it an expectation that every company they inspect will have a SOP that describes how these data are treated.

A summary of the key points of the Barr Ruling is as follows:

• The Court rejected FDA's position that a batch failure occurs when anindividual test result doesn't meet specification. OOS results can be dueto laboratory errors, non-process-related (operator) errors or processrelated (manufacturing) errors. Only non-process or process-related errorsare properly identified as failures.

• The Court ruled that OOS results can be identified only through aninvestigation. The type and extent of the investigation depend on whatcaused the OOS result.

• Failure investigations must be documented and performed within 30business days.

• The Court determined that laboratory errors occur when analysts makemistakes. These types of errors must be reported to and reviewed by asupervisor according to a written procedure. The review should be documented and the cause of the error identified if possible.

• Retesting is permissible if the OOS result was due to laboratory error.Other types of error require further investigation.

• Judge Wolin ruled that if a failure investigation shows that a laboratoryerror didn't occur, or is inconclusive, the investigation has to extendbeyond the laboratory.

• The Court's opinion on outliers was that since, for chemical testing, theUSP does not provide for outliers, the use of outlier testing for chemicalanalyses is prohibited. The Court also agreed that the USP specificallyallows outlier testing for biological and antibiotic assays. (This opinionhas been challenged a number of times on scientific grounds.)

• The Court ruled that re-testing is appropriate if the failure investigationhas determined that re-testing is appropriate. Re-testing is not appropriate if the error was process-related, or for product failures.

• Re-testing must be done on the same sample that produced the original,failing test result. It can substitute for the original result if the error wasdue to an analytical mistake, and it can supplement the original result ifthe investigation is inconclusive. There should be predetermined testingprocedures defining when re-testing ends and
results should be considered in terms of overall batch and product history.

• The Court ruled that companies cannot rely on re-sampling to release aproduct that has failed testing and re-testing unless re-sampling is inaccord with the USP standards (content uniformity and dissolution), orunless the failure investigation discloses evidence that the originalsample is not representative or was improperly prepared.

• The Court ruled that although the averaging of test results can be a rationaland valid approach, as a general rule this practice should be avoidedbecause averages hide the variability among individual test results. Thisphenomenon is most troubling if testing generates both OOS and passingindividual results which when averaged are within specification. Here,relying on the average figure without examining and explaining the individual OOS results are highly misleading and unacceptable.

In September 1998, the US FDA issued a draft OOS guidance for industry. For further comment see [22].

It is obvious that *testing into compliance* (repeating testing until you obtain the answer you want), the averaging of failing results with passing results to give a passing result and the discarding of any data without good reason are all not acceptable practices within the pharmaceutical industry.

1.8.4 System audits

'The organisation of the future must be able to use the information it generates to improve itself.'

This comment was made by a US FDA representative at a conference in December 2001 and further was discussed in issues of the *Gold Sheet*. FDA is warning companies that they expect inspection findings to be acted on not just to fix a situation, but to improve the whole QS. If a large multinational pharmaceutical company receives 483 observations, then commitments for corrective and preventive actions should extend to all similar facilities throughout the company. This

process is getting to the real purpose of QA and audits. It is well understood that things go wrong, equipment fails and people will always do some things wrong, but it is how each company reacts to these findings. FDA audits in future will concentrate on the QS itself. The audits will look at how the system ensures not only that corrective and preventive actions are put in place, but how management is made aware of quality issues, how the systems are improved in response to all audit findings and how management ensures that the improvements are sustained. FDA has become aware over the last few years, that as companies try to save resources, the quality unit (QA and QC) the area that becomes squeezed. Data shows that the number of employees in quality has decreased in relation to those in production and other areas such as marketing. FDA is very concerned with this situation.

System audits will always examine the whole QS in a company and additionally at least two of the other systems listed in the examples given below. These system audits may also be in conjunction with a product specific audit.

QSs would include:

- Change control
- Quality investigations
- Corrective actions
- Preventive actions
- Management review of the quality management system (QMS)
- Out-of-tolerance calibrations
- Out-of-specification results
- Equipment failures
- Training issues
- Internal and external audits.

Other systems could include:

- Facilities and equipment
- Materials
- Production
- Packaging and labelling
- Laboratory controls
- Computer Systems compliance.

FDA pre-approval inspections in the past would normally concentrate on the information submitted in the NDA. There could be (and often are) many other quality problems that would not be uncovered by this narrow focused audit. In fact dual systems could exist in a facility without auditors becoming aware of the fact.

System audits of the type discussed are more similar to the general GMP type of audits performed by the European authorities and also by the author of this work. When there is limited time, such as one or two days, to complete the audit of a large facility, then an audit of the QSs as a whole is a very powerful tool. If this is coupled with a product specific audit to challenge the operation of the systems, then this is even better for assessing the facility.

1.9 Conclusions and the future of regulatory scrutiny

In this chapter, I have attempted to give an overview of the various QSs that are operated within the pharmaceutical industry. Some of these such as ISO 9000 and NAMAS are voluntary and are followed in order to give the company a competitive advantage. Others such as GMP and GLP are mandatory for any company manufacturing materials for human or veterinary use or who supply data to regulatory authorities on the safety of chemicals, whether in an R&D or commercial environment. Hopefully, I have also tried to show that although the R&D environment has different requirements with respect to quality from the commercial manufacture of pharmaceutical materials, quality is still a vital part of most R&D's work.

With respect to where quality and regulation are going, one needs to look at the way MRAs are being introduced between regulatory authorities worldwide. In an effort to control inspection and regulatory submission overheads,there will be an emphasis on harmonisation of standards in all areas. There will be greater reliance on inspections by local inspection authorities and even further acceptance of local registration dossiers as seen within the EU at present. Inspections are certainly becoming more focused on overall QSs auditing and inspection history rather that on pre-approval type of inspections focusing on one product [23]. This approach is also described in the US FDA Compliance Guidance Manual 7356.002 [24]. Areas of scrutiny will continue to be

analytical facilities but additionally with the new USA-EU MRA and the Clinical Trials Directive, R&D facilities can expect to become the focus of increased inspection activity, both for drug products and API production and testing.

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2 DEVELOPMENT OF ACHIRAL SEPARATION METHODS IN PHARMACEUTICAL ANALYSIS

2.1 Introduction

This chapter highlights the latest approaches used in the development of achiral separation methods for the analysis of impurities and degradant molecules relating to active pharmaceutical ingredients (API). This area of analytical chemistry is a diverse, wide-ranging and rapidly developing field, covering numerous analytical techniques, methods and approaches. Examples of the commonest separation techniques include high performance liquid chromatography (HPLC), gas chromatography (GC), thin layer chromatography (TLC) and capillary electrophoretic (CE) methods. Other key-related areas include sample preparation techniques, detection technology, automated approaches and tools for method optimisation. To cover fully the other aspects of each separation technique (e.g. theory and mechanisms of operation, equipment and technical requirements, general applications) is beyond the scope of this chapter, but readers can supplement their reading by referring to the following excellent publications on the individual techniques and areas. For method development in HPLC, there are many good references that offer comprehensive reviews of practical method development and applications of HPLC, particularly in pharmaceutical analysis; GC is covered extensively in, particularly in the areas of practical uses and applications; CE is covered comprehensively in two good publications and. Method development approaches and modern practical applications of TLC are covered in three review documents. Other more recent and less commonly used separation techniques, namely supercritical fluid chromatography (SFC) is reviewed in references.

Many method development strategies have been reported in the literature for different separation techniques. From these, there appear to be common themes and generalised strategies that exist in all the approaches reviewed to date - this will be discussed in the early sections of this chapter in sufficient detail to set the scene for the following sections. Part of this section will also look at why particular separation methods are used, and the importance of sample preparation will be

discussed. A review of the literature over the last five years has indicated that the most popular *from-line*separation techniques are HPLC, GC and CE. The next four sections will focus on each of the separation methodswith particular emphasis on introducing each technique (technical and some theoretical aspects), new developments and some examples of the most up-todate applications in API analysis. The re-emergence of analytical tools such as TLC and SFC will also be mentioned.

Detection and visualisation of analytes, impurities and degradants is a critical component of any separation technique and will be covered by highlighting the latest approaches. Additionally, the enormous utility of combining existing separation techniques with other more specialised systems (e.g. mass spectrometers and other structural characterisation tools) will be discussed briefly.

Other new strategies in modern method development include the use of automation, computer software to optimise separations, and statistical and chemometric tools to enhance existing approaches. The application of these method development tools will be discussed in the final sections of this chapter.

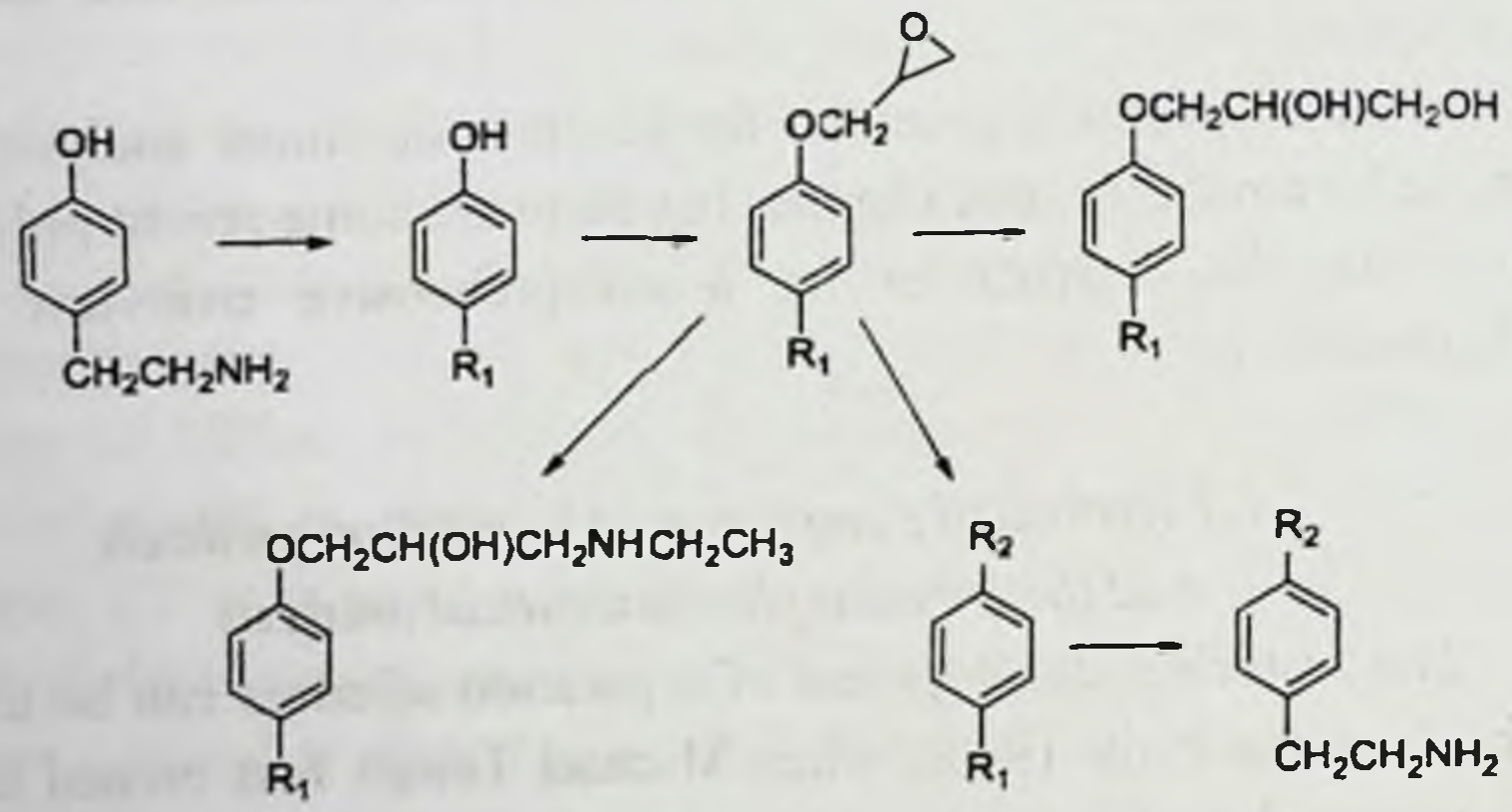
Method development approaches for biopharmaceutical analysis are not going to be covered in this chapter, but there are some recent publications which may be referred to for a comprehensive overview of this application area.

2.1.1 Historical perspective of separation methods and their usesin pharmaceutical analysis

The historical development of separation sciences can be traced as far back as the early 1900s, when Michael Tswett first coined the term chromatography. Since then, a number of major advances in the developmental path of separation sciences have progressed through instrument technology, mechanistic understanding, column technology, the advent of computers and robotic automation. These major developments have established separation sciences as a critical component in modern analytical laboratories. Another key driver in the advancements of separation sciences has been the increasingly demanding requirements of the petrochemical, agrochemical and pharmaceutical industries. In these application areas, there is a continuous and relentless need to

generate reliable, high quality analytical data usually in ever decreasing time frames.

In pharmaceutical laboratories, the use and importance of separation sciences extends throughout almost all aspects of the development process. Separation methodology is used in the very early stages of discovery research, where numerous biologically active chemical entities must be separated to provide information on relative purities and impurities levels. In the pre-clinical and clinical phases of the drug development process, separation methods play a pivotal role in fully characterising chemical entities prior to use in human clinical studies. Throughout the process, batches of API are preparedvia a synthetic route that is continually being improved and optimised. Hence, characterisation of drug substance batches can yield very important information about the synthetic process. A good example of this is that for the antihistamine drug Pafenolol (1). Liquid chromatographic separation of a crude batch of (1) reveals the presence of several important intermediates, by-products from side reactions.



Within the last decade, timelines for developing pharmaceuticals from pre-clinical to clinical phases have become extremely short, typically between 36 and 60 months. A consequence of this aggressive time frame is the requirement for analytical data in the early development phases to be used to make critical decisions. By ensuring that the right decisions are made in these early development phases and backed up by sound reliable data, the likelihood of the successful progression of a drug candidate through the clinical phases and beyond is increased. Separation methods have been applied in pharmaceutical analysis in numerous ways

for drug substances and for formulated drug products in areas such as the assessment of the levels of degradants in batches of drug substance and products - a key regulatory requirement. This information is often used to determine the shelf life of the compounds, to recommended storage conditions and transportation conditions. Degradation can sometimes occur during storage of the drug substance/product caused by interaction between the compound, the storage container, excipients and packaging materials.

2.1.2 Regulatory considerations for separation methods in pharmaceutical analysis

It is important to recognise that all drug substances contain impurities that arise from many different sources. Impurities can originate from raw materials, reagents, as reaction by-products and through degradation during manufacture and storage. The fact that impurities can have implications for safety and efficacy, both the API manufacturer and the regulatory agencies pay particular attention to this subject. Nowhere is this more important than in the development of separation methods to resolve, identify and quantify these impurities. The current guidelines indicate that identification of all recurring impurities at or above the 0.10% level is expected in all batches manufactured by the proposed commercial process. Similarly, degradation products observed in stability studies should also be identified.

The current FDA guidelines for the classification of impurities divide them into two categories: organic and inorganic. The organic category covers starting materials, by-products, intermediates, degradation products, reagents, ligands and catalysts. Inorganic materials will not be covered in this chapter. For drug products, the analytical challenges for identifying impurities are even greater because of the presence of excipient components present in the formulation. Here, impurities can arise through interactions between the API and excipients, packaging materials and through degradation. The registration of a new drug product requires significant documentation for submission to the regulatory agencies including justification and rationale for the methods developed.

With a threshold of 0.10% for the impurities level in most APIs, the implication is that a limit of quantitation (LOQ) of approximately 0.05%

will be required for most separation methods. Hence, to ensure that key impurities are resolved and that main peak homogeneity is maintained, it is becoming essential to increase selectivity, improve sensitivity and enhance the consistency of separation methodology. Current approaches include the use of alternative detection technologies, derivatisation strategies, orthogonal coupling of chromatographic techniques, e.g. HPLC/TLC and HPLC/CE, or the coupling of chromatographic separations with information-rich spectroscopic methods such as HPLC/MS or HPLC/NMR. These topic will be covered in later sections.

2.2 General guidance for method developmentin separation sciences

For most separation techniques (HPLC and GC), technological advances, easeof-use and greater commercial availability have led to separation-scientists having a good practical working knowledge and understanding of chromatographic separation principles. Moreover, knowledge of the effects of making changes to the experimental conditions has significantly improved. This knowledge usually forms the basis of any systematic approach to method development. With this in mind, Snyder *et al.* summarised method development strategy in the following way -*the length of time taken to achieve the desired separation varies from performing relatively few experiments in a short time to exhaustive experimentation*. The strategy should be one that requires as many experimental runs to achieve the desired final result. In this case, each experimental run should contribute towards the desired result.

Nowadays, in the pharmaceutical industry, the need to obtain accurate and reliable analytical data faster and more cost-effectively, has meant that approaches to method development are becoming simpler and more straightforward. These simpler approaches have the benefits of more consistent methods, lower method diversity, reduced inventory costs for separations consumables and more easily transferable methods from R&D laboratories to manufacturing quality control (QC) laboratories. Many pharmaceutical companies have adopted this approach.

A review of method development in separation sciences indicates that individual approaches exist which are based on previous experience

of compounds or previous knowledge; method development often follows the same sequence of events. These are summarised in the flowchart shown in Fig. 2.1. In the following sections, each step is discussed in some detail.

2.2.1 Separation goals/objectives

Before the method development commences, it is important to define as clearly and in as much details as possible what the objectives and goals are for the intended separation. There are many publications that detail the types of questions that should be asked and in many cases, these questions are specific for HPLC. However, by considering these questions in a broader sense, they can apply equally to most, if not all, common separation techniques. The following questions should be asked:

1. Primary purpose

- Qualitative analysis (i.e. for identification purposes)
- Quantitative analysis (i.e. determine impurity levels or purity of the main compound)
- Rugged separation
- Recovery or isolation of a sample fraction or purified material.

Defining the aims and objectives of the separation helps in deciding the choice of separation technique. Moreover, if the aim is to isolate purified material, then separation techniques such as GC and CE are not always easy to use.

- Should all the impurities and degradant be resolved from each other and the main component?
- Is it only necessary to separate the impurities and degradant from themain component?
- Are all the known impurities, degradants and solutes available as standards?

For this question, it may be necessary to separate all impurities and degradants from the main component for a purity assay. However, if the objective is to obtain an impurity profile, then all components will need to be separated from each other. In some cases, it is also possible to obtain both impurity profile data and a purity assessment of the main component from a single method.

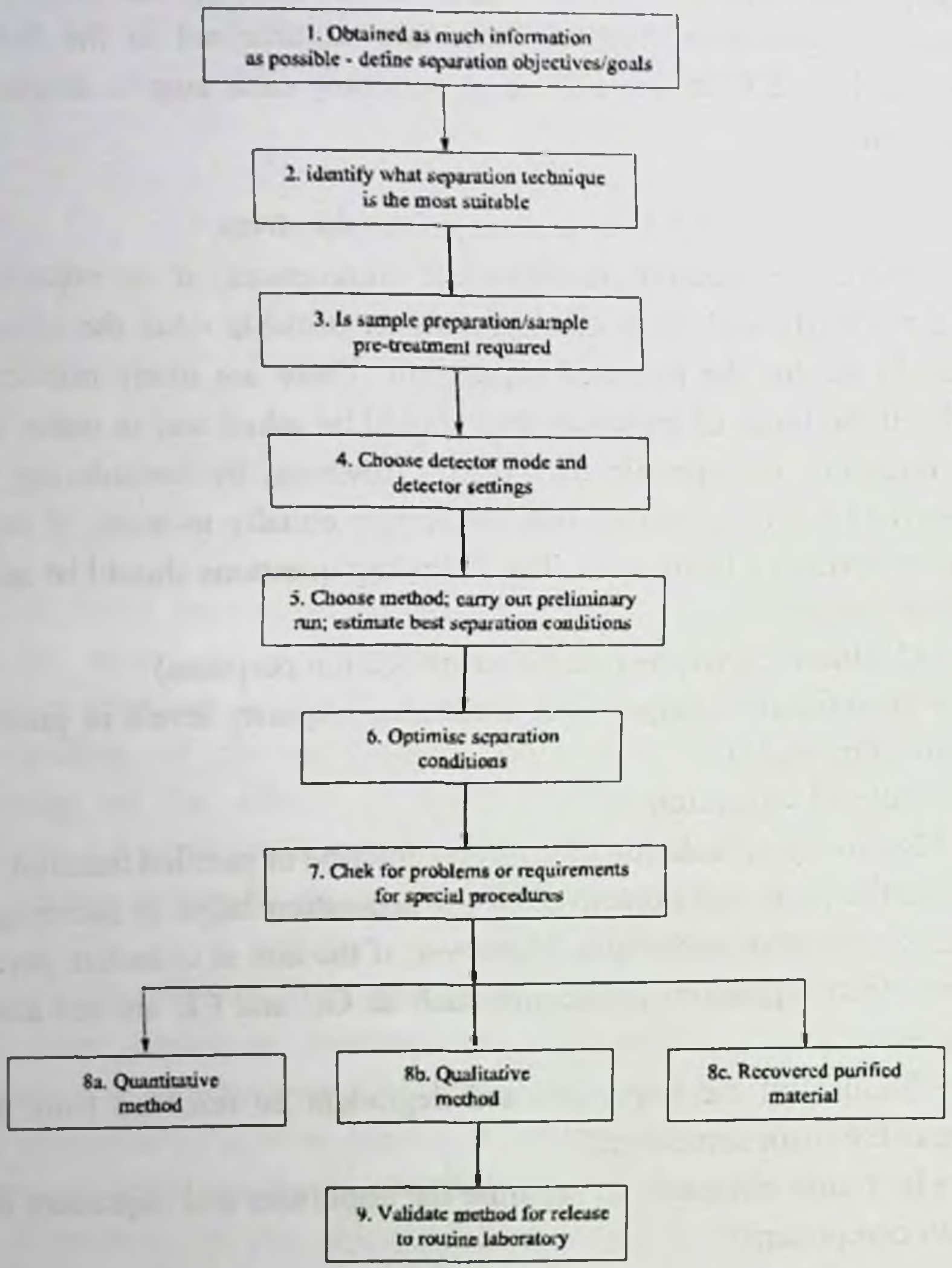


Fig. 2.1 Flow diagram summarising the basic steps involved in developing separation methods.

