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Review

Chromatography of β-adrenergic blocking agents

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CONTENTS

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LIST OF ABBREVIATIONS

BSA	Bis(trimethylsilyl)acetamide
BSTFA	Bis(trimethylsilyl)fluoracetamide
CZE	Capillary zone electrophoresis
FAB	Fast atom bombardment
GC	Gas chromatography
GC–FTIR	Gas chromatography–Fourier transform infrared spectroscopy
GC–MS	Gas chromatography–mass spectrometry
HFBA	Heptafluorobutyric anhydride
HPLC	High-performance liquid chromatography
HPTLC	High-performance thin-layer chromatography
LC	Liquid chromatography
LC–MS	Liquid chromatography–mass spectrometry
MS	Mass spectrometry
MSTFA	N-Methyl-N-trimethylsilyltrifluoroacetamide
PFPA	Pentafluoroacetic anhydride
SFC	Supercritical fluid chromatography
TFAA	Trifluoroacetic anhydride
TLC	Thin-layer chromatography

Other abbreviations are explained in the text.

1. INTRODUCTION

The chromatographic analysis of β-blockers was first described by Ervik in 1969 [1]. Early applications employed gas chromatography (GC) and method development parallelled the technical advancement of commercial GC. The requirement for derivatisation of these basic drugs to facilitate both chromatography and detection increases the complexity of this technique. Since the 1970s advances in liquid chromatography (LC) have resulted in its being established as the method of choice, offering a simpler, more flexible approach to separation. Review articles have periodically summarised progress [2,3], and a more detailed treatise on the determination of β-blockers has recently been published [4]. It is the aim of this review to consolidate previous articles and to highlight more recent perspectives of the chromatography of β-blockers, including developments in associated areas. The review covers a period since 1985, when the subject was most recently reviewed in this journal [5].

2 HISTORICAL PERSPECTIVE

The β-blockers are derived chemically from the adrenergic agonist, isoprenaline (Fig. 1a). Research was initially directed towards the treatment of angina pectoris through the synthesis of a specific adrenoreceptor antagonist. The outcome of several years efforts was propranolol (Fig. 1b). This was the first β-blocker to be formally approved for clinical use in 1965, and to this day it remains one of the most frequently prescribed drugs in clinical practice [6,7].

Refinement of the pharmacological properties of propranolol resulted in the evolution of the series of drugs we now recognise as the β-blockers. Their chemistry is based on the aryloxypropanolamine structure represented in Fig. 1c; the nature of the aromatic group or N-terminal side-chain provides structural differences and the basis for variability or pharmacological action. In his recent treatise Marko [4] catalogued the structure of 50 of the most established members of the group. To assist the reader this catalogue is reproduced in Fig. 2.

The development of the β-blockers has been described as one of the most exciting pharmacologic and therapeutic innovations in recent years [8] and few groups of drugs have been considered to have made such a major contribution to so many aspects of medicine and clinical science [9]. Having been used initially in the treatment of angina and cardiac arrhythmias [9], their application has extended, for example, to the control of systemic blood pressure and relief of intraocular pressure [10].

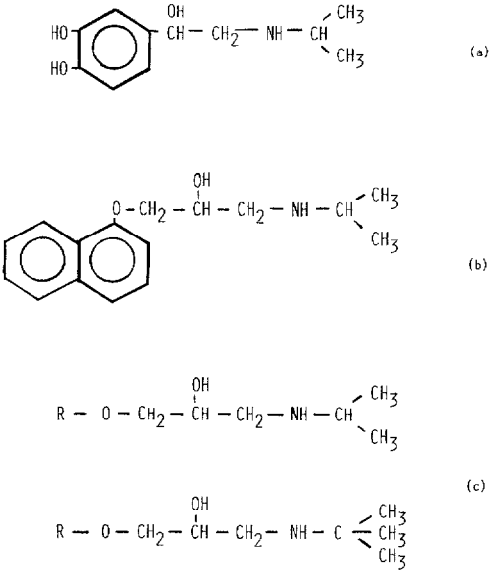
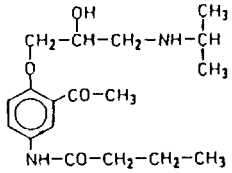


Fig. 1 (a) Structure of isoprenaline; (b) structure of propranolol, (c) general structure for β-blockers containing either N-terminal isopropyl or *tert.*-butyl groups



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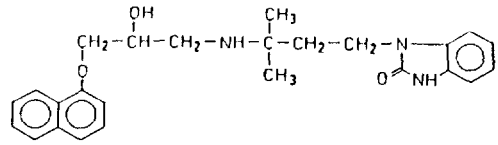

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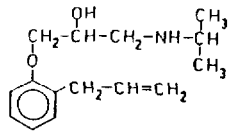

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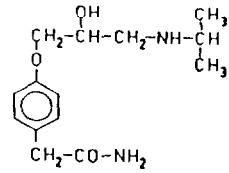

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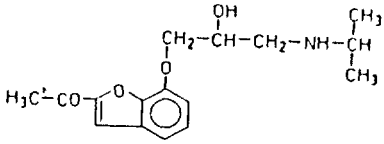

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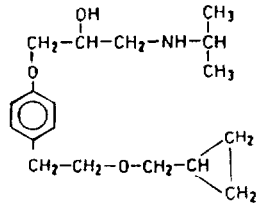

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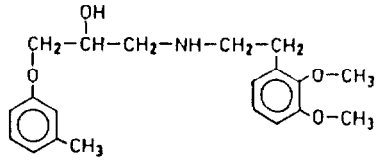

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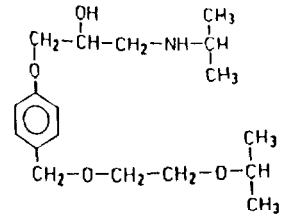

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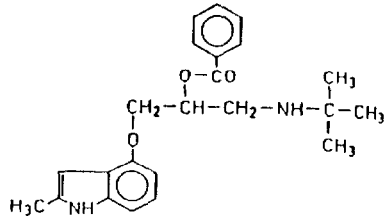

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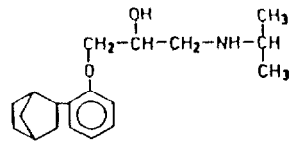

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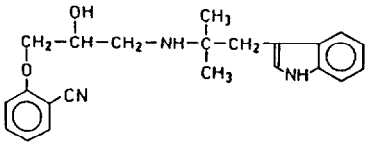

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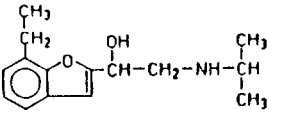

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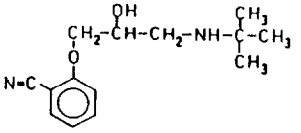

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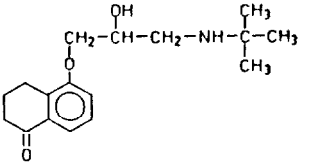

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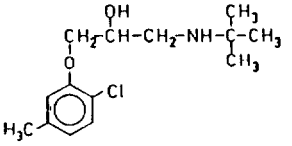

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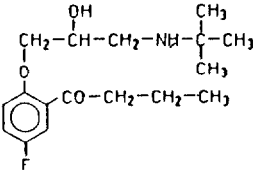

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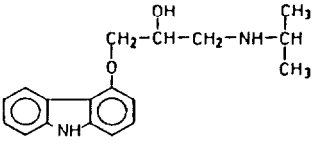

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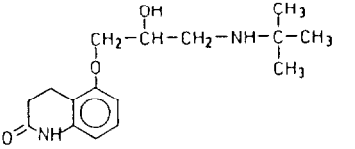

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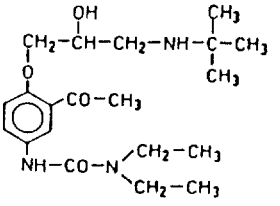

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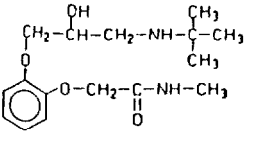

Cetamolol

Fig 2.

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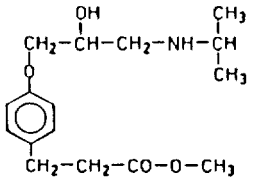

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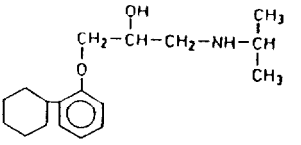

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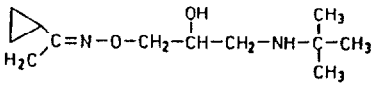

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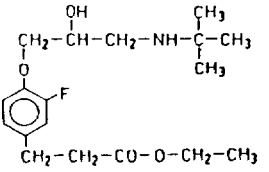

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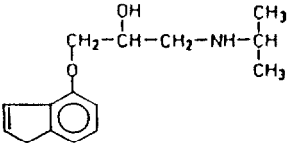

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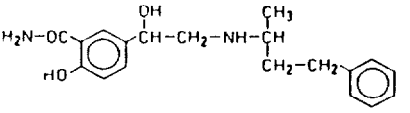

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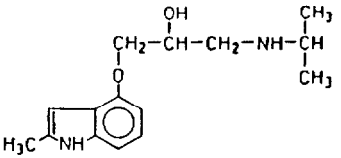

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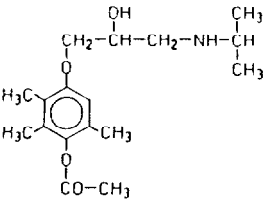

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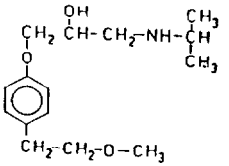

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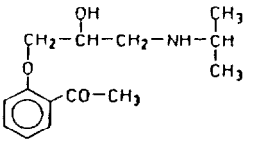

Moprolol

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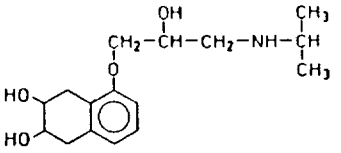

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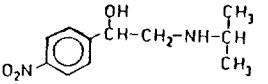

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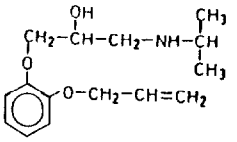

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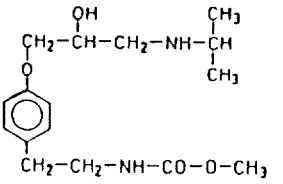

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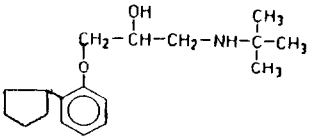

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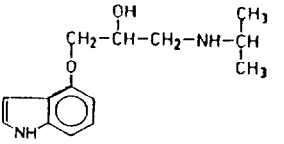

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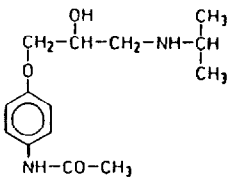