3. Quantitative analysis

- What level of accuracy and precision is required?
- What range of concentrations for the relevant components will be required?
- What is the desired analysis time?

For pharmaceutical analysis, ICH guidelines would normally recommend accuracy value ranges of 95–105% and precision of values less than 10%. Typically, the choice of concentration range would be dependent on the spectroscopic properties of the compound(s), the chosen detection mode and the volume (amount) of sample to be analysed. Deciding the desired analysis or run time is important because certain applications need fast methods for a high sample turnover.

4. Sample matrices

- How many different sample matrices should the method be designed for?
- Will the sample require extraction from the matrix?
- Can the separation be achieved with untreated samples?
- Is sample pre-concentration required?

This question is an important one because the component in the matrix can significantly influence the separation characteristics of the desired components. This is particularly relevant when dealing with biological fluids (where proteinaceous materials can interfere), environmental samples (interfering compounds may be present at very high levels) and in formulations (where excipients can interact with the separation column).

The analysis of untreated samples may also be chosen, however, the risks here are that the separation column can become blocked or damaged with prolonged use. Not surprisingly, this approach is not commonly used. In cases where the levels of matrix component far exceed the compounds of interest, it is important to extract the compounds and concentrate the compound.

5. Number of samples

- How many samples will be analysed at any one time?
- Speed vs Resolution?

When a large number of samples require analysis, then run time becomes very important. This is particularly relevant when the separation method is being used to monitor multiple chemical reactions, and samples are analysed at different timepoints. Quite often, it is desirable to trade in a decrease in sample resolution for a shorter run time. In situations where

the sample numbers are fewer and the information required is quality critical in nature, then longer run times are sometimes required to achieve the desired resolution of critical impurities.

6. End-user of final method

• Will the separation method be used on different instruments makes andmodels?

• Will the separation method be used by different analysts with varying skill levels?

• Will the separation method be used in different laboratories?

In most pharmaceutical analyses, the answer to these questions is quite simple. The separation method will have to satisfy all the above criteria, because by meeting this criteria, the ruggedness of the method is tested. The end-user is most likely the QC laboratory in a manufacturing facility, and the ease with which a separation method can be transferred in most cases, R&D laboratories to a QC laboratory will be highly dependent on the method development strategy used.

2.2.2 Nature of the sample

If some information on the nature of the sample is known before beginning method development, it often provides valuable clues to the choice of separation technique, detection mode, extraction procedure (if required) and initial starting conditions for the separation. Table 2.1 summarises the type of sample-related information that would be required. Quite often, very little is known about the sample composition at the beginning of method development. This is particularly true for compounds presented at the discovery or pre-clinical phases of drug

Table 2.1 Summary of information relating to the physical and chemical properties of the test sample

	Physico-chemical property
1	Physico-chemical property of the sample - b.p., m.p.
2	Number of compounds/components present.
3	Chemical structure or functional groups present – are there anionic, cationic, neutral orzwitterionic moieties present?
4	Molecular weight(s) – are components high (MW >1000 Da) or

low MW?

5 pKa values of compound(s) – is the compound acidic (pKa <4), basic (pKa >7) or neutral(pKa = 7)?

6 UV spectra – does the compound end-absorb (l < 200 nm) or is it coloured (l > 400 nm)?

7 Does the compound(s) fluoresce?

8 Is the compound electrochemically active?

9 Sample solubility in organic and aqueous solvent systems.

10 Concentration ranges of compounds in samples of interest.

11 Is the compound volatile - i.e. b.p. <90°C?

development and it is usually at this stage that separation scientists begin to start collecting critical analytical information on potential drug compounds.

There appear to be two practical approaches utilised for method development by most separation scientists. The first approach is to rely on past experience of the compound or previous separations of a compound with a similar structure and then supplement this information with some information from the literature. This can usually lead to the development of some initial starting conditions. The alternative approach is usually to not consider the sample information and proceed directly to some generic starting conditions. Both of these approaches have been characterised by Snyder *etal.* as theoretical for the former and empirical for the latter. Although both approaches have been shown to work, the optimum strategy for method development is quite often a combination of both approaches.

2.2.3 Choosing the separation technique

One of the first steps in any method development process is to decide which separation technique to use. The choices may be as diverse as GC, LC in its many modes, CE in its many modes, TLC, SFC or some other technique. Within HPLC, choices may include size-exclusion, normal-phase, reversed-phase (RP-HPLC), ion-exchange and/or ion-pairing. Within RP-HPLC, the silica stationary phase can be modified with a range of different functional groups each imparting its own unique chromatographic properties e.g. octadecyl, octyl, phenyl, amino or cyano

groups. Newer stationary phases such as mixed mode, porous graphitic carbon, fluorinated and monolithic phases should also be considered. Figure 2.2 summarises the current choices of HPLC modes available. A further factor to consider is the scale of the separation; if there is very little sample available then techniques such as capillary HPLC are appropriate. If the goal of separation is to isolate material then preparative scale chromatography or at a larger scale, simulated moving bed (SMB) techniques are moreappropriate choices. For GC, TLC, CE and SFC, there are fewer, but still significant choices.

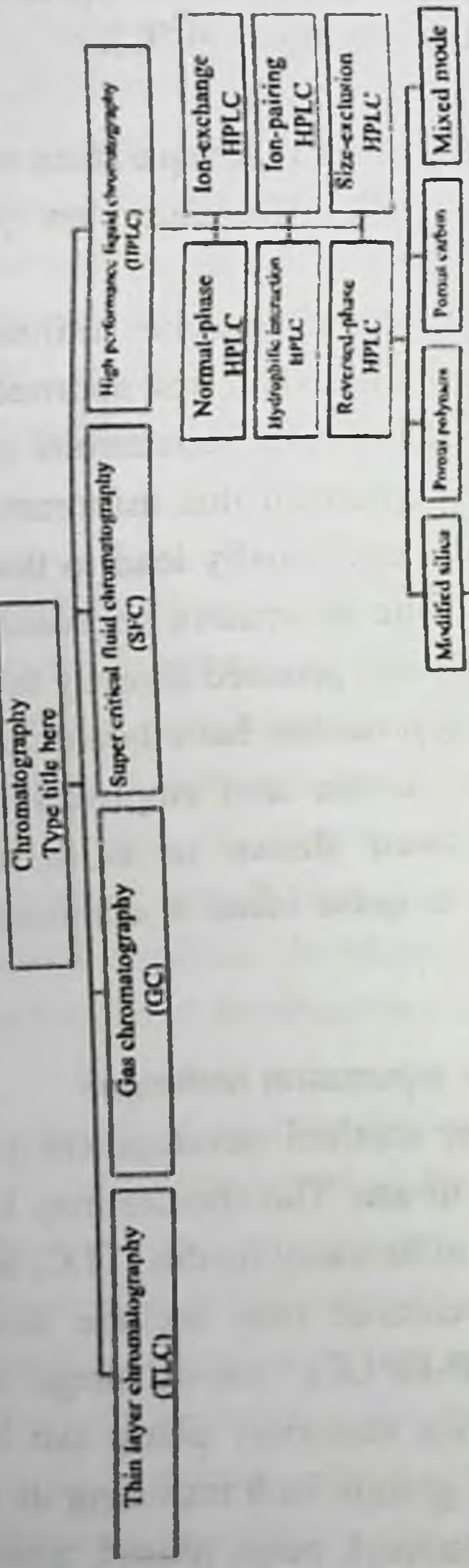


Fig. 2.2 Diagram showing the different modes of chromatography.

To select a separation method for a suitable application, the separation scientist usually relies on a number of factors:

- the intended end-user of the separation method;
- any phyisco-chemical information on the sample;
- personal insights and experiences;
- information from the literature and the manufacturer;
- availability of equipment and consumables;
- ease of use;
- reliability of technique.

There may be other factors to add to the list above, but the seven factors listed are considered to be the most common factors. The first factor relates to who and where the method is likely to end up. The availability of sample information will to a certain extent dictate the most appropriate separation method to use. The use of personal insights and experience is likely to be a dominant one if similar problems have been solved before using a particular separation technique. This is the case for compounds with similar structures. If the separation scientist encounters a completely novel area or analytical solution, then the use of information from published work or from instrument manufacturer is usually of more importance. The availability of instrumentation and consumables in the long and short term is quite often an important requirement particularly if the separation technology is not well established, expensive or is sourced from relatively fewer manufacturers. Quite often, if the separation techniques require very specialised knowledge or experience, then the choice of method would usually be one that is simple, reliable and easy to use.

In pharmaceutical analysis, separation methods are usually developed with a view to ensuring that the technique chosen can:

1. Solve the analytical problem and provide reliable data.
2. The method is robust and reliable enough to be transferred to otherlocations and laboratories.
3. Spare parts and consumables are available for repairs and maintenance.
4. The technique is relatively easy to use by operators with different skill levels.

2.2.4 Sample pre-treatment and detection

Sample preparation is an essential part of the separation process and is one of the key considerations at the start of any method development strategy. Numerous publications are available on this subject. In brief, the primary aim is to provide a reproducible and homogenous solution that isready for injection into the separation system. Sample preparation will produce a sample solution that is relatively free of interfering compounds that can damage the separation column and is compatible with the intended separation method. By satisfying these conditions, the separation characteristics of the method are unlikely to be affected. For most separation techniques, sample preparation is predominantly a manual process and as a result, can sometimes be a time-consuming component of the whole method development process. Despite this, it is still a very important and sometimes challenging area and requires sufficient planning and effort because key method validation parameters such as precision and accuracy are frequently dependent on a reproducible and robust sample preparation strategy for example in weighing, dilution and dissolution. Figure 2.3 summarises the sample preparation choices available. Samples can be presented in various forms as solids, liquids, suspensions and even gases. In the solution form, these samples can either be ready for direct injection into the separation system or require further manipulation, i.e. dilution, buffering or addition of internal standards. When samples are in the solid form, they must first be either dissolved or extracted using a suitable solvent. For samples that consist of complex matrices either chemically based (e.g. formulated drug products) or biological in nature (e.g. blood, urine or plasma), some form of sample pretreatment to remove the interfering components is usually required. Direct injection of these matrix samples will quite often damage the separation column or instrument and should be avoided. In circumstances where the sample is relatively soluble, direct dissolution in a compatible solvent is the preferred mode of sample preparation because it is convenient and offers greater precision.

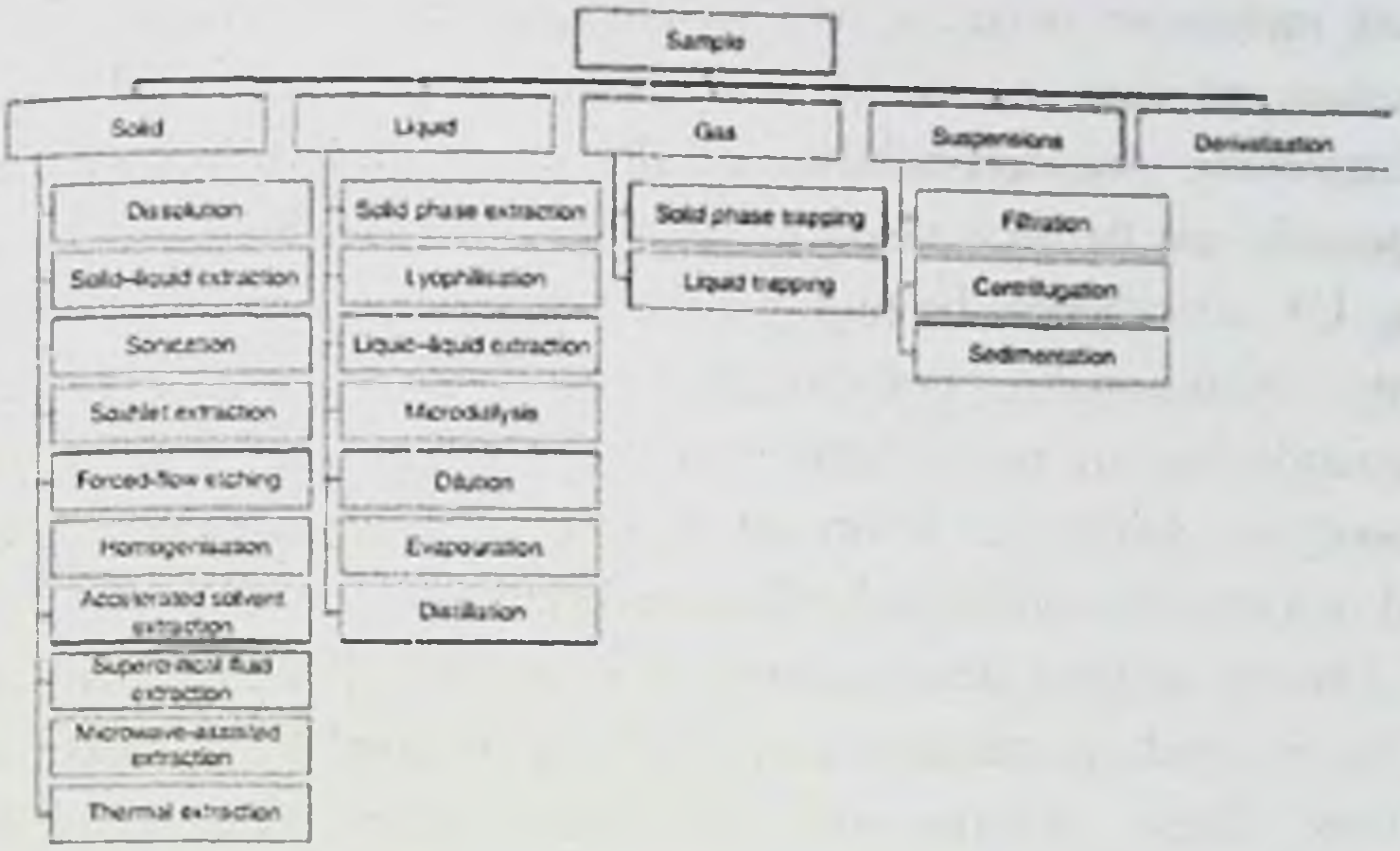


Fig. 2.3 Diagram showing different modes of sample preparation methods currently available.

For these sample types, the choice of solvent is important because choosing a solvent system that closely matches the separation solvent minimises baseline problems and other unwanted separation effects.

There are many sample preparation procedures published in the scientific literature, and within the scope of this chapter, only the most current and popular methods will be discussed. By far, the commonest and most popular method used for pretreatment of liquid samples is solid phase extraction (SPE). For solid samples, several techniques are available including supercritical fluid extraction (SFE), microwave-assisted solvent extraction (MASE) and accelerated solvent extraction (ASE). Solvent extraction methods have long been established as the standard approach to sample preparation, but the increasingly demanding needs of industries like the pharmaceutical, agrochemical and petrochemical for greater productivity, faster assays, and increased automation have led to the development of newer ways of sample preparation summarised in Fig. 2.3.

Derivatisation offers another means of preparing the sample ready for analysis. Derivatisation involves chemical reactions between the analyte molecule and a reagent to change the phyisco-chemical properties of the analyte molecule. By using this approach to sample preparation, the sample's properties can be modified to improve detectability, change its

overall molecular structure and polarity, change the matrix for better separation, stabilise an unstable analyte and change its physical properties. By improving the detectability of the compound(s), poorly detected compounds can be derivatised to form derivatives which can have very strong UV absorbence, be highly fluorescent or possess electrochemical activity. Additionally, derivatisation can also be used to convert compounds that are not volatile into analytes that can be volatilised for GC analysis. Additional information on derivatisation strategies can be found in a recent publication by Blau and Halket.

During method development, it is critical that the derivatisation reaction is rapid, quantitative and produces minimal by-products or side reactions. These criteria are not always easy to achieve, hence, derivatisation as a sample preparation method is quite often chosen as a last resort. In pharmaceutical analysis, the use of derivatisation as a means of sample preparation is not usually the method of choice because the primary objective of any method development is to detect all major impurities and degradants in batches of the drug substance. Quite often, many of these compounds will not have derivatisable functional groups or have impurities that are present at such low levels that the derivatisation reaction is not optimised.

2.2.5 Developing the separation

The final stage in the simple step-by-step approach to method development is to develop the separation. When the separation technique has been chosen andusing the information obtained from the earlier stages, a series of exploratory experimental runs are usually performed. The conditions chosen for these initial runs will depend on the separation method used. The following sections will provide some guidance on some of the new approaches adopted in method development for small molecules in HPLC, GC, CE and other less common separation techniques.

2.3 High performance liquid chromatography (HPLC)

2.3.1 Brief historical perspective of HPLC

The historical development of liquid chromatography has been extensively reviewed and can be traced as far back as the early 1900s, where the Russian botanist Zwett used a variant of liquid chromatography to separate some coloured plant substances. A much more detailed historical assessment of liquid chromatography can be found elsewhere. Here, the focus will be on modern developments in HPLC, a term that was coined in the late 1960s with the advent of more sophisticated instrumentation, better engineered separation columns, and reliable and highly efficient stationary phases and packing materials. These technological advances have been, in part, fuelled, by the need to separate an increasingly larger variety of differing compound classes encountered as APIs, e.g. antibiotic, sulphonamides, nucleotides and nucleosides, fat-soluble vitamins, neutral and non-polar compounds. Additional challenges include developing faster and more consistent HPLC methods requiring higher flow rates, while maintaining peak-to-peak resolution, retention characteristics, peak shape, peak symmetry and efficiencies. Another important analytical challenge is the desire to detect and accurately quantify low levels of impurities at the 0.10% level present in API materials.

2.3.2 Different modes of HPLC

Nowadays, there are many different modes of HPLC available to equip the separation-scientist with the necessary tools to separate compounds that possess either acidic, basic, zwitterionic, oligomeric or aromatic functional groups (Fig. 2.2). Detailed texts are available for each mode of HPLC, along with the descriptions of their operation and applications but in this section, the focus will be primarily on the use and application of RP-HPLC. Although the other modes are routinely used in the pharmaceutical industry, RP-HPLC is the mode of choice. This is because many of the molecules of interest are weak organic bases and the separation is dramatically influenced by factors such as mobile phase pH, ionic strength, organic modifier and possible use of ion-pair reagents.

In RP-HPLC, the stationary phase is less polar than the mobile phase and is usually comprised of spherical silica particles (typically, 3–5 μm in diameter). The acidic functionalities on the silica material have been modified by derivatisation with alkyl (C2 to C18), phenyl, cyano and amino groups. Typical mobile phases used in RP-HPLC consist of mixtures of aqueous buffers mixed with water-miscible organic solvents, such as methanol and acetonitrile. In addition to modified silica stationary phases, other new developments in RP-HPLC are now available, e.g. porous polymeric, carbon and mixed modal phases.

2.3.3 Key developments in HPLC

Over the last decade, the most significant developments in liquid chromatography, specifically in HPLC, have been in the areas of instrumentation and equipment, advances in stationary phase chemistry and their design, improvements in column design and dimensions, use of different additives in mobile phases and sample preparation techniques. All of these factors have helped to significantly improve the way separationscientists develop their methods.

2.3.3.1 Stationary phase and column technology

The column is often called the *heart* of the HPLC separation process, and the availability of stable, high performance stationary phases and columns is critical to the development of rugged, reproducible and robust methods. Modern commercial columns can differ widely among suppliers and these differences can sometimes affect the development process of the desired HPLC method. Specifically, different columns can vary in terms of plate numbers (N), retention characteristics (k') and resolution (Rs). For these reasons, column and stationary phase manufactures have developed technologies to help ensure that these separation materials are produced in a more consistent and reproducible manner. An excellent reference by Snyder *et al*. provides a comprehensive overview of modern stationary phases and column technology.

Most HPLC column packing materials use silica particles as the basis of the support. Silica is known for its reliable strength and rigidity, relative inertness and ability to be modified chemically. Significant scientific effort has been devoted to developing and improving the

morphological and physico-chemical characteristics of these silica materials (Fig. 2.4).

Some of the more modern silicas include micropellicular particles, which consist of solid silica core with a thin outer skin of interactive stationary phase, particles range typically from 1.5 to 2.5 μm. Columns made of these microparticulates generate very sharp, low-volume peaks. Perfusion particles [56] have very large pores (typically 4000–8000Å) throughout the support and also includes smaller interconnecting pores.