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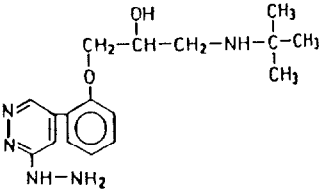

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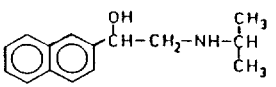

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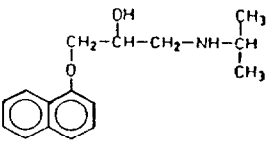

Propranolol

Fig. 2

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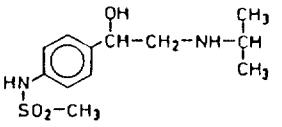

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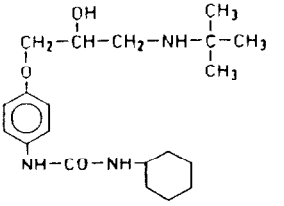

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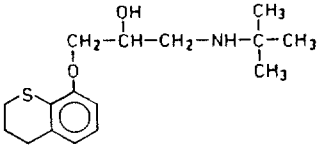

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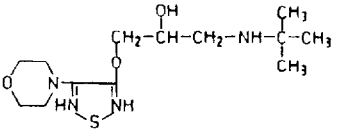

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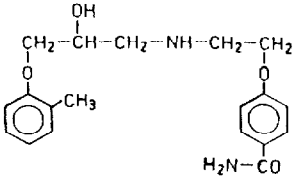

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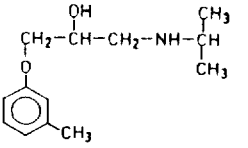

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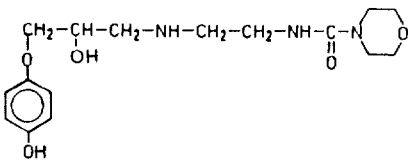
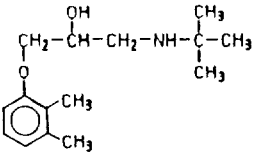
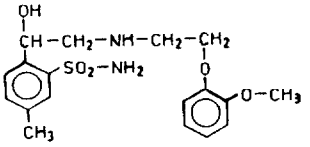


Xamoterol



Xibenolol



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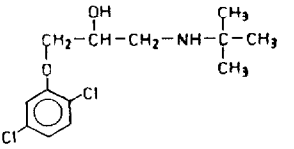

Tobanum

Fig 2. Structures of β-blockers. (Reproduced from ref 21 with permission)

3 ANALYTICAL CONSIDERATIONS

Specific, sensitive analytical methods are fundamental to (a) drug development, (b) drug formulation, (c) pharmacokinetic evaluation, (d) metabolism and disposition, (e) therapeutic drug monitoring and (f) drug screening. Chromatographic methods fulfill all the above requirements and can, for example, provide the sensitivity to quantitate drugs after single dosage and in unbound plasma fractions [11].

A more recent analytical requirement is the resolution of optical isomers of pharmaceuticals. Approximately 25% of the most widely prescribed drugs are sold as racemic mixtures [12]. For propranolol the (*S*)-isomer is 100 times more potent as a β-blocker and has a longer plasma half-life than the (*R*)-form [12]. This stereoselectivity has been demonstrated for all β-blockers and for a given antagonist the (−)/(+)-enantiomer ratio for the binding affinity to the β-receptor may range from as low as 10 for atenolol to as high as 1000 for pindolol [13]. Increasingly, regulatory authorities are becoming aware of analytical information related to the enantiomeric form of candidate drugs [14].

Chromatographic retention data can be exploited to provide information on lipophilic character of drug molecules [15], and estimates of plasma protein binding [16]. Drug screening programmes further explore the qualitative and quantitative aspects of retention. Screening has increased in importance for toxicological studies [6] and, more topically, the control of doping in sport [17–19].

4 SAMPLE PREPARATION

Determination of drugs in biological fluids invariably requires some form of sample preparation, the significance of which is frequently underestimated by the analyst. McDowall [20] conceived sample preparation as a series of unit operations from which analytical objectives could be clearly defined. In the succeeding section, sample processing techniques, as applied to β-blocking agents, are discussed.

4.1. Liquid–liquid extraction

Historically this has been the favoured method of extraction of drugs from biological samples and has been applied widely to β-blockers. Several workers have attempted to "optimise" the selection of extraction solvent [21], but with octanol–water partition coefficients of β-blockers ranging over six orders of magnitude only partial success has been achieved. Judicious selection of the extrac-

tion solvent may nevertheless allow some degree of selectivity to be introduced at early stages of the extraction process [22,23]. Chromatography and manipulations such as the use of emission cut-off filters in fluorescence detection further enhance selectivity. Betaxolol and propranolol, which were co-extracted into methyl *tert.*-butyl ether and incompletely resolved by HPLC, could be distinguished in this way [24]. Compromises may have to be made between safety requirements and solvent selection [25], although in many cases good interference free recoveries are obtained from single-step extractions [26].

Recovery of individual drugs by certain solvents cannot guarantee similar performance for all compounds. Lennard and Parkin [27] reported dichloromethane as a suitable solvent for the extraction of several β-blockers, however, timolol was poorly extracted Poor recoveries can be tolerated provided that the technique is reproducible. Thus, Morris [28] reported only 29.0 and 33.4% efficiencies for the determination of sotalol and atenolol, respectively, but with good precision.

In the case of pharmaceutical dosage forms and animal feedstuffs solvent pre-treatment is simply analyte solubilisation. Bevantolol, used in veterinary medicine for the treatment of hypertension, was isolated from medicated animal feeds and chromatographed after centrifugation to remove undissolved material [29]. Several reports have described the determination of β-blockers in solid dosage forms, either with filtration [30–32] or centrifugation [33] to remove insoluble excipients. Liquid formulations could be simply diluted [30,34,35] and transdermal patches disintegrated by extraction solvent [35].

Solute recovery may be improved by simplification of sample handling procedures and optimisation of pH of extraction mixtures to match the pK_a of the individual drug [36]. This has meant that single-step extractions have frequently proved adequate to meet analytical requirements. An "acid wash" step is often added to the initial organic extraction to control loss of solute and present the sample in aqueous phase suitable for LC. Ion pairs of the acid anion with the basic drug may, however, be retained by the organic phase and reduce recovery The more lipophilic β-blockers such as propranolol can remain partitioned in the organic solvent, so reducing overall extraction efficiency [37]. Acid back-extraction has been reported as an adjunct to solid-phase extraction [38]

The potential selectivity and complexity of solvent extraction was illustrated by Kwong and Shen [39] for the determination of propranolol and its acidic, basic and neutral metabolites (conjugated and unconjugated). Propranolol is metabolised by three different pathways of biotransformation: (i) N-dealkylation, (ii) aromatic hydroxylation and (iii) glucuronidation of the hydroxyl group at position 2 of the side-chain (Fig. 3) Basic and neutral compounds were extracted into organic solvent after alkalinisation. The aqueous phase containing acidic metab-

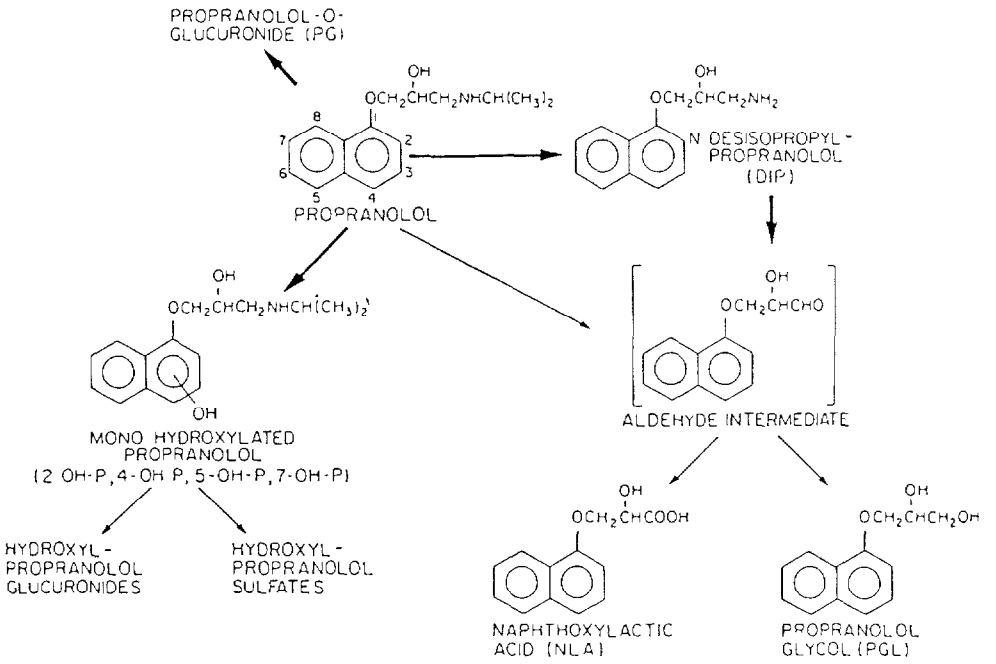

Fig 3 Metabolism of propranolol (Reproduced from ref 39 with permission)

olites was acidified and re-extracted with solvent. Basic and acidic conjugated metabolites were processed following enzymatic hydrolysis of conjugates with β-glucuronidase and arylsulphatase. Using this method the authors were able to determine propranolol and six of its metabolites in serum and urine to provide a comprehensive pharmacokinetic profile of the drug Simpler methods, selective for certain metabolites only, have been reported by other authors [40,41].

Certain polar drugs (*e.g.* atenolol) or metabolites may pose particular extraction problems [42–44]. In the case of levobunolol, the parent compound was subjected to sodium borohydride reduction to dihydrolevobunolol (its major natural metabolite, also possessing pharmacological activity) in order that total active drug concentration could be measured as a single chemical species [45]. Dihydrolevobunol also has the analytical advantage of greater native fluorescence than the parent drug, allowing lower detection limits to be achieved [46]. Extraction of β-blockers from tissues was illustrated by the example of carazolol, a drug with applications in human and veterinary medicine. Its isolation by solvent extraction [47,48] and solid phase [49] was described.

Ion-pair extraction has proved an acceptable alternative to pH suppression in certain applications. The extraction of the sulpho conjugate of alprenolol, using

tetrabutylammonium bromide as the counter ion [50], and the sulpho conjugate of propranolol, using tretrabutylammonium phosphate [41], are examples. Hoogewijs and Massart [51] outlined a standardised analysis strategy for the ion-pair extraction of basic drugs in plasma, emphasising the precautions that should be taken to minimise reaction between the ion-pairing agent and plasma proteins.

Some β-blockers are vulnerable to chemical modification or degradation during extraction. Ultra-short-acting β-blockers used in critical care, and some drug metabolites, *e.g.* 4-hydroxypropranolol [39,52,53], fall into this category. Protection from oxidation may be afforded by reducing agents. Storage times for samples containing labile drugs may be increased by the addition of ascorbate [54], although during the isolation of conjugates of polar metabolites it should be ascertained that reducing agents do not inhibit esterase or glucuronidase activities [53]. In contrast inhibition of enzymic degradation by rapid solvent extraction was an integral feature of the isolation of intact levobunolol [46] and esmolol [55–57]. Esterase activity could be inhibited by the action of sodium dodecyl sulphate (SDS) on whole blood samples. This treatment also haemolysed red cells to provide a homogeneous sample that simplified processing [58]. Tubes containing sodium fluoride as esterase inhibitor were applied to the extraction of esmolol [57] and flestolol [59].

Pindolol and the structurally related bopindolol are thermally and photochemically unstable. Extraction procedures involving these drugs should incorporate some element of protection from light [60]. Spahn *et al.* [61] found that light-induced breakdown of pindolol resulted in a 30% reduction of fluorescence yield of the compound. Pindolol has also been reported to form dimers in acidic solutions [62], indicating caution in the application of back-extraction procedures involving acidification. Bopindolol exhibited similar properties [63].

One solution to the problem of drug stability is derivatisation prior to extraction. It was applied to the determination of prizidilol [64], alprenolol and 4-hydroxyalprenolol [65] and hydroxylated metabolites of metoprolol [66]. Its limitations are that derivatisation must take place in an aqueous environment and that some quantitative estimation of the rate of degradation prior to treatment must be made. In the case of prizidilol the recovery of both the drug and internal standard from plasma was shown to be directly proportional to the time between addition of trichloroacetic acid (the initial process step) and centrifugation of the precipitated protein material. Other β-blockers showed this same effect to a greater or lesser extent [67].

4.2 Solid-phase extraction

The development of bonded-silica phase chemistries for chromatographic stationary phases provided the impetus for advances in sample preparation techniques and the potential of solid-phase methodology for rapid handling of large numbers of samples. Automation of the technique has been commercially exploit-

ed by a number of manufacturers and has further accelerated its growth. For a comprehensive review of the application of liquid–solid separation techniques in drug analysis the review articles of McDowall and co-workers [20,68] and Doyle *et al.* [69] are recommended.

Ruane and Wilson [70] studied the use of solid-phase extraction columns with particular respect to the extraction of β-blockers. As with the chromatography of these compounds, unwanted interactions may occur between the basic drug and residual silanols on the solid phase. The characteristics of two manufacturers' products, Bakerbond (J. T. Baker, Whippany, NJ, U.S.A.) and Bond Elut (Analytichem International, Harbor City, CA, U.S.A.), were compared using canine plasma containing representative radiolabelled analytes. The performance of the two column types differed; however, to obtain maximum recovery from both types it was necessary to supplement the eluent with a competing base (*e.g.* triethylamine). Similar observations were made by Doyle *et al.* [69] working with an experimental β-blocker, SK&F 95018. These effects were consistent with the interaction of the basic drugs with residual silanols on the surface of the packing [71,72].

The influence of secondary ionic interactions on the solid-phase extraction of β-blockers was investigated as a supplement to the above studies [73]. Different solid-phase chemistries allowed for selective separations not possible by solvent extraction, though the batch-to-batch variability of cartridges often resulted in unpredicted changes in column performance and elution characteristics.

A strategy for the determination of β-blockers incorporating solid-phase extraction was devised by Musch *et al.* [67]. Thirteen β-blockers, representative of the group, were included in the study. An earlier report [74] had shown that CN-bonded silica solid-phase columns completely retained basic drugs and that acceptable recoveries from the columns could be achieved on elution with methanol–phosphate buffer or a similar buffer containing 0.15% propylamine. When applied to solutions of β-blockers all the drugs were completely adsorbed onto the column and, with the exception of labetalol, could be desorbed on elution with buffer. Even when the buffer contained propylamine, the recovery of labetalol was still only 50%, an effect which was considered to be due to the relatively low pK_a (7.4) of the drug. When plasma spiked with the test drugs was processed, metoprolol, pindolol, alprenolol, mepindolol, oxprenolol and sotalol were not desorbed if the plasma was deproteinated. Modifications to the pre-treatment stage resulted in good recoveries for all but labetalol, which was better extracted using a less polar extraction column.

The development of small-particle organic resins orgininating from the drug screening programmes of the early 1970s stimulated progress in solid-phase extraction [68]. Delbeke *et al.* [75] described the use of Extrelut C_{18} columns (E. Merck, Darmstadt, F.R.G.) for the qualitative screening of propranolol, atenolol, oxprenolol, pindolol, metoprolol, alprenolol and their respective metabolites in the urine of athletes. In these studies enzymic hydrolysis of conjugates was

preferable to the more rapid procedure of acid hydrolysis because several β-blockers, *e.g.* pindolol and atenolol, were acid-labile [76]. Maurer and Pfleger [76] proposed that acid hydrolysis might be suitable for rapid qualitative screening of urine or the enzymic procedure could be accelerated by raising the temperature of incubation [75]. In combination with thin-layer chromatography (TLC), solid-phase extraction provides a useful means of rapidly screening samples for β-blockers [77]. The potential for automation of solid-phase extraction techniques has been exploited commercially. A description of the various systems available is outside the scope of this article, however, there have been applications of this approach to the determination of β-blockers [78,79].

Early applications of solid-phase extraction for very low levels of drugs (sub-ng/ml range) were not well documented [80]. The technique alone may be insufficient to guarantee specificity for high-sensitivity determinations. The isolation of nadolol at sub-nanogram concentrations from plasma by solid-phase extraction (on CN-bonded silica) incorporated a further automated treatment with XAD resin to prepare the sample material to a level of purity suitable for mass spectrometry (MS) [81].

Solid-phase extraction is of particular value when exposure of the analyte to potentially harmful conditions is to be avoided [42,82]. Purification of the lipophilic β-blockers alprenolol [83], oxprenolol [84], metoprolol [83] and propranolol [53,85] was reported on solid phase. The usefulness of the technique for the extraction of labile compounds was further illustrated for propranolol and its hydroxylated derivatives, the latter being particularly sensitive to alkaline conditions which can be avoided using bonded-phase columns [53]. The selective elution of basic, neutral and acidic metabolites of propranolol was achieved by planned selection of the elution solvent. However, without protein precipitation or treatment of the extraction column with a base, recoveries in the range 60–80% only were achieved. Solid-phase preparation methods have been reported for sotalol using either C_8 [86] or C_{18} [87] extraction columns and levomoprolol using a CN-bonded column [88].

Solid-phase extraction using bonded silicas has achieved a significant representation in β-blocker extraction methodologies, though recoveries of many drugs are reported typically as only 70–80% efficient. It is of importance, therefore, that some understanding of retention mechanisms of basic compounds on bonded silicas should be appreciated by users. The improvements in yields to be gained have been illustrated by some of the work cited above. These difficulties should not detract from the potential advantages of solid-phase extraction. When extraction of a number of metabolites of a particular compound is required, solid phase has the advantage of offering simpler extraction protocol suitability for automation [85].

4.3. Direct injection techniques

Sample processing procedures involving extraction stages add significantly to unit costs [89]. The direct injection of samples, particularly physiological fluids, onto silica-based high-performance liquid chromatography (HPLC) columns was once considered undesirable because of the likelihood of column contamination, however, to use the resolving power of LC both to clean and separate complex mixtures is an attractive concept [90].

Analysts have long sought a solution to this problem and several techniques have been developed incorporating direct sample injection procedures. These have been the subject of recent reviews [91,92]. Their progress and application to the chromatography of β-blockers is discussed below.

4.3.1. Column switching

Applications to the determination of β-blockers are limited. Lecaillon and co-workers [93,94] described column-switching techniques for the HPLC determination of metoprolol from plasma and urine. Although offering potential for automation, column switching may be considered an over-complex operation for routine analysis.

Recently, the chromatography of a wide spectrum of basic drugs, including several β-blockers, was described following direct injection of plasma and preconcentration and clean-up on a 10 cm x 1.5 mm Corasil C_{18} column (Millipore-Waters, Milford, MA, U.S.A.). Performance of the column was found to be superior to that of a comparable octyl-bonded column when used in a column-switching system because it showed a lower affinity for polar plasma constituents [95].

4.3.2. Micellar liquid chromatography

Micellar liquid chromatography (MLC), first described by Armstrong and Henry [96], is best considered as a variant of reversed-phase HPLC rather than a new chromatographic form [97]. The technique has several advantages over "traditional" reversed-phase LC, but two significant assets are its ability to provide rapid gradient capability and to withstand the direct injection of physiological fluids by solubilising the proteins in a surfactant coating [89]. In addition the surfactant monomers appear to displace protein-bound drug, releasing it for partitioning to the mobile phase [98] MLC was applied to the determination of propranolol using fluorescence detection [98]. The use of an emission cut-off filter and adjustment of the micelle concentration enabled the selective separation of propranolol from quinidine in urine. Interference from endogenous material was minimal. In serum samples, the long elution of serum constituent background and the elution of propranolol on the tail of the impurities may limit sensitivity; however, use of a cut-off filter in the fluorimeter may reduce interference and improve the limit of detection (Fig. 4).

The problem of associated interferences when using a direct injection tech-

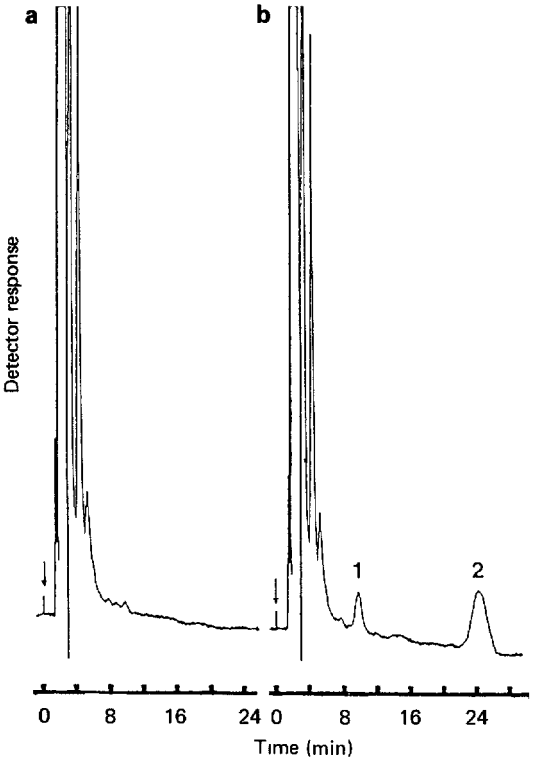

Fig 4. Micellar liquid chromatography of (a) urine blank and (b) urine with added 40 ng/ml propranolol (1) and 400 ng/ml quinidine (2) Chromatographic conditions column, μBondapak C_{18}, mobile phase. 0 02 *M* SDS with 10% propan-1-ol, flow-rate 1 0 ml/min; detector voltage, 700 V, sensitivity, 0 02 μA; excitation wavelength, 215 nm, emission cut-off filter, 300 nm (Reproduced from ref 98 with permission)

nique may be eliminated by combining MLC and column switching. Propranolol was determined in this manner using a programmed micellar solvent cycle [90]. The drug was easily detected at a plasma concentration of 90 ng/ml, within the recognised therapeutic range. The general application of MLC to drug analysis, and β-blockers in particular, has been limited, although it has been applied to therapeutic drug monitoring [99]. The bioanalytical capabilities of MLC have been reviewed elsewhere [100].