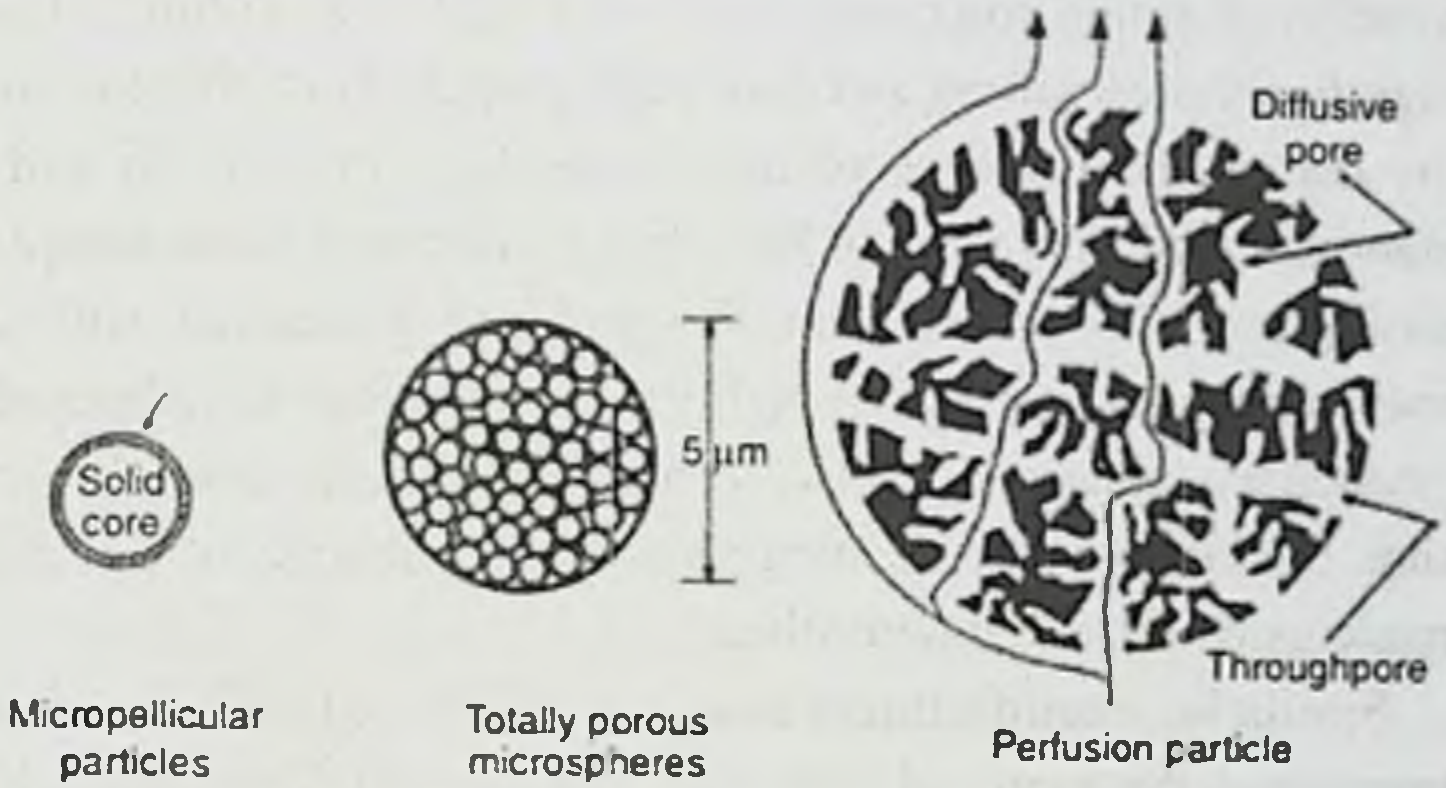


Fig. 2.4 Particle types used in modern stationary phases in HPLC. Figures represent approximate relative sizes of particle used.

These types of highly porous silicaparticles are more suited for separations of biopolymer and not for routine analytical separation of small molecules. Fully porous silica microspheres are the most commonly used because they offer many important benefits in HPLC columns such as good efficiency, high sample loading, durability and wide commercial availability. A recent development is the availability of monolithic silica columns where the column consists of a very porous silica rod with a large surface area for effective separations but with significantly reduced back pressure over a conventional particle-packed column allowing much higher flow rates. Over the next few years as this technology improves and the column types and chemistry becomes more diverse, these columns may well become the most commonly used in the pharmaceutical industry.

One unfavourable characteristic of silica is its solubility at extreme pH values and at elevated temperatures, thus leading to reduced lifetime and deterioration of peak shape and resolution. With the advent of particles formed by the aggregation of silica sols, so-called sol-gel silicas, this property of silica has been significantly reduced. The recent development of hybrid silica particles also address this problem.

Many compounds developed as biologically active drugs within the pharmaceutical industry are amine-containing compounds. In the past, the separation of amino compounds using earlier HPLC columns has resulted in significant peak tailing and poor peak shapes. This effect is now known to be due to the presence of metal ions (e.g. Fe, Al, Ni and Zn) that contaminate and interact with the silica surface and form complexes with silanol groups. To circumvent the problem associated with analysing amine compounds, a range of highly purified silicas have been developed by stationary phaseand HPLC column manufacturers. Nowadays, the column of choice for performing pharmaceutical analysis will most likely be made using ultrahigh pure silica.

Similarly, manufacturers have put significant effort in recent years in improving the range of stationary phases which are available. Apart from the classic reverse phase C18 and C8 phases, other column chemistries are available including amine, phenyl, diol, nitrile and many others which impart selectivity into separations. Phases also containing various polar-embedded groups which modify selectivity and shield analytes from silanol interactions now give much improved peak shapes for polar compounds and prevent phase collapse in high aqueous content buffers. Phases with hydrophilic end capping are also available which permit separations of very polar compounds to be carried out in 100% aqueous mobile phases. The pH range that separations can be carried out at on silica-based columns has also been significantly extended in recent years with improvements in the silica and bonding chemistries. Separations can now be carried out at pH values less than 2 and up to 11 on certain phases. The extension of the basic pH range now permits separation of basic pharmaceuticals under reverse-phase conditions at pHs above the pKa for the molecule.

HPLC column configurations have also undergone important developments over the past few years. Most columns are now made from

lengths of stainless steel with highly polished interior walls because of its mechanical strength and chemical inertness. However, in very few cases, some corrosive chloride-containing mobile phases can slowly erode the steel surface. Some of the newer column configurations include the use of plastic material, glass-lined inner surfaces and outer column surfaces made from the rigid polymer, polyether ether ketone (abbreviated as *PEEK*). Another novel approach is to use a soft polymeric material for the outer surface, and by compressing the column radially, the efficiency of the separation is enhanced as the stationary phase is pressurised.

2.3.3.2 Instrumentation

The manufacturers of modern HPLC systems have adopted a modular approach to the design and utility of the instrumentation. This modular approach is an efficient and flexible means of performing HPLC because, different modules (e.g. different detectors) can be configured easily. Additionally, this set-up allows for easy stepwise tracking of problems that may arise during operation of the HPLC system (e.g. a high back pressure due to a blockage in the system). The modules consist of a pumping system, an autosampler, a column oven and the detector, all controlled by a computer and appropriate software. Although there are small variations in this set-up, all commercially available HPLC systems subscribe to this format. In later sections, the key technological developments that have occurred in HPLC instrumentation will be discussed in more detail.

Some important technological advances in HPLC have been associated with instrumentation. Specifically in the areas of mobile phase pump design, sampling introduction systems, detection systems, connections and detection systems. For supplemental reading, references are recommended. Two areas are worthy of specific mention, namely column ovens and eluent mixing systems. The introduction of column ovens has significantly improved method development by ensuring methods are reliable and more reproducible. This is of particular importance in a pharmaceutical environment. The development of eluent mixing systems in HPLC has meant that efficient mixing of solvents is now possible for isocratic and gradient elution.

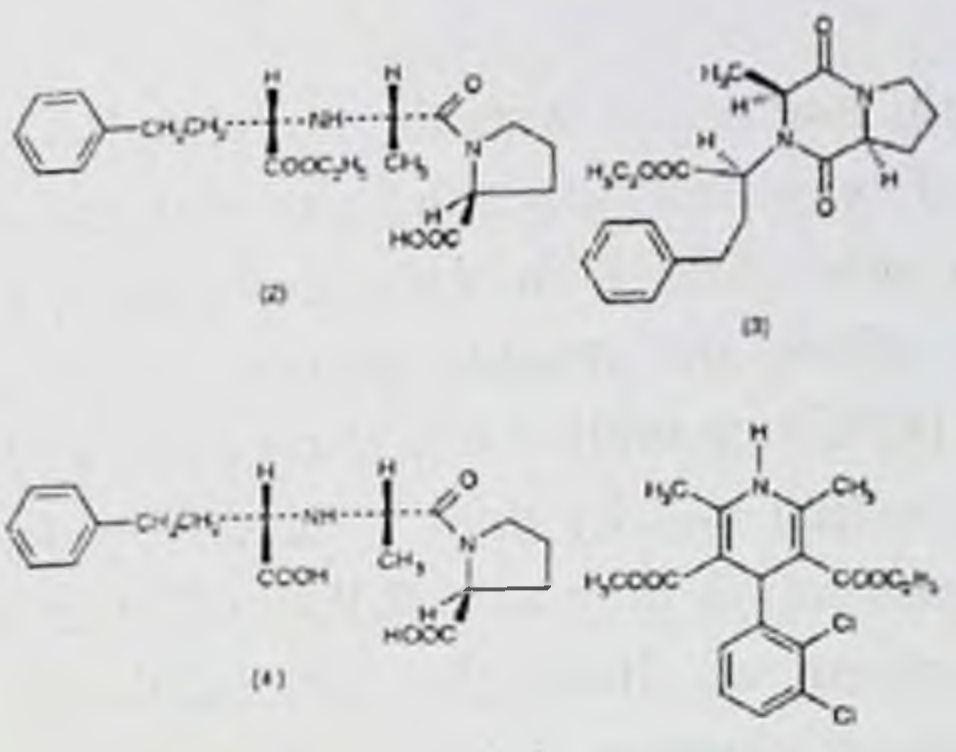
2.3.3.3 Microcolumn liquid chromatography

Any discussion on new developments in HPLC is not complete without mentioning microcolumn liquid chromatography. In microcolumn LC, columns with internal diameters of less than 1 mm are used with HPLC systems equipped with microtubing, a micro injection device and a specialised detector. The most important advantages of microcolumn LC are the ability to work with small sample sizes, lower solvent consumption, small volumetric flow rates and the enhanced detection performance with the use of concentration sensitive detection. A recent review on microcolumn HPLC is recommended for further reading. The main application areas of this technique are bioanalysis, neuroscience and protein/peptide research. However, in small molecule analysis, microcolumn LC is not used as routinely as conventional HPLC. This is most probably due to current instrumentation being less reliable and not as robust as conventional systems. However, this area of liquid chromatography is progressing rapidly, and in the future, the status of this technique in applications such as routine API analysis will change.

2.3.3.4 Combined HPLC methods

To meet the speed and high efficiencies in separations demanded by the pharmaceutical industry, combined HPLC methods have been frequently used to simultaneously determine combination products. A stability-indicating method for the simultaneous determination of aspirin and warfarin in warfarin sodium/aspirin combination tablets has been recently developed and validated. In another example, the simultaneous determination of enalapril (2) and its two degradants, enapril-DKP (3) and enalapril-diacid (4), and felodipine (5) and its degradant, named H152/37 (6) was achieved using combined method approach.

The chromatogram in Fig. 2.5 demonstrates that the retention of (3), (5) and (6) is governed by reversed-phase partitioning processes, whereas for (2) and (4), both reverse-phase and cation-exchange mechanism are operating. Again, this method was validated to be precise, accurate, specific and robust.



(2) (3)

(4) (5)

(6)

2.4 Gas chromatography (GC)

2.4.1 Brief historical perspective

Gas chromatography (GC) was originally developed in the 1950s [70] and commercialised in the late 1950s. The principle as with all chromatography techniques depends on the partitioning of the analyte between a stationary phase and a mobile phase. In the case of GC, the mobile phase is a gas which means that the analyte must be volatile to progress through the column and most

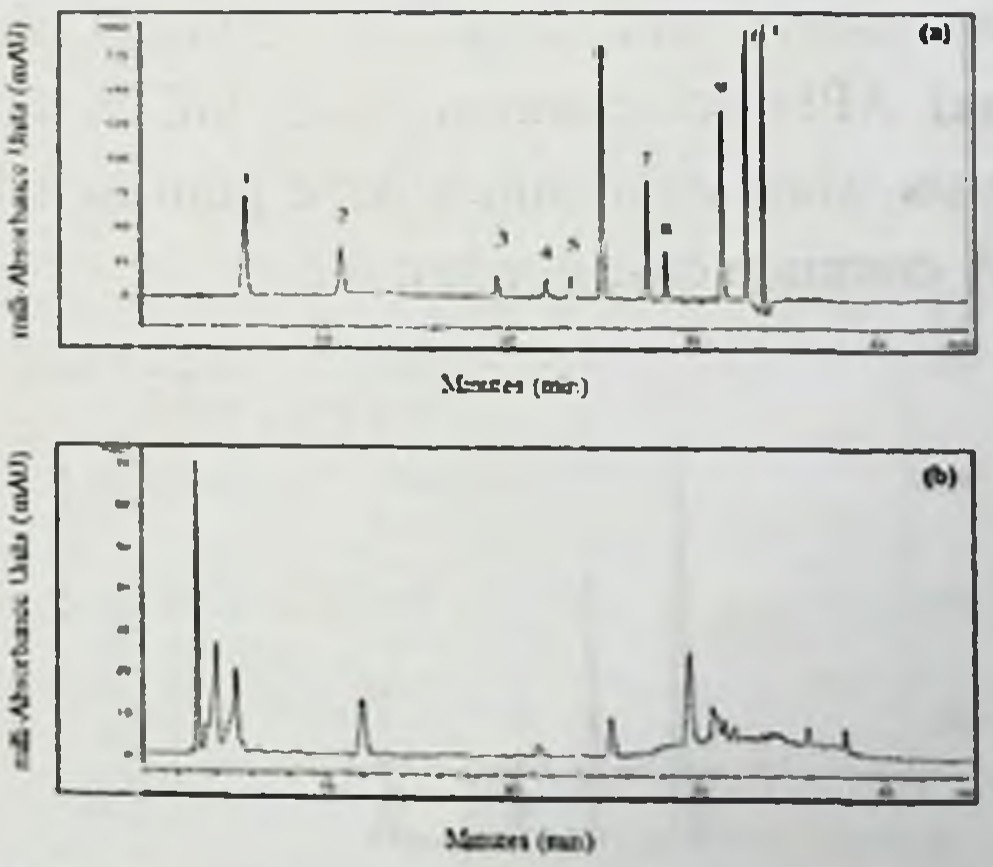


Fig. 2.5 Typical HPLC chromatograms showing the separation of compounds (2) to (6) and a UV-absorbing excipient using the HPLC conditions listed in ref.

analytes have little interaction with the mobile phase. Essentially, there are two modes of operation, gas solid chromatography (GSC) and gas liquid chromatography (GLC). In these early days, the technique used packed columns where the analyte partitioned either with the solid stationary phase (GSC) or with a liquid coating on the solid stationary phase (GLC). Detection was by total conductivity detection (TCD) and later with flame ionisation detection (FID). GC was rejuvenated in the 1980s with the advent of chemically bonded fused capillary columns which offered high efficiencies and thermal stability. GC is used widely for analysis in the pharmaceutical industry and although it is a mature technique, improvements in columns, injection and detection systems, and ovens have permitted the expansion of the technique to analyses previously beyond the scope of GC. Some of these technical improvements are discussed below and are illustrated by analytical examples. In line with most other separation techniques, technical improvements have given rise to the possibility of increasing the efficiency of the technique by reducing cycle time, often with an associated improvement in limits of detection for analytes, and some of these technical innovations will also be discussed.

2.4.2 GC in pharmaceutical analysis

GC is used widely for analysis in the pharmaceutical industry, and applications for assays and impurity profiles for raw materials, intermediates and APIs are commonplace. GC is often chosen as the method of analysis when compounds have poor or widely different UV chromophores. A common case is when the

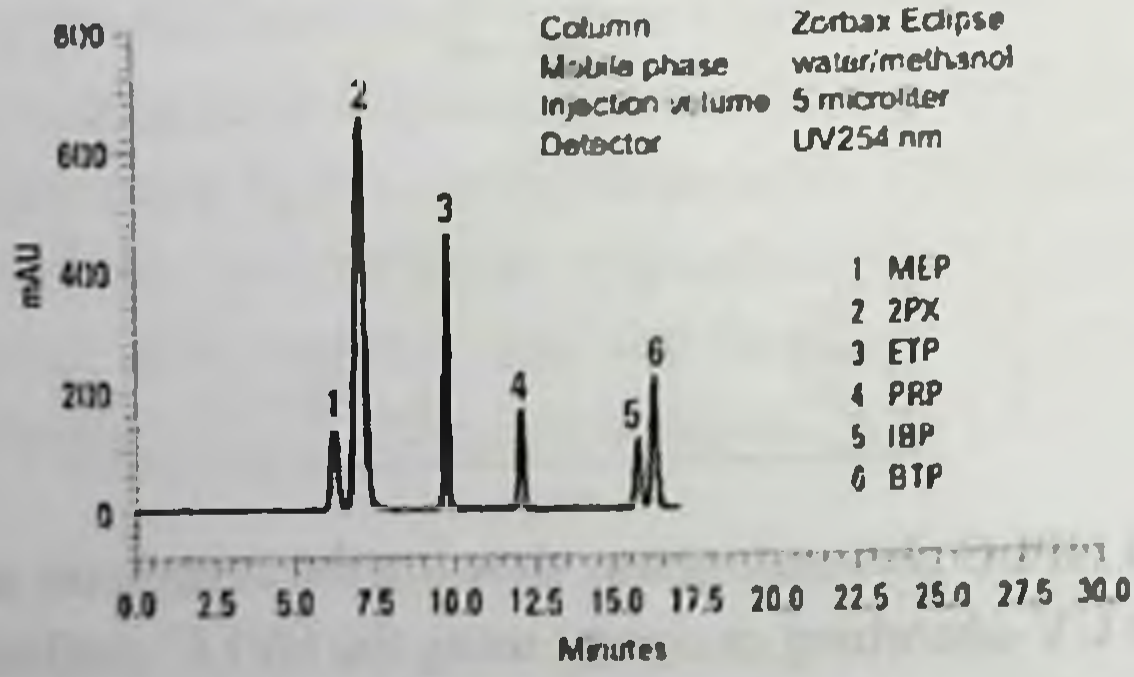
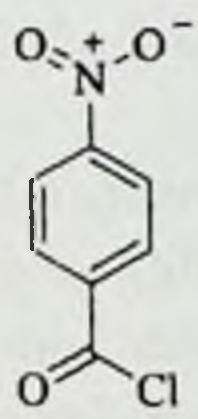


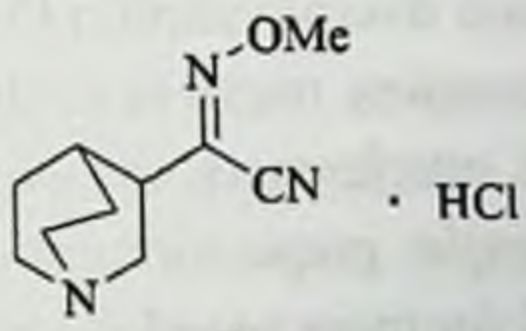
Fig. 2.6 Gas chromatographic separation of 4-nitrobenzoyl chloride (4) and 4-chlorobenzoyl chloride (2) and their regiosiomers.

reduction of a nitrated aromatic species to the corresponding amino species gives rise to large shifts in the UV maxima and hence significant UV response factor differences between starting materials, products and impurities. Another common use is illustrated by the example below, where an impurity profile method was required to analyse (7) and to specifically quantify the levels of the corresponding regioisomers in the samples (Fig. 2.6). The large number of theoretical plates available on a standard GC column makes the technique particularly suited to this kind of separation, especially here in the case of acid chlorides which are difficult to analyse by HPLC.



(7)

GC is the most commonly used technique for residual solvent and organic volatiles analysis in API and has also found application where trace level detection of analytes is required. An airborne monitoring method for SB- 202026-A (8) was required and due to the low occupational exposure limit (OEL) for this molecule it was necessary to be able to quantify (8) in solutions at 100ngml-1. This was achieved readily using a 1 microlitre splitless injection,and detection with a nitrogen phosphorus detector without the need for sample pre-concentration.



(8)

2.4.3 Key developments in GC

2.4.3.1 Sensitivity enhancement with large volume injection

The programmable temperature vapouriser (PTV) inlet allows the enrichment of analytes in the PTV insert rather than relying on extraction procedures and facilitates the use of large volume injection to obtain analysis on low-level analytes. It also permits the coupling of GC with other sample preparation techniques such as solid phase extraction (SPE), liquid–liquid extraction (LLE) or SFE and a method for the coupling of LC to GC. Many applications are recorded in the literature predominantly in the field of environmental analysis. Engewald *et al.* have reviewed PTV techniques and applications. For analytes eluting close to the solvent front, the sensitivity benefits of large volume injections can be obtained using cool-on-column injection with solvent vapour exit (COC-SVE).

2.4.3.2 Thermally labile samples

The requirement for volatility often precluded the use of GC for analysis of thermally labile samples. Injection of the sample directly into the column using cool-on-column injection (COC) with volatilisation occurring on the column has meant such
samples can be readily analysed.

2.4.3.3 Analytes in complex matrices

Often interference effects from either solvents or other components in sample matrices can cause significant problems especially with direct injection of such solutions. Headspace analysis has been shown to be of great value for residual solvent analysis in drug substance and drug product because the drug itself is not introduced into the system. Similarly, residual solvent analysis in pharmaceuticals using thermal desorption and solid phase microextraction (SPME) has been shown to be of benefit. For more complex matrices such asblood or environmental samples, problems with interferences can be more acute and previously required significant sample preparation prior to analysis. SPME has proved to be very useful for these samples especially as the technique can be combined with PTV [80]. SPME can also be used for in situ derivatisation or can be molecularly imprinted to facilitate specific analyte detection.

2.4.3.4 Detection systems

One of the main advantages of GC analysis is that it can be readily combined with a large number of detection techniques and hence a wide range of applications are available. Interfaces connecting GC with the mass spectroscopy to allow structure elucidation have now become commonplace. More recently, the coupling of GC to Fourier transform infra red (FTIR) spectroscopy and computer modelling of spectral features has proved a reliable and fast screening technique. Element-specific detectors such as the nitrogen–phosphorus detector (NPD) have been used to identify low-level components of interest in complex samples especially when combined with GC/MS. The atomic emission detector (AED) has further expanded the detection possibilities to almost any element and provided the ability to give element ratios and hence empirical formulae for analytes of interest. The electron capture detector (ECD) has historically been used in the pharmaceutical industry for the analysis of low-level halogenated compounds but suffers from the disadvantage of the requirement for a radioactive source. For many analyses, the non-radioactive pulsed discharge helium ionisation detector (PDHID) in electron capture mode can replace the ECD with no loss of sensitivity. The PDHID has also proved to be very valuable in normal ionisation mode with excellent sensitivity for a wide range of analytes. An application of GC that is becoming increasingly important in the pharmaceutical industry is that on inverse gas chromatography (IGC). In this technique, the retention of volatile probes is used to determine surface energy information. Changes in surface energy may be used to detect batch to batch differences due to recrystallisation from different solvents and following milling operations. Grimsey *et al*. have reviewed the use of IGC in the analysis of surface energies of pharmaceutical powders.