4.3.3. Modified bonded phases

A novel approach to the direct injection of samples of physiological origin is the use of modified bonded silica stationary phases. Conventional reversed-phase columns may become fouled with denatured proteinaceous material unless some deproteination step has been incorporated into the sample handling procedure.

Three variants of this approach have been proposed, internal reversed stationary phase (ISRP), shielded hydrophobic phase (SHP) and protein-coated ODS.

ISRP and SHP have been applied to the analysis of various types of drugs, but not β-blockers [101–103]. The direct injection of plasma and urine samples containing propranolol onto protein-coated ODS was reported: however, although recovery of the drug was complete, its usefulness to anything other than crude screening procedures was not proven [104].

4.3.4. Unmodified bonded phases

A recent report [105] described the direct introduction of urine, diluted with buffer, onto the chromatographic column. Metoprolol and its α-hydroxylated metabolite were separated on a phenyl-bonded silica column using a mobile phase consisting of acetonitrile–ammonium phosphate. Fluorescence detection was selected, not for its sensitivity, but to reduce interferences. No guard column was used in this procedure and the authors found it necessary to replace the top 5 mm of column packing every 100 injections to maintain column performance. Despite the implied difficulties, resolution remained satisfactory after more than 450 injections.

5 GAS CHROMATOGRAPHY

5.1. General considerations

Although there are reports of the GC of native β-blocking agents in toxicological analysis, the aminoalcohol side-chain confers reactive properties which result in adsorption, peak tailing and poor quantitation in the underivatised state [106] Derivatisation is therefore a pre-requisite of the GC of β-blockers. A summary of derivatising agents and their application was prepared by Ahnoff [106]. The use of halogenated acylating agents such as trifluoroacetic anhydride introduces electrophoric substituents enabling the detection and measurement of therapeutic and sub-therapeutic levels in physiological fluids by electron-capture detection and it is on this foundation that most GC methods are based. The chemical nature of the derivatives and their chromatographic properties should be thoroughly understood. Thus, nadolol, which contains three hydroxyl functions, tends to give a mixture of derivatives on trifluoroacetylation, but predominantly a single derivative when trimethylsilylated [107].

5.2. Applications

The application of bonded-phase capillary columns, which provide greater column efficiency and versatility, has found wide application Bucindolol, one of the newer generation of β-blockers, was determined in plasma by capillary GC after derivatisation of extracted drug with N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) [108]. Silylation, because of its ease of reaction and characteristic fragmentation in the mass spectrometer, is often the agency of choice

TABLE 1

SUMMARY OF CHROMATOGRAPHIC METHODS FOR THE DETERMINATION OF β-BLOCKERS COVERING THE REVIEW PERIOD

Key: LLE = liquid–liquid extraction, SPE = solid-phase extraction, Direct = direct injection, Solution = solubilisation only, RP-HPLC = reversed-phase HPLC, IP-HPLC = ion-paired HPLC; CX-HPLC = cation-exchange HPLC, FL = fluorescence detection, UV = ultra violet detection, ED = electrochemical detection, ECD = electron-capture detection; MS = mass spectrometry, FID = flame ionisation detection, NPD = nitrogen–phosphorus detection; C-S = column-switching

Drug	Extraction	Analysis	Detection	References
Acebutolol	LLE	RP-HPLC	UV	51
Alprenolol	LLE	GC	ECD	65
	LLE	RP-HPLC	FL	205
	SPE	RP-HPLC	FL	83
Atenolol	LSE	RP-HPLC	FL	42,80
	LSE	RP-HPLC	UV	169
	LLE	RP-HPLC	FL	182
	Solution	IP-HPLC	UV	31
	Solution	Various	UV	170
	Direct	RP-HPLC	FL	119
	LLE	GC	ECD	168
	LLE	NP-HPLC/RP-HPLC	FL	37
Betaxolol	LLE	IP-HPLC	FL	24
	LLE	RP-HPLC	FL	171
	LLE	GC	MS	118
Bevantolol	Solution	IP-HPLC	UV	29
Bisoprolol	LLE	IP-HPLC	FL	203
	LLE	NP-HPLC/RP-HPLC	FL	37
Bopindolol	SPE	RP-HPLC	ED	38
	LLE	RP-HPLC	ED	63
Bucindolol	LLE	GC	MS	108
Carazolol	LLE SPE	RP-HPLC	FL	47
	LLE SPE	TLC	UV	48
	LLE	RP-HPLC	FL	49
Carvedilol	LLE	RP-HPLC	FL	206
Dilevalol	LLE	RP-HPLC	FL	157
Esmolol	Solution	RP-HPLC	UV	52
	LLE	GC	MS	56
	LLE	RP-HPLC	UV	55,57
	Solution	RP-HPLC	UV	34
	LLE	GC	ECD	58
Falintolol	LLE	GC	ECD	109
Flestolol	LLE	RP-HPLC	UV	59
Flumolol	LLE	GC	ECD	58
Labetalol	Solution	RP-HPLC	ED	164
	LLE	RP-HPLC	ED	166
	LLE	RP-HPLC	FL	190,191
	LLE	RP-HPLC	ED	165
	SPE	RP-HPLC	FL	79

TABLE 1 (*continued*)

Drug	Extraction	Analysis	Detection	References
Levobunolol	LLE	RP-HPLC	FL + UV	45
	LLE	IP-HPLC	FL	46
Levomoprolol	SPE	LC–GC	UV, ECD	88
Medraxolol	C-S	RP-HPLC	FL	202
Mepindolol	LLE	RP-HPLC	ED	187
Metipranolol	LLE	GC	ECD	36
Desacetylmetipranolol	LLE	RP-HPLC	ED	207
Metoprolol	LSE	RP-HPLC	FL	43,82,83
	LLE	NP-HPLC/RP-HPLC	FL	37
	LLE	RP-HPLC, CX-HPLC	FL	44
	LLE	GC	MS	115,116
	LLE	GC	ECD	25
	LLE	RP-HPLC	FL	184
Hydroxymetoprolol	LLE	GC	NPD, MS	66
Nadolol	Dilution	RP-HPLC	FL	194
	LLE	RP-HPLC	FL	195
	SPE	GL	MS	81
Nebivolol	LLE	IP-HPLC	FL	204
Oxprenolol	LLE	IP-HPLC	UV	22
	LLE	RP-HPLC	ED	193
	SPE	RP-HPLC	UV	84
Penbutolol	Direct	RP-HPLC	FL	50
Pindolol	LLE	RP-HPLC	UV	60
	LLE	RP-HPLC	FL	189
	LLE	TLC	FL	61
Prizidilol	LLE	RP-HPLC	UV	64
Propranolol	LLE	NP-HPLC, RP-HPLC	FL	37
	SPE	IP-HPLC	FL	85
	LLE	RP-HPLC	FL	23,26,39,40, 54,158,175, 177,180,181
	SPE	RP-HPLC	FL	53
	LLE, Direct	RP-HPLC	FL	173
	LLE	RP-HPLC	UV	172
	Solution	IP-HPLC	UV	30
	LLE	CX-HPLC	UV	33
	LLE	CX-HPLC	FL	41
	Solution	RP-HPLC	UV	174
	Solution	RP-HPLC	UV	176
Sotalol	LSE	RP-HPLC	FL	86
	LSE	RP-HPLC	UV	87
	LLE	IP-HPLC	FL	28,197
	LLE	RP-HPLC	FL	198
Tertatolol	LLE	GC	MS	119,120
Tienoxolol	Solution	RP-HPLC	UV	209
Timolol	LLE	RP-HPLC	UV	27,199
	Solution	RP-HPLC	UV	201
	Solution	GC	FID	168

when derivatising β-blockers containing a substituted secondary amine. The steric contribution of the N-substituents not only prevents silylation of the amino nitrogen but also shields the polar nitrogen atom from interaction with the stationary phase.

A general summary of GC applications is given in Table 1, however, there are specialist applications which warrant specific consideration. The geometrical isomers of the novel β-blocker, falintolol, which is characterised by the absence of an aromatic nucleus, were resolved after derivatisation with heptafluorobutyric anhydride (HFBA) [111]. The existence of an asymmetric ketone oxime function confers *syn*- and *anti*-isomerism to the molecule with a natural proportion of 8:2 in favour of the latter, although pharmacologically there appears to be little difference between the two forms [110]. The authors were unable to separate the isomers using a glass column packed with SE-30, but separation was possible on a 50 m x 0.2 mm I.D. SE-30 column (Alltech Assoc., Deerfield, IL, U.S.A.). Regioisomeric products of propranolol metabolism have been separated by capillary GC in conjunction with structural studies on metabolites [111,112]. Structural characterisation was undertaken by combined gas chromatography–mass spectrometry (GC–MS) and a number of metabolites were identified by comparison with synthetic standards.

Though the advantages of using capillary GC for the determination of β-blockers has been stressed by several workers [25,113], packed-column methods are still reported. The determination of timolol in ophthalmic preparations was recently described [114] as was the monitoring of metoprolol by flame ionisation detection and GC–MS [115]. Pharmacokinetic studies frequently require the coadministration of a parent drug in conjunction with an isotopic derivative. Gaudry *et al.* [116] described a chromatographic method in which metoprolol and its deuterium-labelled analogue were derivatised with HFBA and separated on a glass column packed with OV-17. ^{13}C-Labelled metroprolol was used as internal standard and negative-ion MS for detection

The mass-selective detector represents an economic alternative to MS where low-resolution mass spectral data, generated from electron-impact ionisation, are required. Single-ion monitoring, using a mass-selective detector, was applied to the analysis of nadolol [81]. By interfacing the detector to a capillary gas chromatograph fitted with a DB-17 bonded methylphenylpolysiloxane phase (J&W Scientific, Folsom, CA, U.S.A.), the authors were able to monitor levels of nadolol in plasma over a range of 0.6–20 ng/ml, after sample pre-treatment. The selection of low-mass ions, challenged because of its lack of selectivity [117], was justified by its authors on the grounds of the potential increase in sensitivity [107].