2.4.3.5 Efficiency increases in GC

In common with most other separation techniques, technological advances have not only increased the applications of the technique but have also allowed results to be obtained with much shorter analysis times with no loss of sensitivity. In line with HPLC, improved column technology has allowed capillary columns to become commercially available with internal diameters less than 100μm. These columns

combined with the ability for GC ovens to heat at rates of greater than 100°C/min have enabled analysis times to be reduced by factors of up to 10 times with no loss of resolution [A.M. Godwin & J.K. Roberts, unpublished work]. Conversion of existing methods to fast GC methods is also a simpleprocess with the availability of online software to translate the methods. Most modern GC instrumentation is now available with electronic pressure control which will allow flow rate programming to optimise separation efficiency throughout the run. Another technique which can increase the speed of separations is a variant of backflushing where late eluting peaks (e.g. make up solvent in residual solvents analysis) can be backflushed from the column, saving on analysis and re-equilibration time. A relatively recent development has been in the use of resistive column heating in GC separations. In this application, the column is in close contact with a restive heating element and hence very high reproducible heating rates of around 1000°Cmin-1 are possible. Coupled with this, cooling rates are much faster than with a conventional GC oven and limits of detection are significantly lower due to the narrow peak widths. Cycle time reductions for analysis of the order of 5–50 times have been reported.

2.4.3.6 Automation

In today's pharmaceutical industry, the developments discussed above in improving GC instrumentation to reduce cycle times also need to be implemented in an automated and a manner compliant with good manufacturing practise (GMP) to achieve maximum benefit. Most instrument manufacturers now offer data systems which are capable of controlling instruments, storing the data in a compliant manner and allow the use of unattended operation with built-in acceptance criteria. Automated sample preparation is also becoming more important, with the goal being to achieve *round-the-clock analysis* with automated results reporting for samples with minimal human intervention. For some analyses where generic methodology can be used (e.g. residual solvents analysis), most of the above goals are now achievable and systems are in place for total analysis from a solid drug substance. Similarly, systems are also being developed to automate the procedure of method development using GC.

2.5 Capillary electrophoretic techniques

2.5.1 Brief historical perspective

The development of capillary electrophoretic (CE) techniques dates as far back as the late 1960s where, Hjerten separated some UV-absorbing compounds using a 300 μm capillary tube. Since then, the technique has developed further by Virtanen, Mikkers, Everaerts and Verheggen, who use small diameter Pyrex and Teflon tubing to separate inorganic and organic ions. In the late 1980s, Jorgenson and Lukacs used narrower silica capillary tubes (typically <100 μm); this is most likely where the term capillary electrophoresis was popularised. It was not until the late 1980s that interest in CEreally began to take off. This era saw the advent of commercial capillary electrophoresis instrumentation and the transformation of a research technique into a routine technology present in many industrial settings.

In modern pharmaceutical laboratories, CE is still an important technique, but its application in pharmaceutical analysis is less widespread. The rapid developments in LC have to a greater extent overtaken CE techniques, hence the role of CE is either in niche applications such as chiral analysis or as an orthogonal analytical technique to HPLC.

2.5.2 Developments in detection modes in CE

Instrumental developments in CE have largely been focused in two areas: expanding the range of detectors available and the hyphenation to mass spectrometry (this is covered in later sections). UV absorbance is by far the commonest detection mode available in CE and offers sensitive analysis, particularly for the separation of low-level impurities. The low path length of the detection area in the capillary, which is effectively the internal diameter (ID) of the capillary, often limits the minimum amounts that can be detected when compared to other techniques. The use of larger ID capillaries, pre-concentration and focusing effects and the use of low-ionic strength sample solvents has helped to circumvent this disadvantage. Diode array detectors offering wavelength selection between 190 and 700 nm are also available providing good quality spectral analysis. Other sensitive but less common detection systems such as fluorescence, mass

spectrometry, Raman, amperometry, conductivity, radiochemical and NMR have also been used with CE.

2.5.3 Different modes and method development options in CE

Several modes of CE have been described in the literature over past decade. The most common are open tubular or capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC), microemulsion electrokinetic chromatography (MEEKC), capillary electrochromatograpy (CEC), capillary gel electrophoresis, capillary isoelectric focusing and capillary isotachophoresis. Three recent reviews by Watzig, Tagliaro *et al.* and Riekkola *et al.* summarise the current method development options available to manipulate selectivity. In pharmaceutical analysis, CZE, MEKC, MEEKC and CEC are commonly used.

2.5.3.1 Capillary zone electrophoresis

Method development in CE involves optimising the experimental parameters such as pH, organic modifiers, surfactant additives, ion-pair reagents, cyclodextrins, polymer additives, complexation agents and combinations of these additives. The use of pH is a very powerful tool for manipulating and influencing selectivity, particularly for analytes that have small differences in their pKa values.

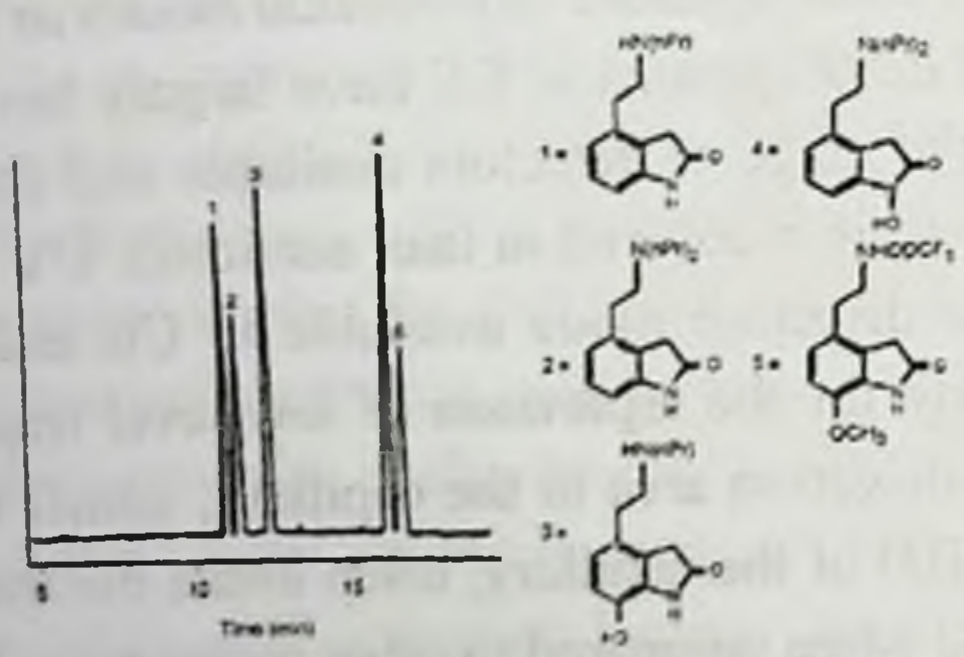
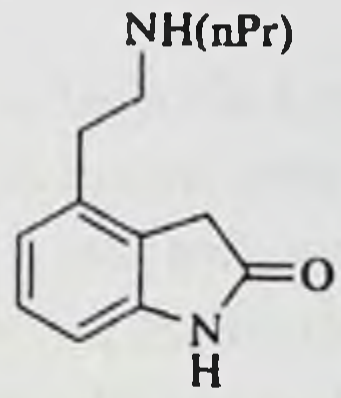


Fig. 2.7 Capillary electrophoresis of the drug ropinirole (peak 2) and a number of related molecules.

A good example of this is in the separation of the anti-Parkinson drug ropinirole (9) from a number of related molecules shown in Fig. 2.7. Here

the pKa differences and overall charge densities between (9) and its impurities are small and by using a phosphate buffer at pH 7.8, all five compounds are baseline resolved. Altering the pH value also changes the magnitude of the electroosmotic flow (EOF), which can affect migration times and the ionisation of the analytes.



(9)

Other experimental parameters to be considered include the nature of the column and its dimensions. The recommended columns are fused silica materials with typical IDs of 50–75 μm and column lengths of between 25 and75cm. Buffers types consisting of phosphates, borates and formates are usually recommended with ionic strengths of 25 and 150 mM. Where the CZE system uses a mass spectrometer as the detector, volatile buffers such as ammonium formates, acetates and bicarbonates are used.

The use of deuterated solvents such as deuterated water has been used to improve the resolution of a number of analytes including pharmaceuticals. This approach has some very unique benefits such as different ionisation equilibria of polar compounds, higher thermal conductivity and higher density, which can give rise to some dramatic improvements in separation.

2.5.3.2 Micellar electrokinetic chromatography

MEKC is used to separate compounds that are neutral and uncharged and cannot be resolved using simple buffer systems. The separation mechanism closely resembles chromatography. In MEKC, different surfactants can be used giving rise to significant differences in selectivity e.g. bile salts, cationic surfactants such as cetyltrimethylammonium bromide and neutral surfactants such as Tween. Further manipulation of selectivity can be achieved by varying surfactant

concentration, the addition of urea, cyclodextrins, ion-pair reagents, temperature, pH, buffer type and concentration. A more recent development in MEKC has been to perform MEKC in the absence of EOF. This is achieved using either coated capillaries or at low pH. This is particularly useful for acidic species which would be ionised at higher pH values and would not interact with the micellar phase.

2.5.3.3 Microemulsion electrokinetic chromatography

MEEKC is a relatively new technique in CE and in many ways is very similar to MEKC except that the solute partitions between the aqueous phase and oil droplets which are moving through the capillary. The microemulsion composition usually consists of a high pH buffer (typically borate at pH 9–10), octane, butan-1-ol and a surfactant (usually sodium dodecyl sulphate (SDS)). The water-immiscible octane forms minute oil droplets that are coated with butan-1-ol and SDS, which reduce the surface tension of the solution allowing a stable microemulsion to form. The same parameters used in MEKC to modulate selectivity can also apply to MEEKC, but it is generally accepted that MEEKC is more applicable to a broader range of solutes and most closely matches the retention and partitioning mechanism described in reverse-phase chromatography. MEEKC has been used to resolve a range of neutral compounds and pharmaceuticals.

2.5.3.4 Capillary electrochromatography

CEC is a relatively new development in CE that strives to combine the best features of HPLC and CE. Over the last decade, publications in the use of CEC have increased significantly signalling the importance ofthis mode of CE and its applications in the area of pharmaceutical analysis. Other modes of CEC have been explored including coupling to mass spectrometry and the use of pressurised flow capillary electrochromatography (PEC) to further extend the applicability of CEC. PEC has been reported to address difficult applications such as the separations of highly basic pharmaceutical compounds. Method development considerations in CEC are the same as those described in Section 2.2 for HPLC and in CE. These include the choice of stationary

phase, choice of organic modifier and concentration, applied voltage, pH, temperature, electrolyte concentration and the use of gradient elution.

2.6 Other separation techniques

Two other chromatographic techniques that are worthy of mention are TLC and SFC.

2.6.1 Thin layer chromatography

TLC is still used in many pharmaceutical laboratories in either manual or semi-automatic operation on conventional, high performance or modified stationary phases. TLC offers a quick, inexpensive, flexible and portable technique that has been the subject of some recent new developments. Despite these advancements, modern TLC has largely served as a complementary technique to other column-based liquid chromatographic methods such as HPLC.

Advances in stationary phase technology have led to commercial availability of adsorbents such as high performance silicas, aluminas, polyamides, celluloses and derivatised silicas. The development of automated method development (AMD) systems now allow multi-step gradients of different elution strengths to be achieved in a relatively short time compared to earlier manual approaches. AMD systems are ideally suited for separation of complex mixtures with a wide range of polarities. Further improvements in sample resolution and reduced method development times in TLC include the use of two-dimensional development approaches and forced-flow development by over-pressure liquid chromatography (OPLC).

The main improvements in qualitative and quantitative detection systems in TLC are centred around the introduction of densitometry. These involve the use of slit scanning densitometers or video or CCD camera (image processing) [130].

Other method development approaches used in TLC include unidimensional multiple development and multi-modal separation techniques, where TLC, in normal phase mode is used in conjunction with reversed-phase liquid column chromatography or GC to provide additional information in separations. This complementary strategy can prove very important even for wellcharacterised mixtures where new and

unexpected peaks can sometimes be revealed. An example of where this complementary strategy has proved successful is the analysis of polar aromatic flavour compounds in vanilla extracts. In this case, the RP-HPLC method was able to separate and quantify most of the principal aromatic compounds in the vanilla extracts. However, by using normal phase TLC, an additional peak was observed, isolated and later identified as 5-(hydroxymethyl)-2-furfural, a known compound found in vanilla extracts.

In the above example, the new impurities were relatively harmless, but in pharmaceutical analysis, the potential dangers are much higher and so there is a need to ensure that all identified and unidentified impurities are accounted for. Other multi-modal approaches in TLC include GC/TLC which has largely been superceded by GC/MS today. Others include SFC/TLC, combining the chromatographic technique to a flame ionisation or flamethermionic ionisation detector (TLC/FID/FTID) or non-dispersive infrared (TLC/NDIR) detection.

2.6.2 Supercritical fluid chromatography

SFC has been undergoing a renaissance in its use in modern analytical laboratories over the last decade, particularly in chiral and achiral analysis of pharmaceutical compounds. Many of the problems associated with implementing SFC have come from the technical and operational difficulties in earlier instruments. New developments in SFC include improved backpressure-regulation, more consistent SFC flow rates, more reliable sample injection systems and improved flow cell designs.

2.7 Hyphenated separation techniques

The need to obtain more information about samples and increased sensitivity for low-level impurities has lead to the coupling of existing separation techniques with specialised detection methods. Nowhere has this been more prominent than in the hyphenation of chromatographic methods to mass spectrometric detectors. A mass spectrometer can offer many significant benefits during method development compared to other detection modes (e.g. UV and diode array). Here, the key analytical challenges are sensitivity, selectivity and speed. The combination to MS

systems can avoid common analytical problems by tracking and identifying individual peaks in the chromatogram between experiments. In systems linked to MS detectors, one can distinguish compounds of interest from minor compounds or interferences. LC/MS and GC/MS represent the most important of these techniques, and this is reflected in the speed of their development and the spread and acceptance of these instruments in analyticallaboratories. In the pharmaceutical industry, LC/MS has become the methodof-choice for analytical support in many stages of drug development. Other separation techniques such as GC, CE, TLC and SFC have been hyphenated to mass spectrometers, NMR, Raman spectrometers and FTIR systems. Table 2.2 summarises a review of the key hyphenated techniques currently being used in pharmaceutical laboratories for impurity and degradant analysis. The developments and applications of separation techniques combined to MS and NMR systems will be covered more extensively in other chapters in this book.

Another new dimension in the area of hyphenated techniques that offers some very significant benefits in pharmaceutical analysis is that of multi-dimensional chromatography. Various set-ups involving coupling GC, HPLC and CE systems together in different configurations have been studied for analysing many different sample types. Examples include coupling RP-HPLC with CZE for separating ionic analytes, ion exchange chromatography with RP-HPLC, size exclusion chromatography coupled to with RP-HPLC and CE and GC coupled to LC. Since RP-HPLC and CE techniques are capable of high resolution separation with orthogonal separation mechanisms, combining both techniques in a two-dimensional mode can produce very high peak capacities and extremely high resolving power, particularly useful for complex mixtures. LC coupled to CE has been successful in a number of applications such as determination of impurities in the antifungal agent fenticonazole

Table 2.2 Summary of hyphenated separation techniques used in pharmaceutical analysis

Separation technique	Hyphenated mode
Liquid chromatography	Liquid chromatography-mass spectrometry (LC/MS)
	Liquid chromatography-Fourier-transform infrared spectrometry (LC-FTIR)
	Liquid chromatography-nuclear magnetic resonance spectroscopy (LC/NMR)
	Liquid chromatography-inductively coupled plasma mass spectrometry (LC-ICPMS)
Gas chromatography	Gas chromatography-mass spectrometry (GC/MS)
	Gas chromatography-Fourier-transform infrared (GC-FTIR)
	Gas chromatography-FTIR-MS (GC-FTIR-MS)
Capillary electrophoresis	Capillary electrophoresis-mass spectrometry (CE/MS)
	Capillary electrophoresis-nuclear magnetic resonance spectrometry (CE/NMR)
	Capillary electrophoresis-surface enhanced Raman spectrometry (CE-SERS)
Thin layer chromatography (TLC)	Thin layer chromatography-mass spectrometry (TLC/MS) Thin layer chromatography-surface enhanced Raman spectrometry (TLC-SERS)
Supercritical fluid chromatography/ extraction (SFC/SFE)	Supercritical fluid extraction-capillary gas chromatographymass spectrometry (SFE-CGC-MS) Supercritical fluid-Fourier-transform infrared (SFC-FTIR)

and the use of capillary-HPLC-CE to resolve hundreds of neutral compounds present in traditional Chinese medicines.

2.8 Use of automated approaches to method developmentin chromatography

Despite the considerable advances in chromatography, the basic approaches to GC and HPLC method development are still based mainly on a trail-and-error experimentation and can be very time-consuming and very inefficient. Within the last few years, there have been many reported examples of strategies that improve method development efficiency, reduce time, produce more consistent methods and ultimately reduce overall costs. These strategies all use automation to varying degrees, ranging from automated method development software programs through to column-switching systems and automated multi-column, multi-eluent approaches and the use of robotic sample preparation systems. Many of these approaches are now available as commercial product that can be retrofitted to existing HPLC systems.

In developing separations methods in the pharmaceutical industry, there exists two contradictory objectives: the first objective is to achieve optimum resolution of critical peaks for the API and related impurities, the second objective is to achieve this separation in as short an analysis time as possible. By using these automated approaches, a balance between resolution and run time should be achievable.

2.8.1 Separation optimisation programmes

There are a number of commercially available computer software packages that have been used recently by many separation scientists in pharmaceutical laboratories. These computer programs are designed to simulate the actions of a practising scientist developing a method. It plans, experiments, collects data, evaluates the results, makes decisions and automatically searches for the optimum conditions. Furthermore, some software systems can learn from previous experiments and observations. To achieve a fully automated system, these software programs are integrated with computers that control the operational parameters of the instrumentation. Conversely, they can also be used as off-line computer assisted HPLC method development. A review of the current literature reveals that the currently available method development software systems can be classified according to its capabilities or function and has been

summarised in Table 2.3. All the software packages listed in the table are commercially available. Many of these expert systems have focused on HPLC because this separation technique is modular e.g. sample preparation, method development and method validation, in which the basic rule is set, and is relatively well defined.

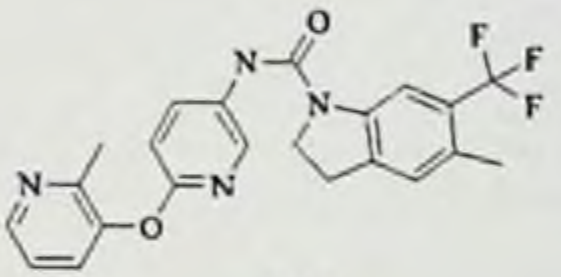
Table 2.3 Summary of method optimisation software currently available

Function/capability	Example
Changes one variable at a time and predicts separation as function of that variable	Drylab
Changes one or more variables at a time and predicts separation as a function of those variables	ICOS, DIAMOND
Changes column conditions (column dimensions, particle size and flow rate) and predicts separation for any column condition	Drylab, ENHANCER
Changes gradient conditions and predicts separation for any gradient condition	Drylab
Changes one or more conditions and examines experimental chromatograms for best separation	PESOS
Expert systems to predict best initial separation conditions on the basis of sample component molecular structures	ELUEX, CHROMDREAM, HPLCMETABOLEXPERT, ProDigest-LC, LABEL

Many of these software systems work (particularly for Drylab™) by using mathematical algorithms to predict separations for a number of other conditions after a few experimental runs have been performed. Typical predictions are made for changes made to the mobile phase conditions, temperature, isocratic or gradient separations, or changes to the column conditions (e.g. column dimensions, particle size and flow rate). The references listed in Table 2.3 provide more specific descriptions of each type of software system.

Presently, Drylab is probably the most widely used method development software package for HPLC and GC in the pharmaceutical industry and has been used to optimise the separation methodology for numerous examples. A good example of the utility of Drylab is in the development of the related substances method for SB-243213 (10).

An initial method was developed to separate SB-243213 from 11 related impurities and degradants which ran for 30 min and utilised a multi-segmented gradient to achieve adequate resolution. Four trial experimental runs permitted the construction of a 3D contour plot displaying critical resolution as a function of gradient time and temperature (Fig. 2.8). Based on these data, eluent starting conditions, gradient slope and temperature were defined to predict an optimised separation in terms of resolution and run time. The predicted and actual separations using the conditions are shown in Fig. 2.9.



(10)

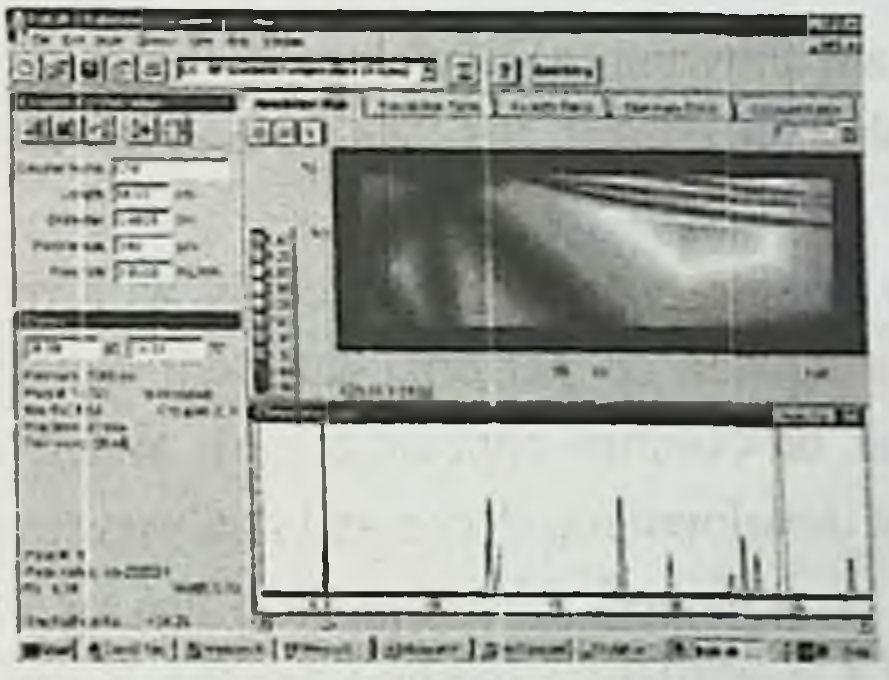


Fig. 2.8 Drylab optimisation chromatograms for the pharmaceutical, SB-243213.