Other authors reported the selection of low-mass ions to increase detection limits [118]. The fragmentation of derivatives of β-blockers in the mass spectrometer produces either ions characteristic of the drug under investigation or of the drug group. In the latter case, β-blockers possessing an N-*tert.*-butyl terminal group, *e.g.* nadolol and timolol, fragment (α) to the secondary amine to form an

intense ion with *m/e* 86. Betaxolol, which has a terminal N-isopropyl function, was assessed in a comparable manner as its trimethylsilyl (TMS) derivative using the fragment at *m/e* 72 generated by electron impact ionisation. It was found that at this low mass, the system was prone to interferences, although for deuterated isomers of betaxolol at *m/e* 84 this was less of a problem, and a sensitivity of 20–50 pg/ml was achieved. Combined GC–MS was also been applied to the investigation of tertatolol and its hydroxylated metabolite [119,120]

Gyllenhaal and Hoffmann [113] introduced phosgene as a cyclisation reagent for the GC of β-blockers. Early investigations using metoprolol favoured the use of the nitrogen-selective detector [121] and this was extended to the determination of alprenolol and its 4-hydroxylated metabolite [65] Cyclization of β-blockers with phosgene has the potential advantage that treatment of the sample may be undertaken prior to any extraction step. The products of the reaction are a series of oxazolidine-2-ones, the formation of which imparts some selectivity as monofunctional units will react more slowly or form unstable derivatives. To determine ring-hydroxylated β-blocker metabolites, such as 4-hydroxyalprenolol, a two-phase reaction system with dichloromethane as the organic constituent was envisaged in an attempt to stabilise the labile chloroformate group formed as a result of the reaction of phosgene with the 4-hydroxyl function. It was found, however, that without further treatment with methanol to form the 4-methyl carbonate, the derivatised β-blocker could not be reliably determined. Chromatography of free 4-hydroxyalprenolol was best achieved by selective solvent extraction prior to cyclisation to prevent reaction of phosgene with conjugates of the drug metabolite [122].

Refinement of this approach was described for the determination of metoprolol and its α-hydroxylated metabolites [66]. Treatment of the dried post-phosgene organic phase with bis(trimethylsilyl)acetamide (BSA) resulted in a series of TMS-oxazolidine-2-ones which, when analysed in combination with capillary GC–MS and GC with nitrogen-selective detection, quantitatively identified three hydroxylated metabolites of metoprolol and quantitated them in urine.

The application of phosgene as a cyclising agent for β-blockers is of particular use in gaining structural information from mass spectra. Other cyclising agents have been utilised, *e.g.* cyclic boronates [106], their utility being their selectivity for bifunctional molecules, where two functional groups are in spatial proximity to form a cycle. Electrophoric cyclisation reagents have also been employed, but with limited success [123].

Recently, the determination of levomoprolol, a β-blocker increasingly being used in the treatment of glaucoma, was reported using on-line coupled HPLC–GC [88]. An earlier method [124], applied to monitoring the drug in plasma and urine at dosages administered for hypertensive treatment, was insufficiently sensitive to the low levels of the drug encountered following topical administration by the ocular route. Although the technique provided increased sensitivity, illustrated chromatograms indicated significant interference which was reflected in the

coefficients of variation reported (15.7–20.8%). The complexity of the LC–GC interface would preclude a routine application: however, one feels that GC with single-ion monitoring or further exploitation of capillary sample introduction techniques (which were not discussed) might provide a simpler solution to the problem.

5.3. Drug screening

Drug screening is required for a variety of reasons, *e.g.* forensic analysis and toxicology. More recently there has been increased activity in the control of drug "abuse" or "doping" amongst athletes [17,19,125] and the β-blockers, as a group, were placed on the International Olympic Committee list of forbidden drugs in 1987 [126]. GC continues to provide a great deal of utility in these programmes.

Delbeke *et al.* [18] extensively studied the use of capillary GC and GC–MS and its application to the detection of β-blocker abuse. Utilising solid-phase and solvent extraction procedures, and trifluoroacetic anhydride (TFAA) as derivatising agent, they detailed a GC–MS screen incorporating selection of two abundant fragment ions characteristic of the isopropylamino N-terminal side-chain at *m/e* 308 and 266. Capillary GC (with split mode injection) was used either with nitrogen-selective detection, which was suitable for detection of sub-therapeutic doses of several β-blockers in urine within 24 h of administration, or with GC–MS for higher sensitivity and positive identification of drugs and metabolites. By selection of additional mass ions for monitoring of the *tert.*-butylamino side-chain all β-blockers could potentially be detected.

Maurer and Pfleger [76] reported a similar approach for their description of a GC–MS procedure for the screening of urine for 23 β-blockers and their metabolites. Extracts of urine or urine hydrolysates (for conjugates) were acetylated with acetic anhydride and subjected to packed-column GC and MS analysis. Retention indices for components were presented and artefacts of the procedure identified. GC screening procedures for β-blockers in urine using electron-capture detection have also been described [127,128]. In the work of Cartoni *et al.* [127] urine extracts were chromatographed as their pentafluoropropionyl derivatives and GC–MS was used for structure confirmation separately. Their identification of double peaks, emanating from isomers of hydroxylated metabolites, confirmed other authors' observations [76]. An improved screening of β-blockers claiming to overcome some of the shortcomings of other methods and able to detect ingested drugs up to 48 h after oral intake was also recently described [128].

The use of capillary GC has meant that is is possible to chromatograph "reactive" drugs without derivatisation. In a method described by Turcant *et al* [129], parallel injection into two capillary columns with separate nitrogen-selective detectors provided sufficient resolution to identify and quantitate over 200 basic and neutral drugs (including propranolol and alprenolol). A similar approach was reported by other authors [130]. Several protocols reported for the general

screening of drugs (including β-blockers) have relied on the establishment of retention indices [131–133] or retention times [129,134] for primary identification. All the general screening methods cited utilised nitrogen-selective detection in conjunction with some means of retention characterisation.

5.4. Conclusions

Although GC is currently less prominent in the determination of β-blockers, its continuing significance should not be underestimated. It remains the principal source of structural information from metabolic studies and with developments in the technology of bonded-phase capillary columns and MS instrumentation (*e.g.* the new generation of mass-selective detectors), this will remain so. Tandem techniques will also extend the use of GC. Recently a GC–infrared–MS system for drug identification was described [135] and forensic analysis is now making use of gas chromatography–Fourier transform infrared spectroscopy (GC–FTIR) for drug screening [136,137].

6 HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

Reports of chromatographic methods for the determination of β-blockers over the review period reflect the predominance of HPLC as the most favoured technique of pharmaceutical analysis. Over 80% of the papers included in this article describe HPLC analytical methods. For a complete background to the HPLC analysis of β-blockers the reader is referred to earlier reviews on the topic [5,138,139].

6.1. General considerations

The physicochemical character of the β-blockers is dominated by two features, the secondary amine function of the side-chain and the aromatic group. The former, with typical pK_a values in the region 9.2–9.6 [21], gives the group its basic characteristics, the latter provides lipophilic character and a feature to be exploited for detection capabilities; the aryloxy group gives high-intensity fluorescence with an excitation maximum below 240 nm [5]. Chromatography of basic compounds on silica-based chromatographic supports can lead to undesired interactions which adversely affect the separation mechanism [140]. The alcohol group also confers some polar character to the molecule, however, within the group as a whole are a variety of substituents, both polar and non-polar which must be considered when selecting columns and suitable solvent systems.

Although lipophilicity dominates the solubility characteristics of the β-blockers, there is wide polarity variation throughout the group. Octanol–water partition coefficients for atenolol, the most water-soluble, and propranolol, the most lipophilic, differ by six orders of magnitude [21]. Thus, as with extraction tech-

niques, this factor features highly in both column and mobile phase selection protocols.

LC of β-blockers, as with other classes of drugs, has favoured bonded-silica stationary phases. Lipophilic phases, predominantly octyl (C_8) or octadecyl (C_{18}), have proved to be the phases of choice, however, as in the case of other basic drugs [141], the performance of different commercial columns in relation to non-specific effects requires the use of phases containing a low level of residual silanols, or whose performance is modified by the addition of a competing base to the mobile phase. The choice of eluent composition is often directed as much to prevent interaction between the solute and free silanols as to elute the solute. The use of ion-pairing agents, *e g.* the alkylsulphonic acids, or the addition of competing bases, *e.g* triethylamine, to reduce interaction of the basic drugs with free silanols is the usual approach [142].

β-Blockers have been used as a vehicle to explore novel approaches to the chromatography of basic drugs. Two of these are the use of unmodified silica and alumina [143]. Hansen *et al.* [144] described the application of silica, dynamically modified with long-chain quarternary ammonium compounds added to the mobile phase, to the chromatography of propranolol and other basic drugs. Unmodified silica may be utilised in what is considered to be a predominantly ion-exchange mode through the application of methanol-rich, high-pH buffers as eluents [145,146]. This approach was shown to be compatible with UV, fluorescence and electrochemical detection of several β-blockers and other drugs [146] and its robustness was confirmed by inter-laboratory studies [147,148]. A more detailed investigation aimed at more clearly understanding the mechanisms of retention was also undertaken [149].

Further understanding of this mode of chromatography of β-blockers was described by Law [150,151]. Retention was shown to be a direct function of the pK_a of the solute and was independent of lipophilicity. This contrasted with reversed-phase chromatography because these silica systems were capable of chromatographing a wide range of basic drugs using only one or two eluents and were particularly suited to drug/metabolite screening. Capacity factors for 25 β-blockers and metabolites were derived [152] and it was proposed that retention characteristics of "unknowns" could be predicted by their "functional group contribution". A variation of this approach, characterised by supplementing ammonium nitrate (or phosphate) with ethylenediamine as the ionic component, promised improved chromatographic reproducibility [153].

The use of unmodified silica as a reversed phase was described by Richardson and Bidlingmeyer [154]. It was applied to the determination of atenolol, metoprolol and bisoprolol using an acidic reversed-phase buffer system with low organic modifier as eluent [37]. At pH values below that of the pK_a of silicic acid, it was suggested that unmodified silica packings show similar retention mechanisms as reversed-phase columns. Elution of the three solutes above, in order of increasing lipophilicity, supported this hypothesis although the behaviour of propranolol

was found to be less predictable. There are reports of other basic drugs being similarly analysed [155,156].

Analagous investigations were performed with alumina columns, using high-pH buffers [95] The retention characteristics of atenolol, pindolol and propranolol were consistent with an ion-exchange mechanism. These alternative modes of chromatography offer practical options when considering separation systems for β-blockers (and other basic drugs). Their application has been limited, in part because of the unawareness and scepticism of chromatographers of alternatives to bonded phase chromatography and also because of the requirement for control of pH and ionic strength to obtain reproducible results. Other alternatives to silica-based phases applied to the chromatography of β-blockers include polymer-based reversed-phase supports [157,158] and, more recently, porous graphitic carbon [159].

Other variants of chromatography on silica-based packings have been discussed in Section 4.3. In the context of chromatography the use of micelles (MLC) offers the opportunity to achieve rapid gradient changes without affecting column or detector stability [97], though there may be exceptions [160]. The pitfalls include reduced column efficiency, potential permanent changes to the stationary phase caused by the high-ionic-strength mobile phase and the necessity to dedicate a single column to the application. Nevertheless, the technique has found application to the determination of β-blockers [97,98].