Actual and predicted chromatograms agree very well with an overall decrease in method run time and a reduction in gradient complexity which will make the method much better suited for transfer to the QC environment.

2.8.2 Column switching devices

Column switching devices fitted to existing HPLC systems can offer significant improvements in method development time in LC [147, 148]. They offer the ability to screen numerous columns and conditions with minimal manual intervention.

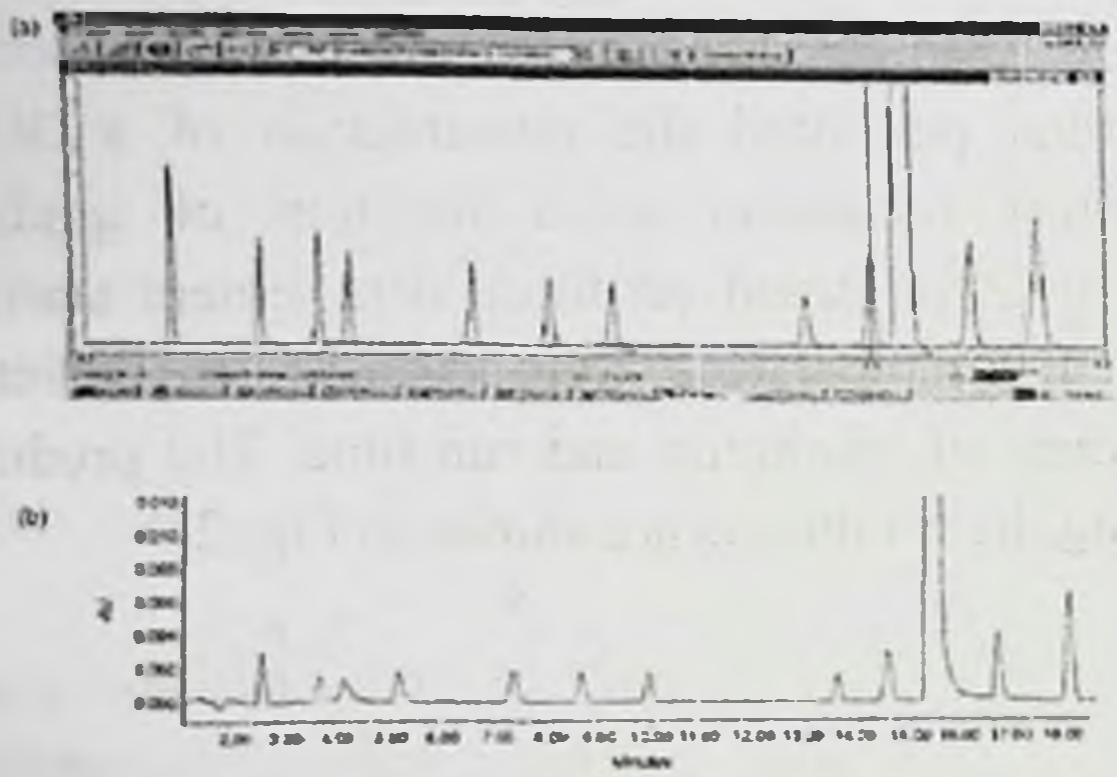
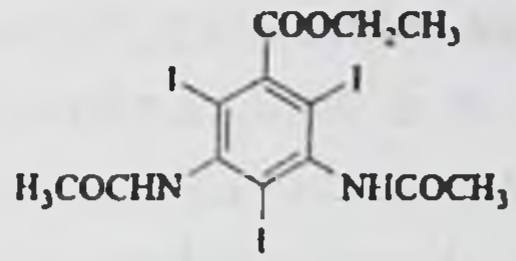


Fig. 2.9 Diagram showing (a) Drylab predicted, and (b) Actual separation for the pharmaceutical, SB-243213 and its related substances.

They are easy to automate, especially with modern HPLC systems and can be configured to perform different functions (e.g. backflush, heartcutting and on-column concentration). When column switching is used for method development, decreased analysis times and higher sample throughput can be achieved. This approach has significant benefits in the development of a quantitative method for the X-ray enhancement agent, 3,5-bis (acetylamino)- 2,4-6-triiodobenzoate (11). Here, six HPLC columns (C-1, C-6, cyano, C-18, amino and phenyl) were screened rapidly (Fig. 2.10). The HPLC set-up is shown in the diagrammatic representation in Fig. 2.3.



(11)

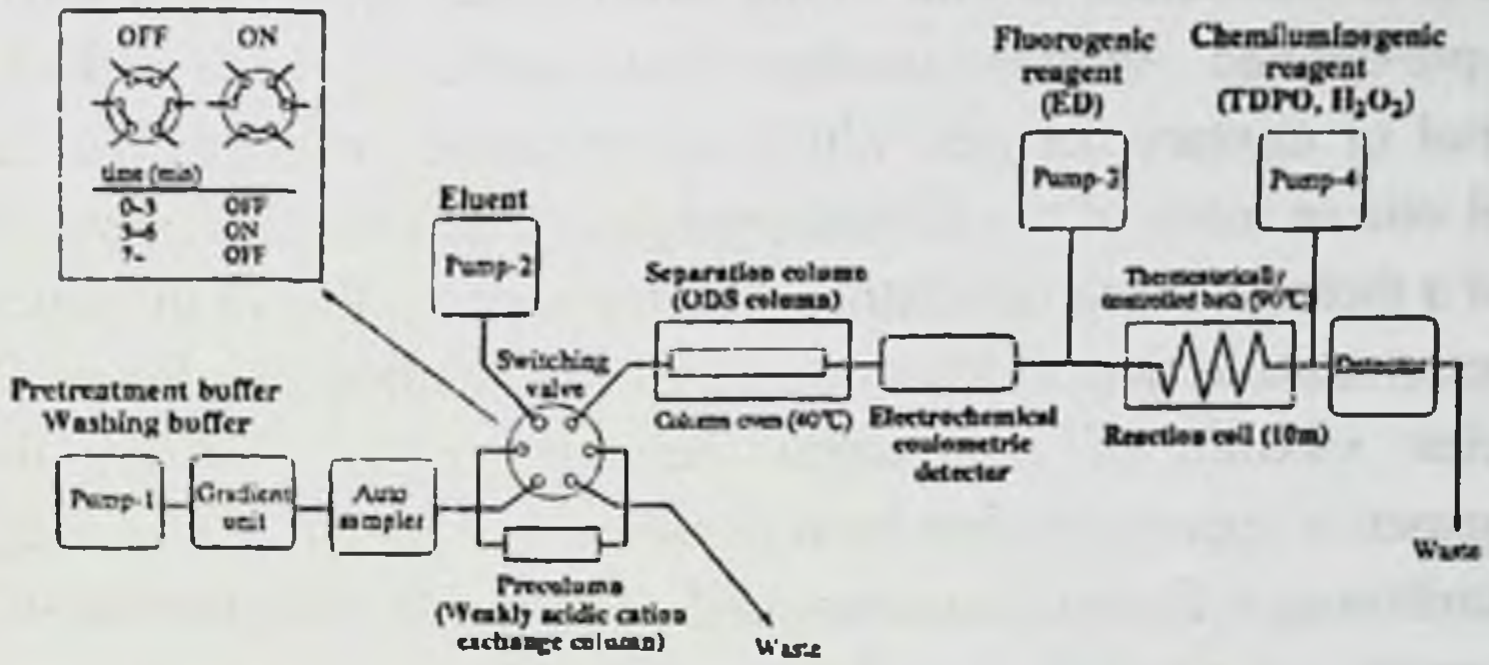


Fig. 2.10 Diagram of instrument layout for column switching screening.

Other applications where the use of column switching devices have offered significant benefits include the direct analysis of the drug indomethacin in rat plasma.

2.9 Use of chemometric approaches to method development

Chemometrics has been defined in some texts as the entire process whereby data are transformed into information used for decision-making. It is this definition that is the most applicable to separation sciences, more specifically in method development and optimisation in liquid chromatography. In this example, chemometrics has been used to predict optimum separation conditions based on empirical data and other separation information. Chemometric approaches to method development can be based on either sequential simplex models or simultaneous fixed factorial designs or interactive mixture designs which combine the advantages of simultaneous and simplex models.

In HPLC, the combination of wide utilisation of the technique, its high degree of automation and computer control, and the high degree of method development processes have made this area particularly attractive to chemometricians. Chemometrics are particularly applicable in mobile

phase optimisation, method development and post-separation data processing. In mobile phase optimisation, the sequential simplex model is most often used because it is easily understood and requires no prior knowledge of the sample and chromatographic system. However, it is limited by its inability to distinguish between local and global optima, hence it is best suited to fine-tuning when global optimum regions have been pre-defined. Another common chemometric approach is the use of factorial or mixture designs, which are regression methods that aim to model one or more of the chromatographic parameters. Examples of the use of a factorial design in method development are that of the separation of metronidazole and tinidazole and for the method development of a complex mixture of monoamine neurotransmitters. Further use of chemometric approaches has been demonstrated by Djordjevic etal. who by combining a factorial experimental design with a sequential strategy and computer simulation software was able to determine retention characteristics for five pharmaceutical compounds.

Other recently developed chemometric approaches include Heuristic evolving latent projections (HELP) which is primarily used for the analysis of qualitative and quantitative data from 2-dimensional data (e.g. HPLC DAD) used for complex mixtures, and HELP lessens the demand on developing complicated separation methods; mixed level orthogonal array design (ML-OAD) has been used to optimise the parameters involved in HPLC detection. The LC methods for determining the purity of tetracycline HCl and resolutionof its impurities were optimised using two new chemometric approaches, namely othoganol projection approach (OPA) and fixed size moving window evolving factor analysis approach (FSW-EFA).

Abbreviations

API	Accelerated solvent extraction
CCD	Atomic emission detector
CE	Automated method development
CEC	Active pharmaceutical ingredient
COC-SVE	Charge coupled device
CZE	Capillary electrophoresis
EOF	Capillary electrochromatography

FDA	Cool-on-column with solvent vapour exit
FID	Capillary zone electrophoresis
FSW-EFA	Electroosmotic flow
FTID	Food and drug administration
GC	Flame ionisation detection
HELP	Fixed size moving window-evolving factor analysis
HPLC	Flame thermionic ionisation detector
ICH	Gas chromatography
IGC	Heuristic evolving latent projections
MASE	High performance liquid chromatography
MEEKC	International conference on harmonisation
MEKC	Inverse gas chromatography
ML-OAD	Microwave-assisted solvent extraction
NACE	Microemulsion electrokinetic chromatography
NDIR	Micellar electrokinetic chromatography
NPD	Mixed level-orthoganol array design
OPA	Non-aqueous capillary electrophoresis
OPLC	Non-dispersive infra red
PDHID	Nitrogen phosphorus detector
PEC	Orthoganol projection approach
PTV	Over pressure liquid chromatography
QC	Pulsed discharge helium ionisation detector
RP-HPLC	Pressurised flow capillary electrochromatography
SDS	Programmable temperature vapouriser
SFC	Quality control
SFE	Reversed phase-high performance liquid
SPE	chromatography
SPME	Sodium dodecyl sulphate
TLC	Supercritical fluid chromatography
	Supercritical fluid extraction

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3 CHIRAL ANALYSIS OF PHARMACEUTICALS

3.1 Significance of chirality in pharmaceutical R&D

That the resolution of the enantiomers of chiral drugs should be considered as a topic in its own right in a treatise on pharmaceutical analysis should come as no surprise. The importance of chirality in many fields of natural and applied science is well established. In pharmaceutical analysis, this topic which commands its own nomenclature (Table 3.1) is especially important, as is apparent from the proportion of drugs on the market that are chiral (Table 3.2).

The chirality of these drugs is a feature which cannot be ignored. While the enantiomers of a chiral drug will have identical physical and chemical properties in an achiral environment, they can be distinguished in a chiral environment provided that there are suitable interactions with a chiral *selector*in a nonracemic form. In molecules such as proteins, enzymes and carbohydrates there is an abundance of such chiral selectors in the body. Drugs might interact with receptor proteins at their site of action. On the way to the site of action, they may be actively transported across membranes (e.g. intestinal membrane or cell wall membrane, leading into - or, in some cases, out of - the site of action) via an interaction with a protein in a so-called protein pump. Binding also takes place with proteins - predominantly albumin and α1-glycoprotein – in plasma. In metabolism, the drug must first bind to an enzyme as its substrate before the enzyme can act upon it. Less obviously, chiral interactions may be involved in binding to tissue, or in secretion of drugs in fatty deposits, for example. Given this range of processes encountered by drugs entering the body which involve these selectors, it is seldom that the body is not able to distinguish between the enantiomeric forms of a chiral drug. Accordingly the drug enantiomers are very likely to have different pharmacological and toxicological properties so that to all intents and purposes the body ***sees*** the enantiomers as different drugs. The potential for the body to be able to distinguish between enantiomers has long been recognised but it was only until the so-called Thalidomide Tragedy (Fig. 3.1) in the late 1950s early 1960s that the issue was brought into stark reality. While the sedative effect of thalidomide was attributed

to the (*R*)-enantiomer and the adverse teratogenic effecy to the (*S*)-enantiomer, it is not always, in fact not often, a simple case of good enantiomer/bad enantiomer. Even in the, by reputation, classic case of thalidomide, things arenot so simple as this.

Table 3.1 Selected nomenclature used in the field of chirality

Chiral molecules	Molecules whose mirror images are not superimposable upon each other.
Achiral molecules	Molecules whose mirror images are superimposable upon each other.
Stereoisomers	Compounds, the molecules of which have the same atoms connected in order but differ from each other in the way the atoms are orientated in space.
Enantiomers	One of a pair of molecular species that are mirror images of each other and not superimposable.
Diastereomers	Stereoisomers with multiple chiral centres that are not enantiomers.
Epimers	Diastereomers differing in configuration at one of two or more chiral centres (less frequently encountered are instances when it is used to describe cases involving chiral axes or planes).
Meso-compound	Diastereomer with two or more chiral centres where the four groups on each of the chiral carbon atoms contains a plane of symmetry within the molecule.
Racemic mixture	1:1 mixture of enantiomers.
Racemates	Strictly speaking this describes a particular solid state which is distinguishable from *conglomerates*and *solid solutions*; now generally used as being synonomous with *racemicmixture*with *racemic compound*being reserved for the description of the distinct solid state.
d-	Dextrorotatory optical rotation at a specified wavelength, solvent and temperature, usually 589 nm (sodium D line emission); now supplanted by (+).
l-	Laevorotatory optical rotation at a specified wavelength, solvent and temperature, usually 589 nm

	(sodium D line emission); now supplanted by (-).
(+)	Dextrorotatory optical rotation at a specified wavelength, solvent and temperature, usually 589 nm (sodium D line emission).
(-)	Laevorotatory optical rotation at a specified wavelength, solvent and temperature, usually 589 nm (sodium D line emission).
D-	Configurational descriptor for carbohydrates and *α*-aminoacids based on correlation with (+)-glyceraldehyde; use for other kinds of chiral compounds is now obsolete (also, for amino acids the *R/S*nomenclature is now more commonly used).
L-	Configurational descriptor for carbohydrates and α-aminoacids based on correlation with (-)-glyceraldehyde; use for other kinds of chiral compounds is now obsolete (also, for amino acids the *R/S* nomenclature is now more commonly used).
Absolute configuration	The spatial arrangement of the stereogenic centres (atoms) in a chiral molecule.
Cahn-Ingold-Prelog (CIP) Rules	Accepted system (1982 version usually used) for the description of configuration of stereogenic centres in organic molecules; generally used to assign a stereogenic centre (atom) arising from e.g. a carbon atom with four different substituents as *R*- or *S*- but there are also rules for describing other types of chiral molecules.
R-	Rectus; stereochemical descriptor in the Cahn-Ingold-Prelog Rules.
S-	Sinister; stereochemical descriptor in the Cahn-Ingold-Prelog Rules.
Chiral	Not superimposable with its mirror image; may be applied to molecules, conformations, and macroscopic objects such as crystals; note also that its use has commonly been extended to describing

	compounds the molecules of which are chiral, even if the bulk sample of the compound is racemic.
Homochiral	Not to be confused with *enantiopure*; its use must be restricted to fundamental stereochemistry e.g. a sample of (+)-tartaric acid is made up of *homochiral* molecules (not a clear term and still controversially discussed and used).
Heterochiral	As counterpart to homochiral →unprecise term.
Enantiopure	Characterization of a compound that does not contain its enantiomer impurity according to available or applied analytical methods (limits of detection and qualification of the minor enantiomer needs to be specified).
Optical purity	Measure of the composition of a mixture of enantiomers determined by measurement of optical rotation (reference compounds and methods needed).
Enantiomeric excess (ee)	The percent excess of the enantiomer over the racemate in a mixture of a pure enantiomer and a racemate.

Compiled from E. Eliel and S.H. Wilen (1994) *Stereochemistry of Organic Compounds*, Wiley-Interscience, New York, USA; G. Helmchen (1996) *Glossary of Problematic Terms in Organic Stereochemistry*,Enantiomer 1; C.A. Challener (ed.) (2001) ***Chiral Intermediates***, Ashgate Publishing Company, Burlington, USA.

Table 3.2 Proportion of single enantiomer chiral drugs in development and in licensed medicines

1996–2000 (of new chemical entities assessed by the UK Medicines Control Agency):

36% were achiral: 48% single enantiomers and 16% were racemates ca. 1997 (of drug substances in all licensed medicines):

30% were natural or semi-synthetic (of these, 98% were single isomers, 1% were mixtures of isomers and 1% were non-chiral)

70% were synthetic compounds (of these, 4.5% were single isomers,

35.5% were mixtures of isomers and 60% were non-chiral)

i.e. the % of chiral drug substances which were single isomers was 99% for natural/semi-synthetic drug substances and 11%* for synthetic drug substances

1998 (of all drugs under development):

80% were single enantiomer chiral drugs

1999 (of value of all dosage-form drug sales worldwide):

33% from single enantiomer chiral drugs

2000 (of value of all dosage-form drug sales worldwide):

40% from single enantiomer chiral drugs

2008 (projected value of dosage-form single enantiomer chiral drugs sales worldwide: projected from

the 2000 figure of $133 billion (*cf* $360 billion for all dosage-form drug sales)):

$200 billion

Complications include *in vitro* racemisation, *in vivo* inversion, appropriateness of the original animal models used and additional toxicological issues other than phocomelia. There are a number of different scenarios which might ensue (Fig. 3.2) and looking at the case of verapamil (Fig. 3.3) it is apparent just how complicated the situation can get. Despite the possibility of encountering such complexity, there are still usually clear advantages in progressing a drug candidate as its single enantiomer. Even if one enantiomer is not significantly more active or significantly less toxic than the other, there may be advantages in one enantiomer being more selective

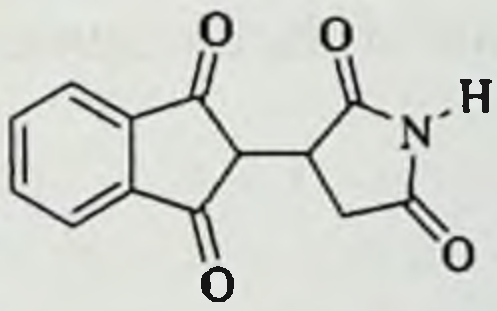
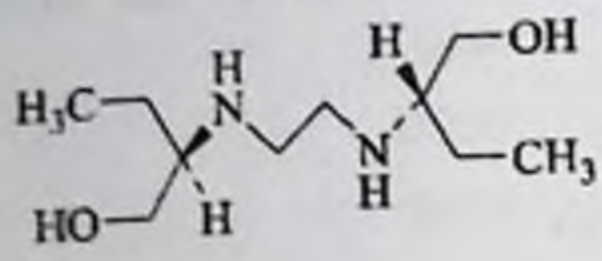
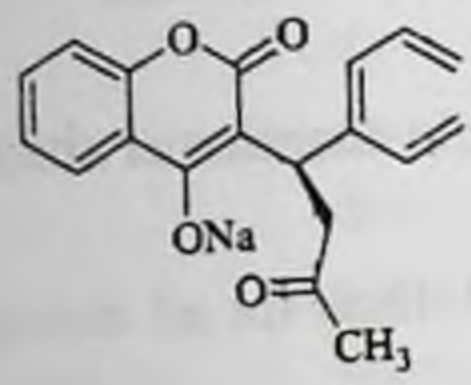


Fig. 3.1 Thalidomide was developed as a non-addictive sedative as an alternative to barbiturates. Following its administration to pregnant women, it was found that the babies were born with truncated limbs (phocomelia). At the time this was perceived as a classic case of the pharmacological activity residing in one enantiomer (R-) and the undesirable toxicological effect residing in the other enantiomer. The

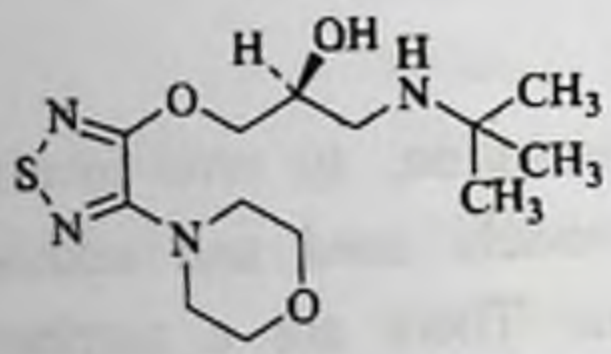
situation is now known to be much more complex than this (see text and reference cited therein).