Shielded hydrophobic phases appear to offer acceptable efficiencies, but their selectivity in comparison to silica-bonded phases is unproven. The same may be concluded of ISRP (or "Pinkerton") columns. Despite question marks over column performance, both these phases at least promise potential advantages in the handling of "difficult" samples, where removal of protein may be a problem.

6.2. Detection

Detection of β-blockers in column eluent is conveniently attained by exploiting their native UV and fluorescence properties [97]. The intense fluorescence of most β-blockers when excited by shorter-wavelength UV radiation means that high sensitivity, sufficient for the detection of most individual drugs in plasma at therapeutic concentrations, is routinely achieved. Over half the applications reported in this review employed fluorescence detection The most significant recent development in UV detection is the commercial availability of the diode-array detector and other rapid-scanning detectors. These detectors offer the development analyst additional scope for obtaining spectral information contained in chromatographic peaks without having to resort to stopped-flow analysis or fractionation. They are also useful in the application of LC to drug screening [160].

Electrochemical detection has found some application to the determination of β-blockers. It has the advantage of sensitivity, selectivity and wide linearity of response [161], but suitably electroactive candidates amongst the β-blockers are

restricted, and in most applications there was no significant advantage over fluorescence detection [162]. Theoretically the secondary amino nitrogen provides a nucleus for electrochemical oxidation, however, with anodic oxidation waves at potentials greater than 1 V [163], this approach is not easily applied. Some β-blockers have electrochemically active substituent groups. Labetalol, with a phenolic hydroxyl group in its aromatic ring [164–166], and bopindolol, containing an indole nitrogen [38], are such candidates. The relatively high oxidation potentials required can, however, generate background signals from the sample and eluent.

The formation of reactive derivatives of poorly electroactive drugs is one practical solution. This approach was adopted by Leroy and Nicolas [163] for the determination of atenolol and propranolol. Secondary amines react quickly with carbon disulphide to give the corresponding N-substituted dithiocarbamates, which form complexes with divalent metal ions such as copper and nickel This complexation had been used previously in the determination of trace metal ions by HPLC [167] and was shown in this study to be suitable in the production of electroactive derivatives of atenolol and propranolol for chromatography and oxidative electrochemical detection. Drug mixtures resulted in the production of mixed complexes which limit the scope of successful application of the method.

6.3. Applications

Having defined the principles of isolation, separation and detection of β-blockers in the preceding discussion, it is perhaps simplest to direct the reader to specific applications in the form of a tabulated summary (Table 1). Nevertheless, many applications warrant more detailed consideration.

Atenolol, the most hydrophilic β-blocker, has been chromatographed on a wide range of bonded silica packings, with and without mobile phase modifiers [31,42,67,82,168,169] Although fluorimetric detection was most frequently reported, Wong *et al.* [169] claimed that UV detection provided sufficient sensitivity, even for single-dose pharmacokinetic studies. Koenigbauer [170] highlighted the problems of reversed-phase chromatography of basic drugs, using atenolol as an example, and suggested practical solutions.

The application of buffered reversed-phase eluents over native silica for the determination of atenolol was described earlier [61]. Reproducibility of propranolol chromatography under these conditions was poor and the authors resorted to acetylation of drug extracts to obtain satisfactory chromatography. Bhamra *et al.* [24] chromatographed betaxolol on normal-phase silica with 1 m*M* D-camphor sulphonic acid in methanol as mobile phase; however, more conventional C_{18} reversed-phase separation is more commonly used to determine this drug [171].

Most recent reports of the chromatography of propranolol have focussed on the quantitative identification of its many metabolites. Some development of

established methods for the parent compound have been noted [30,172–175] and recently there has also been a revision of the United States Pharmacopoeia monograph [176] for propranolol which recommended the use of procainamide as internal standard. This is not the favoured approach and generally another β-blocker is preferred A specific analogue, N-cyclopentyldesisopropylpropranolol, was prepared for this purpose [177].

Chromatographic resolution of propranolol and all, or some, of its metabolites [178,179] has been reported with varying degrees of success [39,40,53,54,180,181]. Radial compression chromatography with its potential for high flow-rates, low back-pressure and relatively high column efficiencies may be one solution to separating large numbers of widely differing components on a single reversed-phase column. Several groups have applied this technique to the separation of β-blockers and their metabolites [23,26,54,182].

Less usual modes of chromatography have been used for the determination of propranolol. Yamamura *et al.* [158] achieved a limit of determination of 1 ng/ml using porous polystyrene gel packing and fluorimetric detection. They were able to apply this technique to determine free and bound drug in plasma. A further approach, suitable for pharmacokinetic and formulation studies, was proposed by Zelikman and Hjerten [33]. They exploited the cation-exchange properties of the sulphate and carboxylic acid groups of agaropectin. This exchanger was considered easy and inexpensive to prepare and provided acceptable results with both UV and fluorescence detection. Belolipetskaja *et al.* [41] also employed cation-exchange chromatography coupled to fluorescence detection and reported the separation of propranolol and a number of its basic metabolites. This method had the advantage of being able to measure drug conjugates without resort to enzymic or acid hydrolysis. Conjugates of penbutolol were directly chromatographed on C_8 silica. Glucuronides were chromatographed in a mobile phase of 0.01 *M* orthophosphoric acid–acetonitrile (gradient) and the sulpho conjugates in a buffer of 0.02 *M* tetrabutylammonium phosphate–acetonitrile in stepped gradient [50]. Unconjugated drug was eluted using an isocratic buffer. The system was reported as being able to separate regioisomeric conjugates.

Metoprolol is another of the longer established β-blockers where recent attention has focussed on the monitoring of metabolites. It is particularly relevant as only 3% of the drug is excreted unchanged in the urine [183] and there is interest in its pre-disposition to polymorphic metabolism. Lennard [44] determined metoprolol and three of its metabolites in urine and liver microsomes, employing C_{18} reversed-phase chromatography for metoprolol and α-hydroxymetoprolol and cation exchange for acidic metabolites. A similar approach was reported by Balmer and *et al.* [43], but other reports have limited their investigation to metoprolol and its α-hydroxylated metabolite only [83,184,185]

The difficulties involved in the sample processing of short-acting β-blockers were discussed earlier. The chromatography of these drugs poses fewer problems. Reversed-phase HPLC was demonstrated to be capable of resolving esmolol

from its degradation products during formulation studies [34,52]. In clinical applications where with a half-life of approximately 10 min, plasma drug concentrations fall rapidly on withdrawal of medication [10]. UV detection following separation on C_{18} silica was adequate to meet most sensitivity requirements [55,57]. Flestolol, another short-acting drug, was similarly treated [59].

β-Blockers containing an indole ring form a sub-group which may be detected electrochemically. Bopindolol as well as being chemically unstable (see Section 4 1) is rapidly hydrolysed on absorption and first-pass metabolism through the liver to the active metabolite hydroxybopindolol, which retains the indole ring, so that both parent drug and metabolite can be subject to sensitive monitoring by HPLC coupled with electrochemical detection. Perkins *et al.* [63] reported selective monitoring of the drug at sub-nanogram levels coulometrically. A sensitivity limit of 25 pg/ml (100 pg on-column) was reported by Humbert *et al.* [38] but assay precision was not good. Aellig *et al.* [186] monitored hydroxybopindolol by similar methods to a limit of 0.5 ng/ml in plasma.

The closely related drugs pindolol and mepindolol were investigated using ion-pair chromatography and coulometric detection [187,188], but methods based on fluorimetric [189] and UV detection [60] have also been described. Shields *et al.* [60] claimed that by careful optimisation of detection wavelength, a sensitivity for UV detection equivalent to that obtained for fluorescence could be obtained.

Labetalol with its phenolic grouping provides another sub-group suitable for electrochemical detection (see Section 6.2). This is a useful feature as the molecule exhibits poor native fluorescence, although Luke *et al.* [190] demonstrated that enhanced fluorescence response could be obtained by the post-column introduction of ammonium hydroxide and the use of a high excitation wavelength Rather than alkalinise the post-column eluate, Bates *et al.* [79] chromatographed labetalol on a polymeric reversed phase which tolerated high-pH eluent. It may, however, be possible to obtain enhanced fluorescence of labetalol at neutral or slightly acidic pH values [191]. Wang *et al* [164], using amperometric detection, found that the pH of the mobile phase affected the peak current as well as chromatographic retention. Plavsic *et al* [166] also reported an amperometric method but other workers used coulometric detection to provide pharmacokinetic data.

Dilevalol, the (*R*,*R*)-isomer of labetalol, has a quite different pharmacological profile to the racemic mixture. Its determination was previously reported on a polymeric stationary phase in conjunction with fluorimetric detection [157]. The method was capable of detecting therapeutic levels of the unchanged drug in blood and urine [157] and breast milk [192].

HPLC with electrochemical detection was reported for the determination of oxprenolol Like other β-blockers, it is electroactive only at high oxidation potentials. To overcome operating difficulties at high potentials, Gregg [193] proposed preanodisation of an amperometric electrode as one solution, and demonstrated its application in human pharmacokinetic studies The pretreatment was found to

offer only limited detector stability, but results did correlate well with plasma levels determined by GC–MS. Coulometric detection might have provided a more practical alternative, but there were no reports of this application to the determination of oxprenolol.

A more conventional combination of HPLC and fluorimetric detection of oxprenolol, described by Devi *et al.* [84], did not improve performance. Significant interference was apparent in the chromatography and it is doubtful whether the quoted detection limit of 5 ng/ml in plasma obtained from pre-clinical studies in animals could be reliably attained in human pharmacokinetic investigations. An alternative method employing UV detection was less sensitive [22].

The high-potency β-blocker, levobunolol, is rapidly metabolised in blood to dihydrolevobunolol. This reduction product shows high intrinsic fluorescence which can be exploited for high-sensitivity determination of parent drug and metabolite. Applying a two-stage procedure incorporating inhibition of *in vivo* reduction and then chemical reduction, Hengy and Kolle [46] were able to measure drug and metabolite by difference. Tang-Liu *et al.* [45] improved the sensitivity of the method for direct measurement in animal studies, but still required a two-stage procedure to quantitate unchanged drug and metabolite.

The hydrophilic β-blocker nadolol, because of its large volume of distribution and low bioavailability presents low plasma drug levels Two groups reported methods employing reversed-phase HPLC on C_{18} columns. Using a mobile phase containing an ion-pairing agent. Moncrieff [194] improved sensitivity and selectivity by optimisation of fluorescence response. A sensitivity limit of 5 ng/ml in serum was attained using only a protein precipitation pre-treatment. Liu and Robinson [195] with triethylamine in their eluent and employing a single-stage extraction, established a sensitivity limit of 0.5 ng/ml for the drug in plasma, serum and urine.

Several reports have described the HPLC determination of sotalol, which like nadolol and atenolol is excreted largely unchanged in urine [196]. It is extremely hydrophilic and, like atenolol, is not highly bound to plasma proteins. Several assay procedures have been reported in the past, but a recurrent pitfall has been low recovery of this amphoteric drug [197]. One method currently reported [28] only recorded an overall recovery of 30% by solvent extraction. The detection limit was quoted as 25 ng/ml in plasma provided that larger (unqualified) volumes of extract were chromatographed. Hoyer [87] improved the recovery of sotalol to 76% by solid-phase extraction. A more recent HPLC–fluorimetric procedure described by Poirier *et al.* [198] featured a CN-bonded column phase and fluorimetric detection resulting in complete chromatography in less than 6 min, but there was no improvement in overall recovery or detection limit over previously reported methods. Gluth *et al.* [197], achieved almost complete recovery of sotalol from body fluids (see Section 3) and described separation incorporating ion-pair chromatography.

Timolol, which has gained widespread acceptance for reducing interocular

pressure in glaucoma [10], had previously been determined by HPLC with electrochemical detection [5] Lennard and Parkin [27] reported a reversed-phase method using UV detection which was of equivalent sensitivity, while Wu *et al.* [199] described a less sensitive method using UV detection, suitable for experimental studies on aqueous humour after drug administration. The application of dynamically modified silica to the chromatography of β-blockers [35] improved chromatographic quality (see Section 6.1), and better reproducibility was claimed. Using this technique timolol was separated from three potential degradation products. The separation of timolol pro-drugs (timolol esters) from timolol was also reported [200,201].

Levomoprolol, another β-blocker administered by the intra-ocular route, was measured quantitatively in pharmacokinetic studies by coupling a liquid chromatograph to a capillary gas chromatograph fitted with an electron-capture detector [88]. By this method an analytical limit of 0.2 ng/ml in plasma was detected following administration of 540 μg of drug. In this application HPLC was performed on CN-silica in normal-phase mode for better solvent compatibility with the gas chromatograph.

Many other applications of HPLC to the determination of β-blockers have relied invariably on a standardised approach. Medroxalol was determined following column switching on a C_{18} phase and fluorimetric detection. The column-switching method correlated well with solvent extraction [202]. Bisoprolol was separated on a silica column [37] (see above), but a simplified procedure with equivalent sensitivity for pharmacokinetic studies was also reported [203]. Nebivolol, a novel β-blocker containing a pyran ring, retained the native fluorescence characteristic of β-blockers [204] and was chromatographed on C_{18} silica with a mobile phase containing diethylamine and tetrabutylammonium hydrogensulphate. Other applications reported include alprenolol [205], carvedilol [206], desacetylmetipranol [207], indenolol [208], tienoxolol [209] and experimental β-blockers [155,210,211], which were all determined using the principles discussed above, although in the case of one experimental compound, pre-column dansylation was necessary because of a lack of native fluorescence [210].

6.4. Determination of physicochemical properties

HPLC has been used to provide a direct estimate of the binding of propranolol to plasma proteins [212]. Using a Lichrosorb Diol size-exclusion column (E. Merck) eluted with a mobile phase containing a saturating concentration of propranolol, plasma protein binding was calculated using the method of Hummel and Dreyer [213]. The result was shown to have good correspondence with that obtained from equilibrium dialysis and was considered to have wide application for other drugs. An alternative approach to estimation of drug protein binding using HPLC has been proposed [214], but no application to β-blockers was alluded.

Lipophilicity of drug molecules is regarded as a measure of membrane permeability, an important indicator of structure–activity relationships in drug design. Two methods of determining lipophilicity are popularly adopted, derivation of octanol–water partition coefficient by shake flask method and retention on reversed-phase HPLC [215]. Vila *et al.* [15] determined the lipophilicity of 18 β-blockers at three pH values and ranked lipophilicity by means of k' values. They found good correlation between their results and relative lipophilicity calculated from partition coefficients.

6.5. Conclusions

HPLC offers the greatest versatility for the separation of most drug groups and the example of the β-blockers bear testimony to this statement. In most cases reversed-phase separation on C_{18} silica-based columns is the method of choice, and provided that due recognition of the basic characteristics is made and the mobile phase modified to accommodate this (*e.g.* ion-pair agent, competing base), this approach suffices in most applications.

Fluorescence, by manipulation of excitation and emission frequencies, usually provides the most sensitive and specific mode of detection, but other modes may be applicable in specific cases. Interferences in chromatography were not a problem in most applications reported, but may be encountered [60]. A generalised analysis scheme for β-blockers by HPLC was recently summarised by Barnhill and Greenblatt [138].

7 THIN-LAYER CHROMATOGRAPHY

The complementary relationship between TLC and other modes of LC have been highlighted by recent advances in the technique TLC has been instrumentalised, and availability of bonded-phase layers and high-performance TLC (HPTLC) plates have contributed to the progress which has been reviewed by various authors in general terms [216] and with specific reference to β-blockers [217].

Toxicological screening is well suited to TLC. Bonicamp and Pryor [6] described a protocol for the screening of β-blockers in urine, which although not specific (pindolol and timolol had the same R_F value), provided the required selectivity by a sequence of colour development stages. Harper *et al.* [218], in a general screen for basic drugs, were able to positively identify several β-blockers. Musumarra *et al.* [219] included β-blockers in a scheme for the preliminary identification of almost 600 drugs by Principal Component Analysis, while Singh *et al.* [77] screened for a variety of doping agents, including propranolol, by TLC. A simpler drug-specific screening procedure (for carazolol) by two dimensional HPTLC silica gel plates was described by Haagsma *et al.* [48]. Ojanpera and Ruohonen [139] recently reported the application of Fast Black K salt as a sensi-

tive and partially specific visualising agent for β-blockers, with a typical detection limit in urine of 100 ng/ml, significantly more sensitive than the procedure of Bonicamp and Pryor [6].

Bonded-phase TLC plates with chemistries analagous to HPLC packings have been available for some time. Ruane and Wilson [220] investigated the use of octadecyl-bonded plates for the TLC of β-blockers. Three different types of commercial C_{18} bonded gel, their own paraffin-coated silica gel and unmodified silica gel were eluted with various mobile phases containing either heptanesulphonic acid or SDS as ion-pair modifier. In the absence of ion-pair agent, the paraffin-coated gel and the unmodified gel gave poor results, however, the three bonded phase C_{18} plates performed well. Coating of the unbonded plates did not improve their performance. Coating of the bonded phases produced results for the test substances consistent with ion-pair retention mechanisms. R_F was decreased and spot shape was improved with some inter-packing variation of absolute performance. Changes in pH of the elution buffer did not substantially modify the R_F values for any of the packings, in contrast to observations with HPLC.

Spahn *et al.*[61] investigated a specific qualitative TLC procedure for pindolol in plasma for patient compliance studies. They explored intrinsic fluorescence and *o*-phthaldialdehyde-derivatised fluorescence, the former giving a detection limit of 2 ng/ml in plasma after enhancement of native fluorescence by immersion of the TLC plate in 4% (v/v) liquid paraffin in cyclohexane. Spraying the plates with *o*-phthaldialdehyde did not improve this limit, because of the presence of background fluorescence from sample impurities. Volgram [221] also reported optimum conditions for TLC of a number of β-blockers on normal-phase silica sorbents.

In tandem with other developments in LC, TLC has been applied to assess the lipophilicity of β-blockers. Jack *et al.* [222] treated reversed-phase TLC plates with octan-1-ol prior to chromatography and elution in a phosphate buffer saturated with octan-1-ol. The R_M (equivalent to k' for column chromatography) correlated well with previously published values of the octanol–water partition coefficient.

8 RESOLUTION OF OPTICAL ISOMERS

8.1. Background

The interest and progress in chiral separations is reflected by the appearance of several reviews and authoritative texts [223–228]. The stereoisomerism of β-blockers arises from the presence of a chiral carbon centre at the 2-carbon of the arylpropanolamine side-chain. This feature is common to all β-blockers, and drugs such as labetalol may contain additional chiral centres. The ability to perform quantitative enantiomeric separations is potentially of great significance in drug disposition studies. The determination of optical isomers of β-blockers has

been the subject of recent review articles [227,228]. In this discussion it is intended to summarise recent developments and highlight some specific applications.

Although the basic requirements for enantiomeric separations have been understood since the turn of the century, it is the past decade which has proved to be the turning point in the field [229]. There are three recognised approaches to the chromatographic resolution of enantiomers, each of which has found application to the determination of β-blockers.

(a) Use of optically stationary phase and achiral mobile phase (chiral stationary phase, CSP).

(b) Reaction of the chiral solute with an optically active derivatising agent to form diastereoisomers, which may be separated on a non-chiral stationary phase (chiral derivatising agent, CDA).

(c) The use of chiral additives to the mobile phase to form "dynamic" diastereoisomers (chiral mobile phase, CMP).

(a) and (b) apply to both GC and LC, (c) to LC only.

LC has almost completely superseded GC as the preferred method for chiral separations of β-blockers (and other drugs) A single separation method describing the separation of metoprolol enantiomers as optically active oxazolidine-2-ones on a chiral capillary column was described by Gyllenhaal *et al.* [230]. The following discussion therefore refers only to LC techniques. A summary of applications is presented in Table 2.

8.2. Chiral stationary phases

The rapid commercialisation of CSPs has resulted in the recognition of four distinct types: (a) the "Pirkle" phase, consisting of (*R*)-N-(3,5-dinitrobenzoyl) phenylglycine attached to γ-aminopropylsilanised silica gel; (b) chiral polymers, *e.g.* cyclodextrin-bonded silica gel; (c) affinity phases, and (d) chiral ligand exchange phases [228,231,232]. No single type is capable of resolving all forms of enantiomers, but there are examples of separation of the enantiomers of β-blockers on all but the ligand-exchange phase [227].

The Pirkle-type phase has been found to be particularly useful following cyclisation of the β-blockers on reaction with phosgene (see Section 5.2). Derivatisation of solute is frequently required for successful chromatography on this CSP [232] and Wainer and Doyle [233] reported the quantitative determination of propranolol isomers in human serum using this system. The reaction of phosgene with propranolol could be carried out conveniently with nanogram to milligram quantities of propranolol. No racemisation was observed and intrinsic fluorescence of the drug was retained. Pirkle phases are commonly used with mobile phases composed of hexane and an alcohol as a polar modifier [232], in this application isopropanol and acetonitrile were used. Urea CSPs with a mechanism of selectivity thought to be similar to that of the "Pirkle" phases have had some limited success in separating β-blocker enantiomers [234].