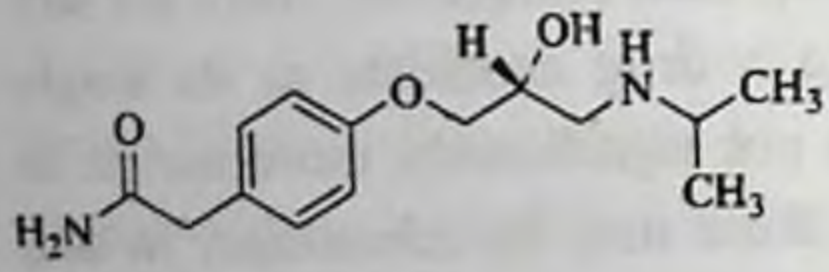
Have adverse toxicological effects e.g. ethambutolantituberculosis, while enantiomer may induce blindness (also, by reputation, thalidomide (*R*-sedative, *S*-teratogenic) but see qualification in text and references cited therein)



Have the same desired effect, but at a lower level e.g. warfarin (sodium) (anti-coagulant) (also propanolol (β-blocker)).



Have differing activity for more than one therapeutic effect e.g. timolol (*R*- is ¼ as potent at reducing intraocular pressure and 1/50 as active as a β-blocker so that in principle it might be a safer drug for the treatment of glaucoma than the *S*-).



Be totally ineffective and harmless e.g. atenolol (β-blocker).

Fig. 3.2 Illustrations of some of the differences in activity of unwanted enantiomers that may arise.

S-enantiomer

- *More potent Ca2+ channel blocker*
- *Both vasodilating and cardiac depressant*
- *More rapid clearance.*

R-enantiomer

- *Less potent Ca2+ channel blocker*
- *Principally vasodilating*
- *More bioavailable*
- *Na+ channel and other cell pump actions.*

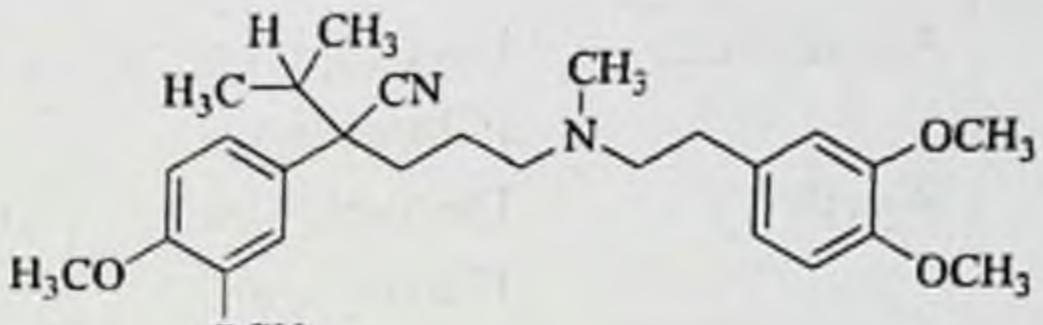


Fig. 3.3 Pharmacological action of verapamil enantiomers.

in its therapeutic action. Also, in the rare case of an enantiomer that, if it is possible at all, is considered completely inactive there is still a preference for developing a single enantiomer. The reason for this is that there is no justification for administering an equal amount of *isomeric ballast*to the active pharmaceutical ingredient when there is no need for it to be there. While there may be exceptional cases when the progression of a racemate or, for that matter, a mixture of enantiomers in a ratio other than 50:50 may be justified, it is now the case that, irrespective of the exact nature of the regulations, it is usually mandatory to develop a new chiral drug candidate as its single enantiomer. Given this strong preference for single enantiomer drugs, it is not surprising that racemic chiral drugs already on the market have been revisited. The replacement of a marketed racemic chiral drug by one of its enantiomers as a superior drug is known as a chiral switch (or occasionally but less commonly as a racemic switch). In a recent review on all aspects of the chiral switch approach a case was made that there are still opportunities in this area, e.g. single enantiomers of therapeutically active metabolites that are produced *in vivo*from a racemic drug. Nonetheless it is fair to say that the possibilities of gaining advantage from making a simple chiral switch are becoming exhausted with most of the obvious opportunities (Table 3.3) already having been exploited.

Table 3.3 Illustrative marketed chiral switches

Drug (trade name)	Company	Single enantiomer (trade name)	Company
Omeprazole (Losec)	AstraZeneca	Esomeprazole (Nexium)	AstraZeneca
Bupivacaine (Sensorcaine)	AstraZeneca	Levobupivacaine (Chirocaine)	Chiroscience
Ketoprofen (Orudis)	Wyeth	Dexketoprofen (Enantyum)	Menarini
Ofloxacin (Floxin)	Ortho-McNeil	Levofloxacin (Levaquine)	Ortho-McNeil
Cetirizine.HCl (Zyrtec)	Pfizer	Levocetirizine (Xyzal)	Sepracor

Source: Agranat et al. (2002).

Under the circumstances described above it is no surprise that the shift in proportion of chiral drugs is swinging back to single enantiomer drugs (Table 3.2). In the early days of the pharmaceutical industry most drugs came from natural sources and hence single enantiomer drugs predominated. As the proportion of synthetic drugs increased, the proportion of achiral and racemic drugs increased. Now, the proportion of single enantiomer drugs will continue to increase, irrespective of the exact nature of the regulations.

Therefore the main emphasis with respect to analysis is that stereoselective separative methods are needed primarily to resolve any unwanted trace enantiomeric impurity from the main enantiomer present in a drug substance or drug product sample. Chiral separative methods may also be required in drug discovery in order to isolate sufficient quantities of each enantiomer for pharmacological screening. Preparative scale chiral separations are even becoming a commercial proposition for the production of single enantiomer drugs. With respect to drug bioanalysis, stereoselective separative methods are still required, for example, to check whether or not *in vivo* chiral inversion takes place, as in

the case of ibuprofen, or perhaps to check the enantiomeric distribution of metabolites.

With all these applications a chiral separation must be developed, but, after, under two decades of rapid progress in commercially available chiral separation technology, the situation has now been reached that developing a suitable chiral separation is often the easiest part of the overall method development.

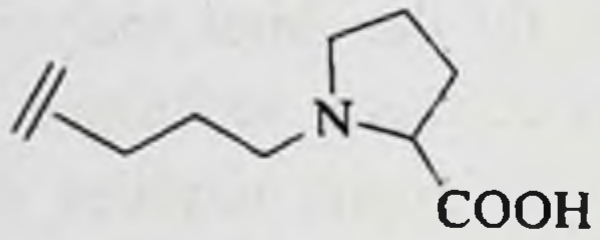
3.2 Evolution of methodologies for chiral resolution

Just as modern liquid chromatography (LC) is the dominant separative technique in the analysis of pharmaceuticals, so, in the evolution of methodologies for the chiral analysis of pharmaceuticals, LC has emerged as the pre-eminent technique. As already intimated, discrimination between enantiomers requires the presence of a chiral selector. In the determination of enantiomers by LCthis may be by a chiral derivatisation agent, a chiral stationary phase (CSP) or a chiral mobile phase additive (CMPA). A further prerequisite for discrimination is that the chiral selector must be able to form three point-to-point interactions with one or both enantiomers [8]. While this *three-point interaction rule*is a simplification in that the interactions will not involve *points*it was nonetheless very useful in aiding the design of early successful LC chiral selectors and rationalising others. CSP had clear advantages over the use of chiral derivatisation and CMPA, which, for example, involves costly consumption of the chiral selector. Also the presence of the additive in the mobile phase might cause detection problems in that (a) for example, a UV-absorbing additive would give rise to high background noise for a UV detector, and (b) each enantiomer would pass the detector on average, at least in part, as its transient diastereomeric complex with the CMPA so that potentially the detector response arising from each of the enantiomers could be different. Accordingly, the vast majority of the commercial development was in CSP such that derivatisation and mobile phase additives are now only used for a few specific applications to which they are well suited.

The first wave of development of commercial CSP was in the 1980s at a time when analytical technology had advanced to the point when it

was at last able to meet the need to address the study of the individual enantiomers of chiral drugs that had been sharply brought into focus by the thalidomide tragedy some two decades earlier. Progress made in the commercial development of CSP during the latter half of the 1980s has been well chronicled (ancillary reading list). The features of the eight classes of chiral selector that were brought into routine use during the 1980s are summarised in Fig. 3.4 a–h. Wainer was able to reduce these to five different *types*. By the end of the 1980s, then, there were a number of well-defined classes of commercially available CSPs for LC and for each class of CSP there was a reasonable possibility of predicting what types of compounds, the enantiomers of which, could be resolved using that class.

(a) Ligand exchange



• Cheap, readily available pure, natural amino acid chiral selector, in this case proline;

• organic-aqueous buffer, pH 7.5–9.5 containing metal ions, often Cu^{2+};

• may be carried out on an achiral reversed-phase LC column, using a hydrophobic amino acid as a mobile phase additive.

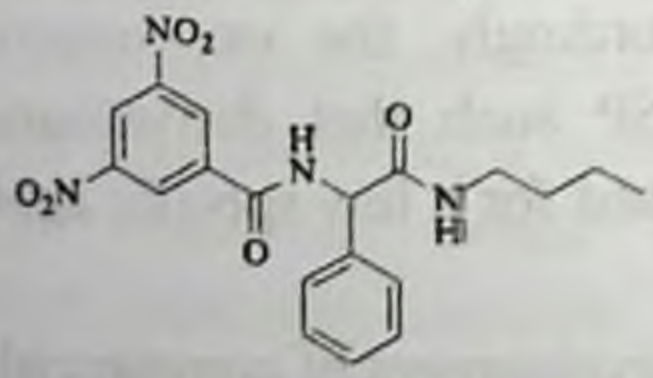
Features

• High enantioselectivity for α-amino acids and α-hydroxy amino acids;

• several options for manipulating experimental variables to optimize resolution;

• occasionally poor chromatographic efficiency is encountered at ambienttemperature because of slow mass transfer: in such cases LC at elevatedtemperature may be advantageously carried out.

(b) Synthetic multiple interaction (Pirkle-type)



• Modified natural amino acid chiral selector, in this case N-(3,5-dinitrobenzoyl) phenylglycine;

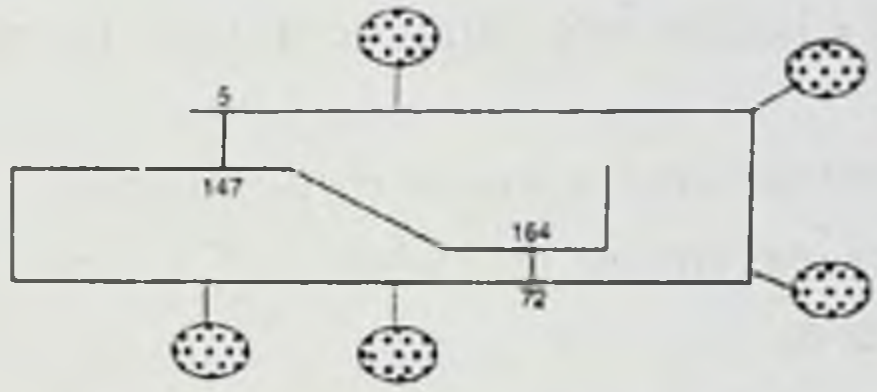
• straight-phase solvents used, typically propan-2-ol–hexane.

Features

• Initially claimed to be broad spectrum but essentially π-acidic selectorsworked well for neutral compounds containing a π-basic group andessentially π-basic selectors worked well for neutral compounds containinga π-acidic group.

• Very good resolution may be obtained if use achiral derivatisation toadd a π-acidic or π-basic group to the analyte as appropriate.

(c) Proteins



Schematic representation of α1-acid glycoprotein showing the peptide backbone, two disulphide bridges and five carbohydrate units.

• Large molecular weight, natural chiral selectors; importantly, capable of

chiral discrimination in vivo.

• α_1-acid glycoprotein and bovine serum albumin CSP first to be introduced and most commonly used.

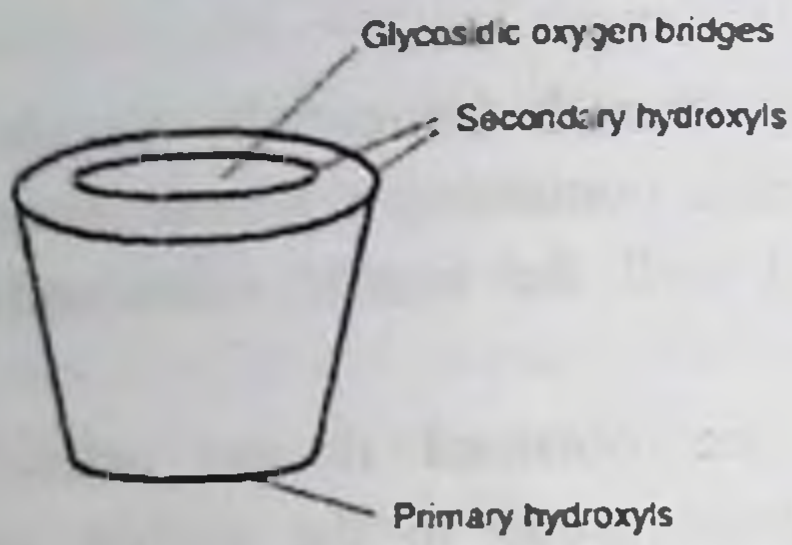
Features

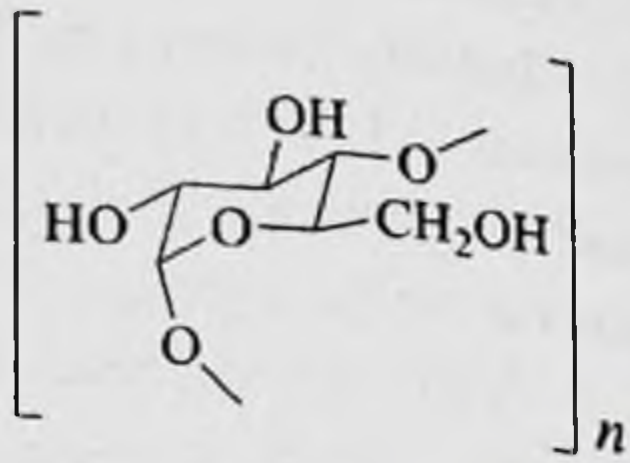
• Broad spectrum, especially when considering AGP and BSA together since AGP is particularly successful for basic analytes and BSA is best suited for acidic analytes.

• Physical and chemical stability issues, not the problem that many perceive but best not to stray too far from physiological pH of 7.4 and not to use too high an organic content in the mobile phase.

• Because of slower mass transfer arising at least in part from the size of the chiral selector, the chromatographic efficiency is reduced compared to that obtained when using for example, conventional achiral reversed-phase columns.

(d) Cyclodextrins





General cyclodextrin structure (e.g. for α-cyclodextrin, n=7); the glucose units form a bucket-like structure suitable for molecular inclusion complexation.

• Low/medium molecular weight chiral selector;

• obtained by enzymatic degradation of a larger, readily available naturalchiral molecule.

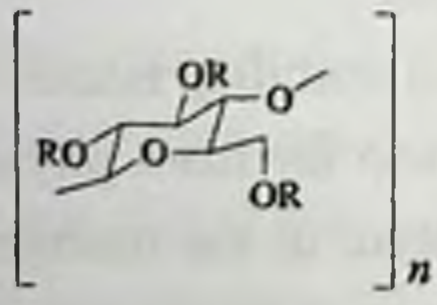
Features

• Originally used only in the reversed-phase mode with reasonably goodbreadth of spectrum;

• good chromatographic efficiency;

• given that, in the reversed-phase mode, chiral recognition involves the inclusion of a hydrophobic group of appropriate size into the hydrophobic cavity of the cyclodextrin bucket (e.g. a naphthalene ring into a β-cyclodextrin), there may be a sharp difference in enantioselectivity arising from a slight difference in the structure of the analyte.

(e) Derivatised cellulose



Cellulose triacetate
and benzoate

• Derivatised natural chiral selector;

• acetyl and benzoyl phases introduced initially.

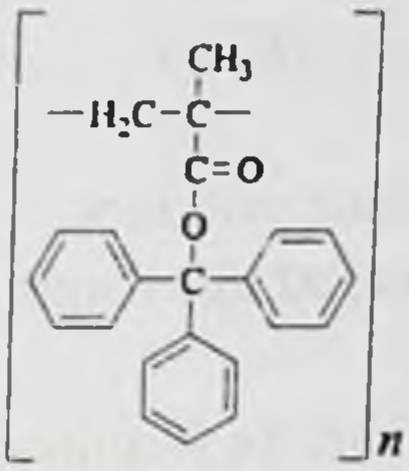
Features

• Used with straight phase mobile phases;

• acetyl and benzoyl phases successful for aryl-neutral hydrophobic molecules;

• need to avoid chlorinated solvents.

(f) Synthetic polymers



Synthetic polymer, poly(triphenylmethyl methacrylate) forms a helical structure similar to that of cellulose; chirality arises from the twist of the helix with one enantiomer being formed because of the use of a chiral initiator in the polymerisation.

• Synthetic, large molecule chiral selectors;

• designed to mimic derivatised cellulose phases.

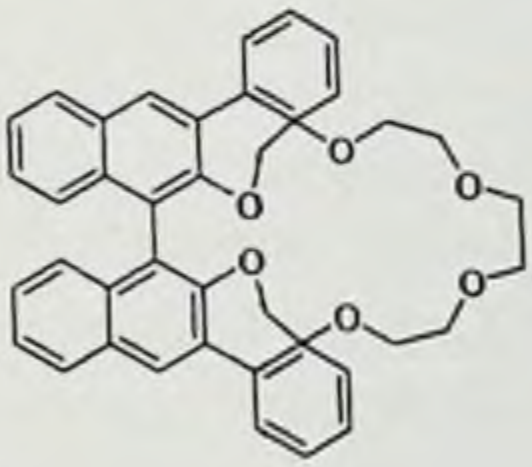
Features

• Very similar properties to derivatised cellulose phases;

• used with *straight phase*mobile phases;

• successful for aryl-neutral hydrophobic molecules.

(g) Crown ethers



Crown ether; with this particular example the chirality arises from molecular overcrowding in the binaphthol moiety.

• Synthetic chiral selector;

• used with a very simple aqueous perchloric acid mobile phase.

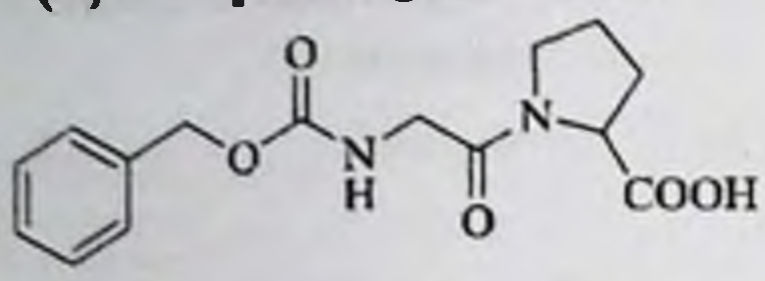
Features

• Very limited breadth of spectrum; only works for primary amines asthere is an ammonium ion;

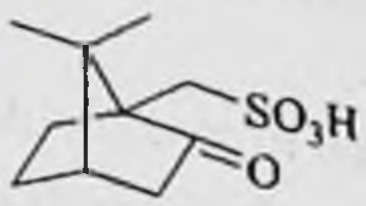
• little scope for mobile phase optimisation;

• good chromatographic efficiency.

(h) Ion-pairing reagent



N-benzoxy-glycyl-proline (ZGP)



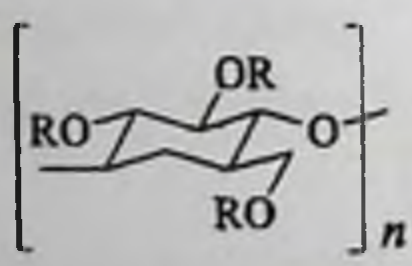
D-10-camphorsulphonic acid

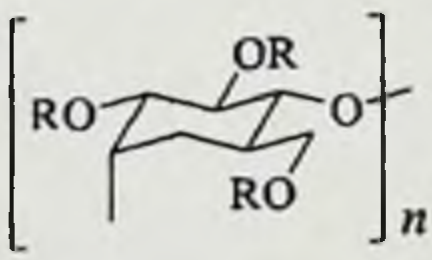
- Modified natural chiral selectors;
- used as mobile phase additive under straight phase conditions.

Features

- Successful separations for β-amino alcohols;
- careful control of trace water content of mobile phase needed.

Following the rapid advances made during the 1980s, there followed a period of proliferation of CSP and the use of chiral selectors developed for LC in other analytical techniques. The proliferation, though, was not entirely without purpose. By designing additional complexity onto existing types of chiral selector it was hoped to bring about new chiral selectors that would still have good chromatographic efficiency but greater breadth of spectrum with respect to the range of compound classes for which they could bring about a chiral separation. A good illustration of this was the development of aryl carbamates (Fig. 3.5) of not only cellulose but also amylose to form coated stationary



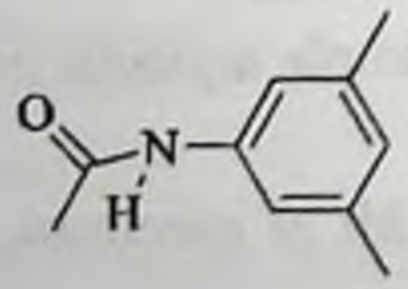


When R=H,

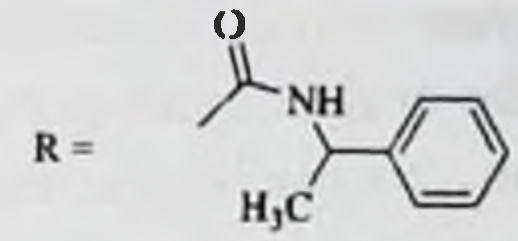
cellulose amylose

cellulose tris (3,5-dimethylphenyl carbamate) Chiralcel OD

amylose tris (3,5-dimethylphenyl carbamate) Chiralpak AD

R = 

amylose tris (*S*-phenylethyl carbamate) Chiralpak AS



cellulose tris (4-methylbenzoate) Chiralcel OJ

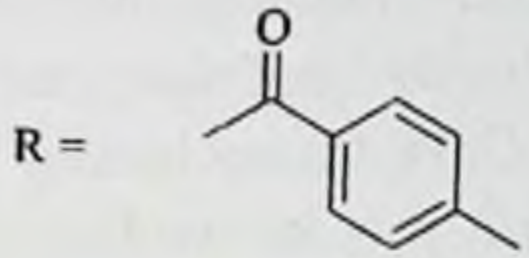


Fig. 3.5 Popular Daicel derivatised polysaccharides.

phases that were highly successful when using *straight-phase*mobile phases such as *n*-hexane-propan-2-ol. These CSP gave the desired good chromatographic efficiency and chiral resolution for a much broader spectrum of compound classes than the earlier cellulose triacetate and cellulose tribenzoatecoated CSP. A similar strategy was adopted in attempting to enhance the performance of cyclodextrin CSP by derivatising the secondary hydroxyl groups on the *rim*of the cyclodextrin *bucket*(see Fig. 3.4(d) for the general shape of cyclodextrins) to produce naphthylcarbamate groups. This CSP gave excellent enantioselectivity for specific analytes such as dinitrobenzyl-*R*,*S*-1 (1-naphthyl)ethylamine for which the dinitrobenzoyl group gives a π–πinteraction with the naphthalene rings on the CSP, and the naphthalene rings on the analyte fit into the hydrophobic cyclodextrin *bucket* in the CSP. Different enantioselectivity was therefore obtained from the equivalent native cyclodextrin CSP but there were no general improvement in breadth of spectrum. Similarly, the earlier generations of Pirkle-type or synthetic multiple interaction CSPs were modified by introducing additional charge-transfer facilitating groups and structural rigidity. In general, these CSPs were an improvement on the earlier such CSPs but they did not have the same breadth of spectrum as the derivatised polysaccharide CSP. These developments and the introduction of macrocyclic antibiotic CSP in which the additional structural complexity is natural but which are smaller than proteins and so give better chromatographic efficiency have been well reviewed (see Ancillary reading list or for an overview).

The CSPs that have been introduced are in the main, more effective, and taken together offer the analyst much greater diversity of options in deciding which CSP to use to resolve the enantiomers of a particular compound. To a limited extent these benefits are offset by the fact that with these newer CSPs it is more difficult to predict what chiral compounds may be separated into their enantiomers unless sophisticated approaches such as molecular modelling are adopted. As discussed, simple classifications of CSPs by mechanism of chiral recognition and types of chiral analyte that may be resolved, as could be made in the 1980s, can no longer be made. Therefore the current trend in the practice of chiral separations is towards a screening approach whereby a number of different CSPs are evaluated in an automated system. These approaches are so successful that, in fact, in terms of simply being able to develop a chiral separation, it could be argued that technology has progressed as far as it needs to progress. Certainly the field of chiral separations is now relatively mature. Despite this, the practices of chiral separation continue to evolve in light of the changing needs for methods for the determination of enantiomers, particularly in the pharmaceutical industry, and the ongoing proliferation of new commercially available chiral selectors. This will continue to be the case given the strategic importance of chirality in drug development and the sheer size of the chiral drugs market. That technology,that has progressed as far as it needs to, could not be said for specific applications that arise in pharmaceutical R&D or similar environments. The determination of a trace enantiomeric impurity in a sample of a single enantiomer drug substance in the presence of a range of other structurally related impurities and a large excess of the major enantiomer remains challenging. Similarly, in chiral drug bioanalysis being carried out now more in discovery rather than in development and in preparative chiral LC being considered for production, there will always be a case where there will be a need for new developments to try to improve throughput and drive down costs. These issues are discussed in more detail later.

3.3 Recent developments in commercial CSP for LC

3.3.1 Polysaccharide-based CSP

The polysaccharide-based CSP manufactured by Daicel Industries [C1] have been amongst the most successful and useful ever since the early 1990s. Within the wide range of these phases there are a few that can be identified as being the most successful. This has been acknowledged by Daicel by coining the phrase gold medal to describe their OD, AD, OJ and AS CSP (Fig. 3.5). However, almost all of this success was achieved using organic straight-phase mobile phases. A reversed-phase version of the OD CSP was introduced but it was only until recently that a range of reversed-phase compatible derivatised polysaccharides was developed. It is now possible to purchase CHIRALPAK® OD, OJ, AD, AS CSP in -RH versions which are not only suitable for use with polar-organic and reversed-phase organic-aqueous mobile phases but are supported on 5 µm particles, thus warranting the high resolution tag to distinguish them from earlier Daicel materials based on 10 µm and 20 µm (primarily for semi-preparative work) particles. -H versions, based on 5µm particles, but only suitable for straight-phase use, are also available. It remains to be seen just how successful the CHIRALPAK-RH CSP will prove to be since they have not been on the market long enough to have been comprehensively evaluated. However, although the driving force for their development seems to have been a simple me-too attempt to obtain a slice of the lucrative reversed-phase chiral market, there is already enough evidence to suggest that they represent a useful addition to the range of commercial chiral LC options. Increasing retention is usually accompanied by increasing resolution. Accordingly, chiral resolution may be increased by decreasing the proportion of organic solvent in organic-aqueous mobile phases. Enantioresolution may be increased for basic chiral drugs by increasing mobile phase pH. Enantioresolution may be increased for acidic chiral drugs by decreasing mobile phase pH. Similarly, as with the earlier introduced CHIRALPAK OD-R CSP, retention and resolution may be increased by increasing ionic strength. Interestingly, the nature of the salt usedto regulate ionic strength, e.g. sodium perchlorate vs sodium phosphorus hexafluoride, may also influence retention, suggests an

element of ion-pair interactions contributing to the retention mechanism. Reduction in temperature too may be used to increase retention and resolution but, as in most other cases, the use of sub-ambient temperatures would not be the first option to go for in developing a method for routine use.

This all seems like very good news and would be especially good if the newer RH-versions could be used for all applications. However, it seems that these phases exhibit a memory effect such that it is recommended that one column should be used for one mode and another for the other mode (i.e. reversed-phase or straight-phase). This memory effect has been noted by users and is definitely not just a ploy to boost sales.

3.3.2 Macrocyclic antibiotic CSP

Just as Daicel Industries have been responsible for the commercialisation and success of polysaccharide CSP borne out of the work of Okamato, Advanced Separation Technologies Inc. [C2] has been the commercial conduit for CSP and chiral selectors developed by the American chiral separations pioneer, Armstrong. This began with the exploitation of cyclodextrin products, but the later introduced macrocyclic antibiotics from Astec are now more popular. These have been well established for some time now and, like the cyclodextrins, may be used in both reversed-phase and polar-organic modes. As such they regularly feature in the chiral method development screening strategies employed by pharmaceutical R&D analysts, particularly when enantioselective separations for polar compounds are being sought.

One significant commercial development which has been taking place is that it has been demonstrated that macrocyclic antibiotic CSP may be used successfully with normal phase solvents, mixtures of *n*-hexane and ethanol being the most frequently used. Most of the illustrative examples cited in the commercial literature feature non-polar analytes not all of which are of pharmaceutical interest (Fig. 3.6) but nonetheless it is a good selling point that these CSP may be used with the complete range of mobile phase polarities.

The most recent addition to the suite of commercialised macrocyclic antibiotic chiral selectors is teicoplanin aglycone, which, as its name

suggests, is teicoplanin with the three carbohydrates removed (Fig. 3.7). This selector does genuinely offer something different particularly in comparison to teicoplanin itself. As might be expected, there are differences such as some acidic molecules showing increased enantioselectivity and a number of neutral molecules showing enhanced resolution, unusually, in single solvent mobile phases such as methanol, ethanol and acetonitrile. However, the most characteristic advantages are the significantly enhanced resolution displayed for many of the amino acids and

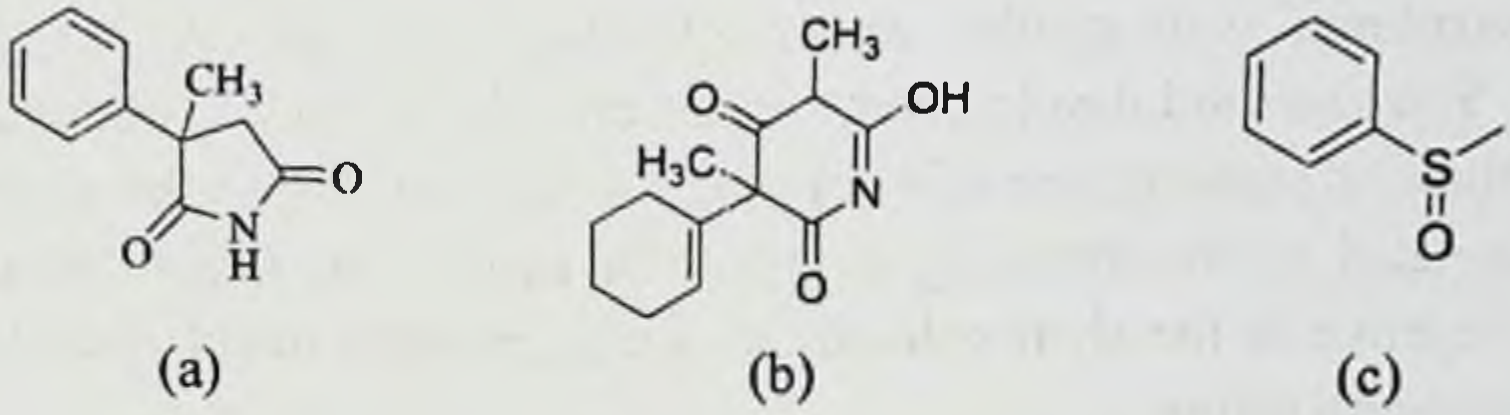


Fig. 3.6 Illustrative analytes resolved on macrocyclic antibiotic CSP in the straight phase mode (a) phenyl-α-methyl-α-succinimide, hexane–ethanol (30:70, v/v) on teicoplanin CSP (Chirobiotic T) (b) hexobarbital, hexane–ethanol (40:60, v/v) on vancomycin CSP (Chirobiotic V) coupled to teicoplanin CSP (Chirobiotic T) as part of a coupled short column method development screen (c) methyl-phenyl sulphoxide, hexane–ethanol (40:60, v/v) on teicoplanin agylcone CSP (Chirobiotic TAG).

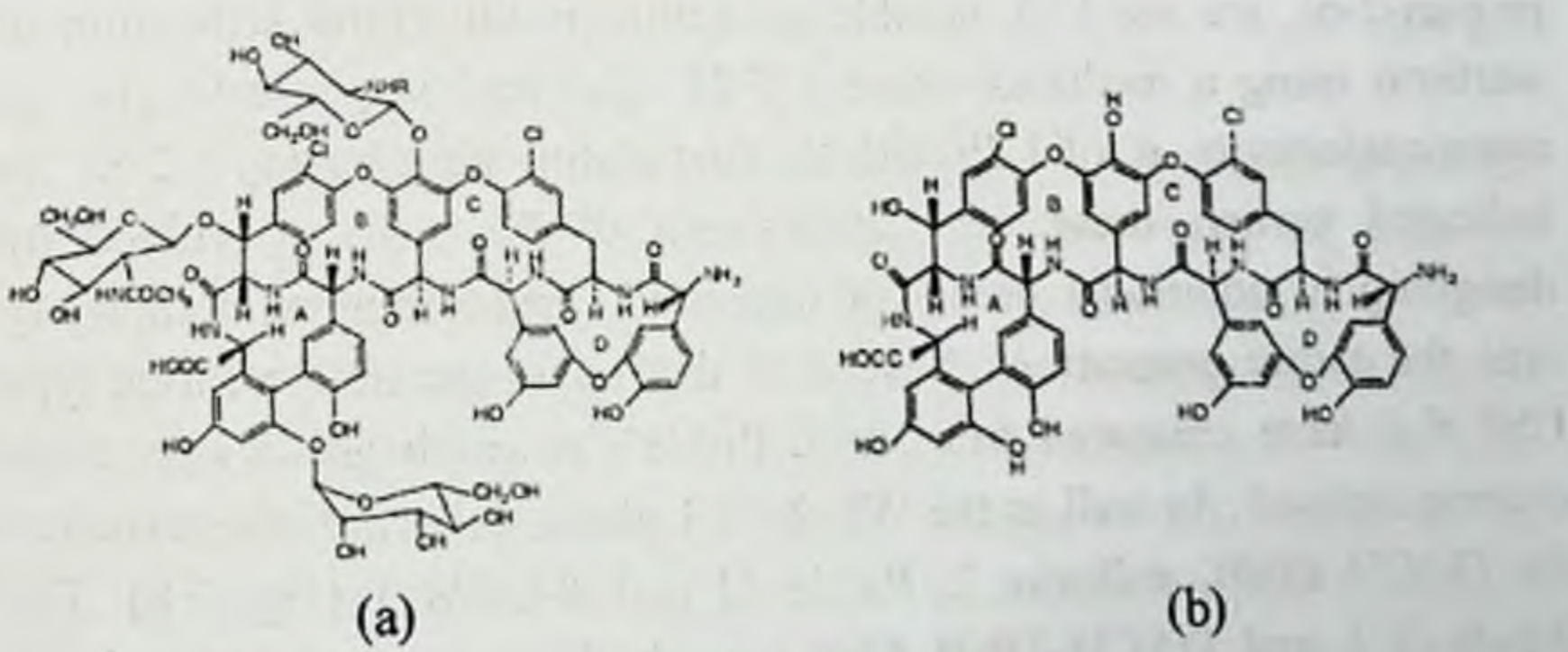


Fig. 3.7 Structure of chiral selectors (a) teicoplanin, and (b) teicoplanin aglycone.

for sulphur-containing compounds including the amino acids, methionine, histidine and cysteine. Another notable development in the use of macrocyclic antibiotic CSP is that it is being advocated [C2, C3] that in screening for method development using these phases three short columns (e.g. vancomycin, teicoplanin, ristocetin) in series are used instead of three conventional length columns in parallel with automated column switching between runs. The short columns in series approach might be faster and be carried out at lower cost but there is a possibility, surely not a remote one, that a chiral separation on one column might be missed because of the resolution being cancelled out by a separation on anothercolumn with similar enantioselectivity and opposite retention order. Since method development screens are often carried out overnight on automated systems, speed is not often an issue so long as the exercise is completed by the following morning. Given this, the minor risk of a false negative in the short columns in series approach might often be a risk not worth taking.

3.3.3 Synthetic multiple-interaction CSP

The *flagship* of the synthetic multiple-interaction or Pirkle-type class of CSP is the Whelk-O 1 phase. This has a highly suitable shape and electronic distribution to resolve naproxen and accordingly naproxen enantiomers may be separated on it using reversed-phase conditions [C4, C5]. However, in almost all the other applications that have been reported, straight phase conditions, typically using *n*-hexane in combination with propan-2-ol, are used. A notable exception is the chiral separation of warfarin using a methanol–water (75:25, v/v) mobile phase to give an enantioselectivity, α, of 1.49 with the first eluting peak having k 2.53. As indicated earlier, these new generations of CSPs have evolved by designing in additional points of interaction and structural complexity onto the earlier generations. Several of the higher generation Pirkle-type CSP that have emanated from Prof. Pirkle's research group have been commercialised. As well as the Whelk-O 1 phase, there are others such as the DACH–DNB, α-Burke 2, Pirkle 1J and β-GEM 1 (Fig. 3.8). The Whelk-O 1 and DACH–DNB CSP have both π-acceptor and π-donor properties while the α-Burke 2, Pirkle 1J and β-GEM 1 CSP are π-acceptors. The Whelk-O 1 CSP has the broadest spectrum of applicability

but each of the others is claimed to be particularly suitable for certain applications. DACH-DNB is recommended for sulphoxides, phosphonates and phosphine oxides. α-Burke 2 is recommended for benzodiazepines. Pirkle 1J is recommended for non-steroidal anti-inflammatory drugs (NSAIDs). β-GEM 1 is similarly recommended for NSAIDs. It is also recommended for a wide variety of carboxylic acids and for anilide derivatives.

The number 1 in the title of a Regis CSP designates linkage of the chiral selector to the stationary phase via a single Si-O linkage while 2 designates a *trifunctional* version with each silicon atom on the selector terminus being linked to three, in theory, oxygen atoms on the silica surface. This latter linkage is said to improve resistance to hydrolysis while using strong organic modifiers such as trifluoroacetic acid (TFA). The Whelk-O, α-Burke and β-GEM in particular seem to be available in both 1 and 2 forms.

The synthetic multiple-interaction or Pirkle-type class of CSP is not covered by patents in the same way that some other classes of CSP are. Accordingly researchers other than Pirkle's group have made Pirkle-type CSP worthy of commercialisation. Lindner has made tartramide coated phases, as well as derivatives, phases derived from cinchona alkaloids utilizing ion–ion attraction as one of the multiple interactions, and was involved with co-workers' development of a CSP derived from (*R*,*R*) or (*S*,*S*)-1,2-diphenyl- 1,2-diaminoethane that was to be named ULMO (Fig. 3.9) by Regis. The ULMO CSP works well for many of the compound classes for which the Whelk-O CSP is used and works especially well for resolving the enantiomers of aryl carbinols.

It is ranked in order of preference by Regis just behind the Whelk-O and ahead of the DACH–DNB, α-Burke, β-GEM, Pirkle 1J, leucine, phenylglycine (i.e. Pirkle-type 1A) and naphthylleucine in that order.

Just as Pirkle does not have a monopoly on synthetic multiple-interaction CSP, similarly Regis do not have a monopoly on their commercialisation. Kromasil [C6] market a range of CSP based on the work of Allenmark's research group in which a derivatised tartramide chiral network polymer is covalently bonded to silica. As discussed later, these products are geared towards the preparative chiral LC market.

Fig. 3.8 Popular CSP manufactured by Regis (a) α-Burke 2, (b) β-Gem l, (c) Pirkle 1J, and (d) DACH–DNB.

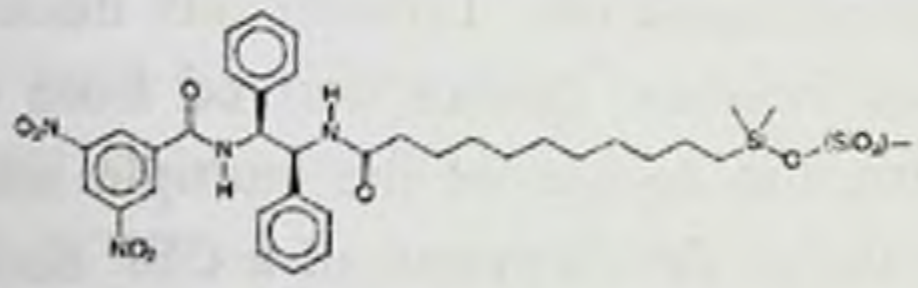


Fig. 3.9 The ULMO CSP manufactured by Regis.

In this market the advantage of all synthetic multiple-interaction CSP that the retention order of enantiomeric analytes may be switched by switching from the CSP based on the *R*-chiral selector to that based on the *S*-chiral selector or vice versa is particularly pertinent. This feature will ensure that there remains a future for synthetic multiple-interaction CSP even in the face of successful developments of CSP with a broader spectrum of enantioselectivity.

3.4 Role of *historical* CSP

The current success and dominance of the commercial CSP market by derivatised polysaccharide, macrocyclic antibiotic and, to a lesser

extent, synthetic multiple-interaction Pirkle-type materials is such that it could be considered questionable whether or not there is a need to maintain an awareness of the capabilities of some of the earlier developed CSP. Indeed in a rather shrewd marketing move some Diacel products have been dubbed *historical*CSP. This suggests, at the same time, that the newer CSP represent a major advance in technology but that the older ones are well worth preserving. The latter point is especially true. No matter how successful multi-column screening approaches for chiral separations - method development have been, there is no one who claims a success rate greater than 80%.

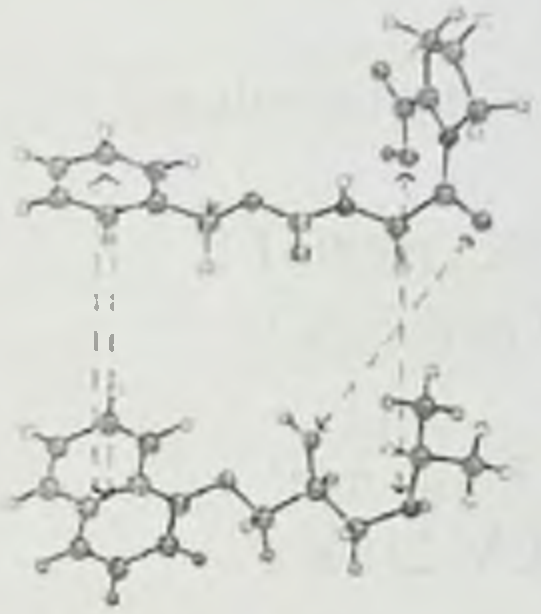


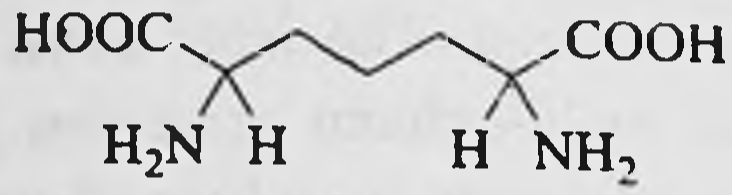
Fig. 3.10 Possible three points of interaction between N-benzoxy-glycyl-l-proline, ZGP, and propranolol. There will be a strong attraction between the acidic functional group of the chiral selector and the aminogroup of the analyte and a potential H-bonding interaction between a carbonyl group of the ZGP and the hydroxyl group of the propranolol. A third interaction is possibly a π-stacking interaction, or steric repulsion, between the naphthalene ring of propranolol and the benzene ring of the ZGP.

This suggests that for the time being there will remain a need for these older CSP as alternatives. More importantly, given that the mechanism for older CSP classes has been very well characterised and it is known which types of compound may be well resolved on them, it ought often to be possible to proceed to using one of them right away without needing to resort to conducting a screen.

The enantiomers of β-amino alcohols such as the β-blocker drugs are well separated by chiral ion-pair chromatography using either benzoxyglycyl-proline (ZGP) or D-10-camphorsulphonic acid. There is a

strong attraction between the acidic functional group of the chiral selector and the amino group of the analyte and a potential H-bonding interaction between a carbonyl group of the chiral selector and the hydroxyl group of the analyte (Fig. 3.10).

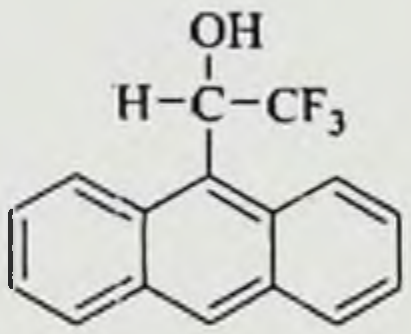
diaminopimellic acid (and potential enzyme inhibitor analogues)



CrownPak CR(+), aq. perchloric acid, pH 1.3 αII/dd 5.12 (4.82 for diethyl ester) (while only partial resolution obtained in one set of conditions in a multi-column screen)

Fig. 3.11 Example of high enantioselectivity using crown ether CSP.

Column:	TSK Enantio L1
m. phase:	1 mM CuSO4
flow:	(aq.)
detector:	1.0 ml min^1
	UV, 215 nm



Substituents		k1	k2	α
3-H,	4-H	0.79	1.25	1.58
3-H,	4-OH	0.68	0.82	1.21
3-H,	4-Cl	1.40	2.32	1.66
3-CH 3O,	4-OH	0.72	0.94	1.31
3-OH,	4-CH3O	1.28	1.79	1.40

Fig. 3.12 Chiral resolution of mandelic acids by ligand-exchange LC.

Accordingly there is little needed by the way of a third interaction to bring about the required chiral separation. Similarly the CROWNPAK CSP from Daicel are very well suited for the separation of the enantiomers of any chiral compound that contains a primary amino group. The mobile phase required, aqueous perchloric acid, is very simple, efficiency is good, and enantioselectivity is generally high. For example, these conditions give good selectivity between d,d- and l,l-diaminopimellic acid (Fig. 3.11). There is no shortage of CSP that will

resolve the enantiomers of amino-acids but the ligand-exchange CSP which were amongst the first to be commercialised are very reliable. They are also very reliable for the enantioresolution of α-hydroxy-carboxylic acids. As can be seen from the data for mandelic acid and related compounds (Fig. 3.12) the selectivity obtained is fairly consistent given that in each case the transient diastereomeric complex is formed via the hydroxyl group and the carboxylate anion acting as a bidentate ligand.

3.5 Chiral drug bioanalysis

As suggested earlier, there is still a need for chiral drug bioanalysis even although chiral drugs are now being developed almost exclusively in single enantiomer forms. There is a need to support some preliminary pharmacokinetics in drug discovery at the point where the decision is made as to which enantiomer is to be progressed. Also, during development it is necessary to ascertain whether or not there has been any in vivo racemisation or inversion of stereochemistry. Such phenomena are more common than imagined, arising for example in such well known drugs as ibuprofen and thalidomide.

Since the mid-1980s when the whole question of chiral drug bioanalysis was first seriously addressed by pharmaceutical companies, a perfectly satisfactory range of approaches to such assays has been developed. Chiral derivatisation strategies have frequently been used and there are an abundant number of cases reported in the literature in which chiral derivatising agents incorporating a strong UV chromophore or a fluorophore have been used in order that, as well as there being a separation of diastereomers, detection at low levels of analyte is facilitated. It must be noted that some of the critical disadvantages of using diastereomer formation, such as enantiomeric purity of the chiral derivatising agent, do not have the same potentially detrimental impact on assessing the ratio of diastereomers in a biological fluid as they would in the case of accurately quantifying a trace enantiomeric impurity in a single enantiomer drug substance. An approach that was prevalent when large numbers of samples from pharmacokinetic and other studies on racemic development and marketed drugs were still being processed, was to carry out an achiral separation followed by an chiral separation on-line

using column switching technology. In this way the expensive chiral column is exposed only to the drug or its metabolites' enantiomers and not to the many polar endogenous compounds that might pass down the achiral column. Later, when chiral drug bioanalysis was being carried out in studies involving fewer samples, it could be argued that it was important to have chiral drug bioanalysis methods that could be easily developed. The use of achiral derivatisation, for example, with the use of derivatisation by achiral fluorogenic agents and subsequent LC on a Pirkle 1A column, fulfils this criterion. Conventional sample clean-up may be used (the resolution of enantiomers when exploiting π–πinteractions between dinitrobenzene and naphthalene or higher polyaromatics very rarely fails) and very low limits of detection may be obtained with both UV and fluorescence detection. In the context of speed, Krull demonstrated that pre-column achiral derivatisation could be carried out on-line. The simplest solution of all is of course to carry out a sample clean-up that is more extensive than would normally be needed so that a fairly clean sample of the drug enantiomers may be injected onto a chiral column. The degree of clean-up might even extend to eliminating drug metabolites since, with hydrophobicity not always beinga major factor in retention on CSP, a drug and its metabolites might elute closely together in LC using a chiral column.

While the methods mentioned above may be fine in their own right, they do not address the fact that today a very high proportion of drug bioanalysis is carried out by LC-MS often with minimal sample preparation and using short columns. Accordingly, much of the recent development in chiral drug bioanalysis is orientated around the need to have LC-MS compatible methodology. For example, in the use of the polar-organic mode for cyclodextrin and macrocyclic antibiotic CSP, it is not only being acknowledged that there is more than one (formerly anointed *magic*) mobile phase that will give polarorganic or *new* polar-organic separations, but also the use of alternative acidic and basic additives and varying ratios of acidic additive to basic additive ratios is being advocated as a means of enhancing sensitivity in LC-MS. With these modifications it is possible to carry out rapid short column LC separations on cyclodextrin and macrocyclic antibiotic CSP with MS detection. However, it does not follow that it is possible to carry our rapid

chiral drug bioanalysis on such CSP. It remains to be seen. A more pragmatic approach to chiral drug bioanalysis using LC-MS has been adopted by Imrie and Noctor (poster presentation at 14th International Bioanalytical Forum, Guildford, Surrey, UK (2001)).

3.6 Preparative chiral separations

There may not have been any recent revolutionary new developments in preparative chiral LC but practice has evolved to the point that it can be viewed as an option for the production scale isolation of enantiomers of drug substances or intermediates as well as for the smaller quantities required in discovery and development. The use of 10 μm particles and techniques such as *recycle and shave* are still being advocated [C2]. Similarly it is still worth considering using unusual solvent compositions in order to optimise enantioselectivity and, often more important, analyte solubility. In illustrations of preparative LC on the relatively new range of macrocyclic antibiotic CSP it has been pointed out that since chiral separations on these CSPs in the reversed-phase mode may be carried out with mobile phase compositions often containing under 15% organic solvent, fractions eluting from the chiral column may be concentrated up by passing through a C18 column (and subsequently eluted in a small volume of methanol which is easy to evaporate off). This methodology is similar to *on-column sample focusing* which may be used in drug bioanalysis and even more reminiscent of the preparative work using β-cyclodextrin as a CMPA carried out by Cooper and Jefferies in the early 1990s. Like cyclodextrin CSP, macrocyclic CSP may be used in the polar organic or new polar organic mode. This mode is especiallycommended for preparative scale use. Methanol is suitably volatile and the use of trifluoroacetic acid as the acidic additive and ammonium hydroxide as the basic additive at compositions ranging from 0.5 to as low as 0.005 parts per 100 parts of methanol is advised.

An important factor that must always be taken into account in preparative chiral LC is that, because of peak tailing, the second eluting enantiomer is always more likely to be contaminated with the second eluting enantiomer than vice versa. Because of this, despite the popularity of the Daicel derivatised polysaccharide CSP and to a lesser extent the macrocyclic antibiotic and cyclodextrin CSP for preparative work, there

will always be a place for Pirkle-type CSP. Because these columns are available in their different enantiomeric forms, one enantiomer of the analyte may be purified as the first-eluting component on one column and the second enantiomer may be freed from its enantiomeric contamination by passing it through the column of opposite chirality (on which it will be the first eluting enantiomer). Preparative LC on the Regis range of Pirkle-type or synthetic multiple-interaction CSP is well established but of more recent prevalence the use of aroyl tartaric acid based network polymeric phases on Kromasil silica has been demonstrated. The use of the chiral network polymer is purported to give rise to a long lifetime through high chemical stability, high capacity through a large number of chiral sites, high enantioselectivity and universality through the three-dimensional structure and high efficiency and enantioselectivity through a reduction of nonchiral interactions. While the principle is sound, and certainly a high loading capacity would be expected there is insufficient evidence as yet to suggest that these materials may be used successfully for as wide a range of compound classes as Daicel and Regis CSPs for straight phase conditions. Indeed it is difficult to make a comparison at all as the preferred mobile phases used, typically *t*-butylmethyl ether-ethyl acetate, are quite different from the more familiar *n*-hexane-propan-2-ol.

As in analytical chiral LC, Daicel derivatised polysaccharide CSPs are the most frequently used materials in preparative scale chiral separations. Recently CSPs have been prepared in which derivatised polysaccharides have been covalently bonded to the solid support rather than coated on as in the Diacel materials. The rationale for this is that it is advisable to reduce the chance of the chiral selector leeching off the column in trace amounts to contaminate samples of chiral drugs isolated by production scale LC. However, the extent to which the Daicel coated CSPs are now used in production scale chiral LC would tend to suggest that such a problem, if it exists, is not a very significant risk.

The most discernible recent trends in preparative chiral resolution have been the increasing use of supercritical fluid chromatography (SFC) and simulated moving bed (SMB) chromatography, and the fact that Daicel through its US and European Chiral Technologies subsidiaries have offered these as a custom service has played a role in this.

Generally in SFC supercritical carbon dioxide is used as the mobile phase along with a small fraction of modifier solvent to enhance analyte solubility. Analyte solubility in such mobile phases is good, the low viscosity of supercritical carbon dioxide gives rise to good efficiency and allows for higher flow rates, occasionally enantioselectivity might be improved by dipole interactions with the carbon dioxide and, probably most importantly, the bulk of the mobile phase is very easily removed instantly by evapouration at atmospheric pressure. SFC is not uniquely carried out on Daicel derivatised polysaccharide CSP. It may also be carried out on Pirkle-type synthetic multiple-interaction CSP and macrocyclic antibiotic CSP as promoted by PDR-Chiral, using a Berger SFC and a laser polarimetric detector as well as UV to aid method development [C7] and by Berger Instruments themselves [C8].

To quote the exact words used to promote the Separation Services of Chiral Technologies in their own brochure [C9], 'SMB chromatography is a continuous, counter-current separation method and, as a binary separation technique, is much more efficient in the use of stationary and mobile phases than a batch chromatography system'. Thus, SMB separations produce more concentrated product streams while consuming less solvent. Typically, a chiral separation with SMB is achieved with five to eight columns of short length (~10 cm). A contributory factor to the success of this technique is the very fact that Chiral Technologies, and to a lesser extent others, have built up a high level of expertise in this technique and offer it as a service rather than there being a case of every potential new user having to build up their own expertise.

The logical conclusion of these recent trends in preparative chiral chromatography ought to be that the next step would be to carry out SFC in an SMB system. Unfortunately it seems that the technological difficulties would almost certainly outweigh any potential benefits (personal communication [C10]).

3.7 Present and future perspectives

3.7.1 Alternatives to chiral LC

LC is undoubtedly the most important technique in the chiral analysis of pharmaceuticals. However, that is not to say that chiral LC of pharmaceuticals is synonomous with chiral analysis of pharmaceuticals. The chiral selectors utilised in LC may be usefully deployed in a range of other analytical techniques. Despite this, chiral LC is more dominant now than it ever was. The early commercial developments in chiral analysis were in LC. The application of similar selector systems was demonstrated in other techniques but now with the notable exception of chiral capillary electrophoresis (CE) there is a strong reliance again on LC.

Chiral GC was under development and was being used successfully, for example for chiral drug bioanalysis, at the same time as commercialised CSP for LC were breaking through. The technique received a boost when cyclodextrin GC phases came onto the market. However, with most drug bioanalysis being carried out by LC, and LC-MS in particular, chiral GC is more the preserve of application areas such as the analysis of odourous compounds and the field of insect pheromones.

Successful methodology for chiral LC can be adapted for use in TLC more readily than for other analytical techniques. All CSPs and CMPAs used in LC can in principle be used in TLC systems and many have been used in practice. TLC plates for chiral ligand exchange using a CSP are the longest established of the commercial products and cyclodextrins have frequently been used as CMPA for TLC. Despite the advantages of TLC over LC in certain situations (cheap, easy to use, all sample components may be detected therefore making it suitable for investigation of mass balance problems, many samples may be analysed simultaneously) it is now being used much less frequently in analytical support for pharmaceutical development. To exacerbate matters, this less frequent use has led to a loss of expertise in the technique. In chiral TLC there are even fewer reasons to prefer it over chiral LC, especially since, spots are visualised against a chiral background and so may not give an equal response, unless the fortuitous situation arises in which the chiral selector can be eluted off the plate leaving the mixture components

behind. Things are moving towards the situation where chiral TLC will only be done in the situation where the TLC enthusiast wishes to demonstrate that it can be done.

Chiral NMR is a different matter. While chiral selectors that are used in LC, particularly cyclodextrins, may be used quite effectively in NMR, the chiral selectors used are usually ones that are especially suited to NMR. Large chemical shifts were produced by *lanthanide shift reagents*but these are not used so frequently nowadays because of the signal broadening that accompanies the induced shifts. With modern high-field instrumentation the differential shift between enantiomers brought about by chiral solvating reagents such as 2,2,2-trifluoro-1-anthrylethanol (Fig. 3.13) is sufficient to give chiral resolution. Provided the instrumentation is available in the first place, it can be a very simple means of obtaining quantitative information. However, it is more likely to be used for monitoring a chiral resolution or analysing a drug intermediate in which the levels of enantiomeric contamination are greater than 2% than for determining trace levels of enantiomeric impurity in a single enantiomer chiral drug substance.

In chiral CE, screening approaches can be so facile that there is often little need for a rational approach. Just how successful CE screening can be may be gauged by studying the work of Nussbaum who used a screen involving five cyclodextrins, each at a high or low concentration, andwas able to obtain a 100% success rate for his own class of basic development compounds. In terms of simply being able to develop a separation, it could be argued that chiral CE methodology has progressed as far as it needs to progress.

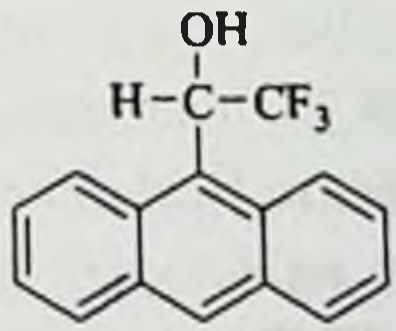


Fig. 3.132,2,2-Trifluoro-1-(9-anthryl) ethanol, a chiral solvating agent commonly used in 1H NMR. Because of the strong aromatic ring current, a shift is induced in compound to which it is added in the NMR tube. The single enantiomer solvating agent gives different shifts for enantiomers for a wide range of compounds even when no obvious 3-point interaction may be observed.

Without recourse to the scientific literature, it is usually possible, similar to Nussbaum, to set up a quick screen using a few cyclodextrins that are available in a laboratory to achieve one or more chiral separations which may easily be optimised. Within a few simple experiments one can obtain one or more chiral separations, which may easily be optimised. One possible limitation is that with some pharmaceutical analytes having a weak UV chromophore, special measures (e.g. *bubble*cells, extended path length cells, high resolution with high loading perhaps via stacking techniques) might need to be taken to be able to detect down to 0.05 or 0.1% of a trace enantiomeric impurity. However, this is not always an issue, and often low enough limits of detection may be obtained with a conventional 50 μm capillary and normal loading.

Chiral CE is used primarily in the early stages of the development phase of pharmaceutical R&D with the degree of uptake often depending on the expertise and preferences of the individual analysts involved. However, despite the obvious advantages of chiral CE including its speed of method development, low cost (one capillary and small quantities of a range of chiral selectors, usually cyclodextrins, does it all) and versatility, its use seems to have reached a plateau with little penetration of chiral CE into methods included in regulatory submissions and even less in methods used in production sites. The reason for this is more to do with the overall uptake of CE in industry for the analysis of small molecules than to do with chiral CE itself. Generally LC is doing a perfectly good job and for most applications any advantages that might be had by using CE are insufficient to warrant the wholesale replacement of LC equipment with CE equipment.

This limitation applies even more so to chiral capillary electrochromatography (CEC). This technique is essentially LC in a fused silica capillary using an electric field rather than a pressure drop to drive the flow. As such the separations may be achieved by chiral derivatisation, by use of a CMPA or a CSPjust as in chiral LC. Greater efficiency may be had in chiral CEC compared to chiral LC and very low amounts of CSPs or CMPAs are needed. However, the technique does not share the convenience of ease of use that is enjoyed by chiral CE and has not been developed to the point where it could be considered suitable for routine use.

Both CE and CEC would stand a better chance if the tendency to reduce dimensions in LC had progressed to the point where the standard LC column was at least of 1mm i.d. and could be used in a system that was also compatible with running CE and CEC. Such a hybrid system has existed as a prototype but was probably ahead of its time.

3.7.2 Fit for intended purpose?

One thing that is certain with the developments in chiral selectors and their applications that have been described is that they will continue, whether it be by gradual evolutionary fine-tuning in an already mature area or by some yet unforeseen major advances. What is of more importance is how these developments currently shape up with respect to the applications presented by the chiral analysis of pharmaceuticals.

The most common application of chiral LC is the determination of a trace enantiomeric impurity in a sample of a single enantiomer chiral drug. There are certainly enough options when it comes to developing the required separation in the first place. The success rate of screening approaches to chiral LC method development using a selection of the most effective CSP is usually reckoned to be in the order of 80%. It will generally be possible to take care of the other 20% by using the CSPs that would not be included in those chosen for the screen. Obtaining sufficient resolution to carry out the determination is therefore not normally a problem. Nor is it normally a problem achieving a limit of detection low enough to quantify 0.05–0.1% w/w of the enantiomeric impurity. (The guidelines set by the International Conference on Harmonisation (ICH) (www.ich.org) levels down to which related substances in samples of active pharmaceutical ingredient should be qualified, identified, or reported are about to change, and new guidelines should be in place by 2004.) If the resolution is a problem, then there are plenty options for optimising the separation including using a CSP of opposite chirality in order to reverse the retention order. In fact, a more common problem is that, due to the successes in developing CSP with high chiral recognition properties, excessive chiral resolution is obtained. For example, the first enantiomer might have a very low k value while the second enantiomer might elute with a very high k. The former occurrence might lead to specificity problems with respect to the first eluting peak and peaks

arising from other fast eluting sample exponents. The latter occurrence leads to unnecessarily long analysis times. What is needed is to *de-tune* the separation. A solution for this for synthetic multiple interaction, ligand-exchangeand crown ether CSP is to use the chiral column in conjunction with the racemic version of the phase. While this strategy is sound in principle, it is rarely used. For work on derivatised polysaccharide and macrocyclic antibiotic CSP detuning may be carried out by altering the ratio of acidic to basic mobile phase additives.

Perhaps there is scope for further developments in CSP in order to more frequently achieve high enough enantioselectivity for facile preparative isolation to be carried out. However, most of the current developments in terms of preparative chiral separations seems to be in developing technolgies for SFC and SMB and, to a lesser extent, CSP in which there is not even an ultra-trace leakage of the chiral selector.

Chiral drug bioanalysis is an area that could be better served by existing technology. There is still work to do, on achieving good mass spectrometric detector response when using some of the mobile phases that are needed to obtain good separations of enantiomers on certain CSPs. Further, rapid analyses will not be possible without attention being given to the sample preparation step. In general, especially considering column prices, having a relatively *clean* sample solution to inject is more of an issue when working with a CSP than when working with an achiral phase such as a C18 bonded phase.

3.7.3 The future

Future developments should follow on automatically from current needs. Hopefully this will be the case in chiral drug bioanalysis, where rapid, sensitive methods still cannot be easily developed. This might come, for example, through a marriage of chiral LC technologies with biofluid-compatible LC technologies or this might be an area where CE, with analyte focusing methodologies and/or sensitive detection systems, can play a greater role.

What is more likely to happen is that despite the maturity of the field, there will be further developments in new CSPs. Even now there could still be a market for new types of broad spectrum CSPs. What would be more useful would be the design of easily constructed cheap

chiral selectors tailored to specific analytes in order to give such high enantioselectivity that preparative isolation of the individual enantiomers was a simple one-step batch process. Again looking a bit further into the future, it is worth noting that the determination of the enantiomeric impurity in a single enantiomer chiral drug is almost always a separate assay from the determination of other structally related substance impurities. With a continuing drive to achieve *more for less* in pharmaceutical R&D it would be useful to have methodologies or new stationary phase technologies that would allow these determinations of the enantiomeric impurity and other impurities to be carried out from the same chromatographic runs. While the chiral analysis of pharmaceuticals is a mature area which is no longer the major challenge that it once was, there is still more to come.

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ACKNOWLEDGMENT

The study guide was written on the basis of the practical project number ALM-202310062530 on the topic "Organization of the laboratory for the creation of anticancer drugs" that is being carried out at the Samarkand State University named after Sharof Rashidov.

FOR THE RECORDS

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"Bilig-ilmiy faoliyat" nashriyoti

Muharrir: Fayzullayeva G.
Texnik muharrir: Xujakulov Sh.
Nashrga tayyorlovchi: Abdullayev F.

№ 098355

ISBN: 978-9910-9184-4-5

"Bilig ilmiy faoliyat" nashriyoti, Joylashgan mazili Samarqand viloyati, Samarqandshahar,
Zavod ko'chasi 9-uy, 10-xona. Faoliyat manzili Samarqand viloyati,
Samarqandshahar, X.Obiddinov ko'chasi 7-uy.
tel.: +998 97-925-97-91

Terishga berildi: 18.07.2024-yil. Bosishga ruxsat etildi: 08.10.2024-yil.
Bichimi 60x84 1/16, "Times New Roman" garniturasi.
Bosma tabog'i 9. Adadi 100 nusxa. Buyurtma № 2024/ UQ 66
Bahosi kelishilgan narxda. Noshirlik litsenziyasi: № 098355
Samarqand viloyati pedagoglarni yangi metodikalarga o'rgatish
milliy markazi bosmaxonasida nashr etildi

ISBN 978-9910-9184-4-5