TABLE 2

CHIRAL SEPARATION METHODS BY LIQUID CHROMATOGRAPHY AND THEIR REPORTED APPLICATION TO THE RESOLUTION OF β-BLOCKER ENANTIOMERS

Key CSP = Chiral stationary phase, CDA = Chiral derivatising agent, CMP = Chiral mobile phase, AAG = α_1-acid glycoprotein, CTE = Cellulose tri-ester; MCF = Menthyl chloroformate, bSA = bovine serum albumin; RP = reversed phase, NP = normal phase, ZGP = N-benzoxycarbonyl-glycyl-L-proline, BPFN = *S*-(+)-benoxaprofen; BDLA = *tert*-butoxycarbonyl-L-leucine anhydride. Other abbreviations as used elsewhere in text

Drug	Method	Reagent/column	References
Alprenolol	CSP	Phosgene/CTE	249
	CSP	Phosgene/AAG	237
	CDA	NEIC/RP/TLC	273
	CMP	ZGP/NP	270
Atenolol	CDA	MBIC/RP	259
	CDA	DATAAN/NP	253
	CDA	MCF/RP	264
Betaxolol	CSP	Phosgene/CTE	249
	CSP	CTE	241
Bisoprolol	CSP	Phosgene/CTE	249
Bopindolol	CSP	bSA	235
Bunitrolol	CDA	NEIC/RP/TLC	273
Bupranolol	CSP	Phosgene/CTE	249
4-Chlorobupranolol			
Dechlorobupranolol			
Dimethylbupranolol			
3-Hydroxybupranolol			
Isopropylbupranolol			
Carazolol	CSP	Phosgene/CTE	249
Carvedilol	CDA	GITC/RP	258
Celiprolol	CSP	CTE/NP	248
Flavodilol	CDA	MCF/RP	263
Labetalol	CSP	AAG	238
	CMP	Cyclodextrin/TLC	273
Metoprolol	CDA	Phosgene/CTE	249
	CDA	PEIC/RP	261
	CDA	NEIC/RP/TLC	273
	CDA	GITC/RP	256
	CDA	MCF/RP	264
	CDA	BPFN/TLC	271
	CDA	FLEC/RP	256
	CSP	Phosgene/AAG	237
	CSP	CTE/NP	246,247
	CSP	bSA&AAG	240
	SCP	AAG	238
	CMP	ZGP/NP	270
Nadolol	CSP	AAG	238

TABLE 2 (*continued*)

Drug	Method	Reagent/column	References
Oxprenolol	CSP	Phosgene/AAG	237
	CDA	BPFN/TLC	271
	CDA	NEI/RP/TLC	273
Penbutolol	CSP	Phosgene/CTE	249
Pindolol	CSP	Phosgene/AAG	237
	CSP	bSA	235
	CDA	GITC/RP	258
	CDA	NEI/RP/TLC	273
Propranolol	CSP	Phosgene/CTE	249
	CSP	Phosgene/AAG	237
	CSP	Pirkle/NP	233
	CSP	CTE/NP	242
	CSP	Cyclodextrin	250
	CDA	MCF/RP	263,264
	CDA	BPFN/TLC	271
	CDA	BDLA/TLC	251
	CDA	DATAAN/RP	252
	CDA	NEIC/RP/TLC	273
	CDA	PEIC/RP	262
	CMP	ZGP/NP	269,270
	CMP	CSA	266
Sotalol	CDA	MCF/RP	264
	CSP	AAG	236
Toliprolol	CDA	MCF/RP	264
	CSP	Phosgene/CTE	249

Kusters and Giron [235] separated underivatised enantiomers of pindolol and bopindolol on Resolvosil BSA-7 (Macherey-Nagel, Düren, F.R.G.), a CSP containing immobilised bovine albumin. Using a phosphate-buffered eluent modified with isopropanol, peaks were broad and tailed, but resolution at 1.81 was sufficient to ensure baseline separation. Delee *et al* [236] obtained similar performance for the separation of sotalol on an LKB Enantiopak (Pharmacia-LKB, Uppsala, Sweden) column; however, the phosphate buffer mobile phase required the addition of an anionic modifier to achieve resolution (propan-2-ol was ineffective) and resolution was sensitive to pH adjustments (Fig 5) Bound-protein affinity columns have also been used to separate the oxazolidine-2-ones of β-blocker enantiomers [237]. Derivatisation with phosgene retains the optical activity of the parent compounds, but increases separation factors on these relatively low-efficiency phases. As illustrated above some improvement of retention and other column parameters may be exercised through the control of pH and the addition of organic modifiers to the mobile phase. The chromatography of metoprolol enantiomers on an α-acid glycoprotein column was enhanced by the addition of tetrapropylammonium bromide to the mobile phase [238]

A more interesting separation on an α-acid glycoprotein CSP was the resolu-

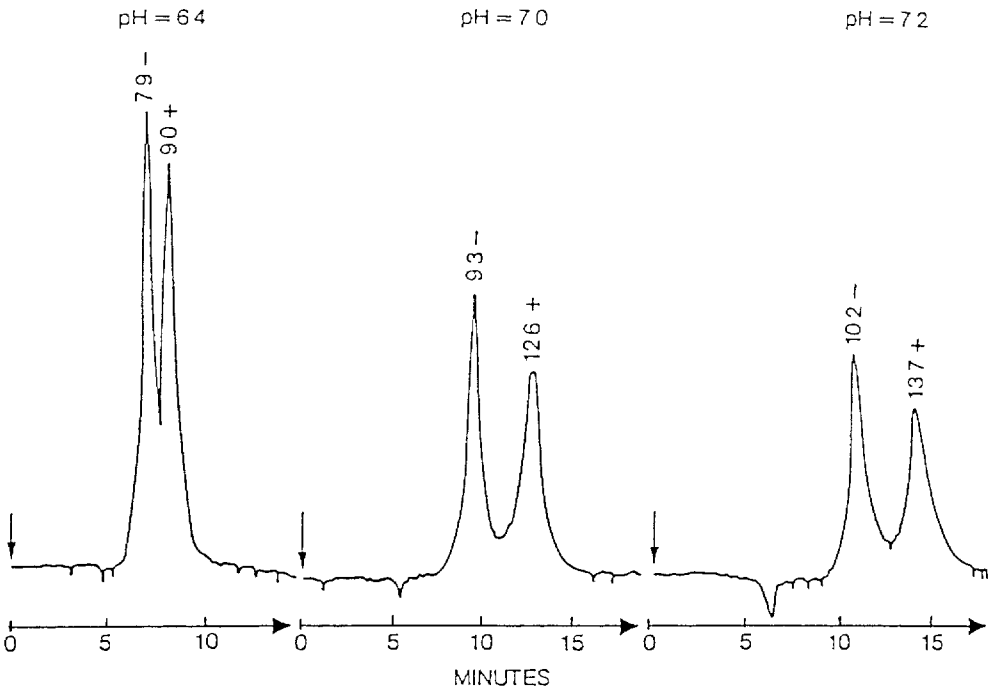

Fig 5. Effect of mobile phase pH on resolution of sotalol enantiomers on an immobilised protein chiral stationary phase (Enantiopak) (Reproduced from ref 236 with permission)

tion of the four stereoisomers of labetalol [239]. This could also be applied to assess the stereochemical purity of dilevalol, which was not possible using a previously described procedure [157].

To overcome the low efficiency and restricted chromatographic conditions of immobilised protein CSPs, Walhagen and Edholm [240] coupled an immobilised bovine albumin column to two "concentrator" reversed-phase guard columns. They were able to resolve metoprolol enantiomers by application of a complex valve-switching mechanism, collecting each enantiomer on the separate concentrator columns. These two columns were then selectively eluted onto a reversed-phase analytical column. In this way analytical efficiency was increased from approximately 3500 plates to 15 000 plates.

Cellulose triesters, *e.g.* cellulose triacetate, coated on macroporous silica, form diastereoisomeric complexes with chiral solutes within the pores of the gels. Mixtures of hexane and modifying alcohols, or polar solvents, may be used for elution and many β-blocker enantiomeric pairs can be separated in this way [232]. A polar solvent may require the addition of a competing base to protect against residual silanols [241], but drugs may be chromatographed underivatised [242,243]. This system was evaluated against a chiral separation system requiring the formation of diastereoisomers [244], using propranolol as the study drug.

Excellent correlation was found, and the limit of detection, using fluorescence detection, was at least 3 ng/ml, comparable with many conventional achiral HPLC separation methods Betaxolol enantiomers were similarly separated [241], while Krstulovic *et al.* [245] applied these phases to confirm the optical purity of enantiomerically resolved β-blockers. Other recent applications include the separation of metoprolol [246,247] and celiprolol [248]. These columns show relatively high efficiencies and short retention times compared to other chiral phases [239].

Modified monosaccharides bound to silica gel have also found use as CSPs for the separation of enantiomeric β-blockers and other drugs [249]. As with other chiral phases, good resolution was achieved against the background of low column efficiency.

A detailed study of the stereochemical requirements for chiral recognition of drugs by cyclodextrin-bonded CSPs was conducted by Armstrong *et al.* [250]. Using two 25-cm columns, containing a β-cyclodextrin-bonded phase, coupled in series, they were able to partially resolve propranolol isomers, but metoprolol isomers were poorly separated. Using computer simulation they projected the putative sites of interaction of propranolol isomers within the cyclodextrin inclusion complex, identifying the site of chiral recognition. The stereochemical constraints for chiral selectivity of β-blockers within the complex indicated that propranolol was perhaps a better candidate for resolution than most drugs of the group. Cyclodextrin-bonded phases have subsequently found limited application to the separation of β-blocker enantiomers.

8.3. Derivatisation

The resolution of optical isomers by chemical derivatisation to diastereoisomers has provided the mechanism for one of the "classical" means of obtaining optically pure materials. In the chromatographic context diastereoisomers, with different physicochemical properties, can be separated using achiral chromatographic systems, frequently to lower limits of detection [228] The limitations of the method arise from the optical purity of the derivatising agent, the potential for different rates of reaction between enantiomers and the chiral reagent and the possibility of racemisation of chiral centres during the derivatisation reaction [232]. A summary of chiral derivatising reagents is shown in Table 3.

The potential sensitivity of this mode of chiral separation was recently illustrated by Guttendorf *et al.* [251] They used *tert.*-butoxycarbonyl-L-leucine anhydride to form diastereoisomeric propranolol-L-leucine derivatives which were then separated on an Ultasphere ODS (Beckman Instruments, Fullerton, CA, U.S.A.) microbore column eluted with a methanol–water mobile phase, adjusted to pH 2.8 with ammonium phosphate. From 200 μl of rat whole blood they were able to determine individual enantiomer levels as low as 2.5 ng/ml. There was no evidence of any racemisation.

TABLE 3

OPTICALLY ACTIVE REAGENTS USED TO CONVERT β-BLOCKERS INTO DIASTEREOMERIC DERIVATIVES

CP = chromophor; FP = fluorophor, HPLC systems: predominantly reversed-phase. (Reproduced from ref. 252 with amendment.)

No.	Reagent	Abbreviation	Chromophore	Resolution	α Value	References
1	(−)-N-Trifluoroacetylprolyl chloride	(−)-TPC	–	1.4	1.2	11–13
2	*tert.*-Boc-L-Leu anhydride		–	1.7	1.3	14,15
3	(+)- and (−)-Phenyl isocyanate	(+)-PEI	CP	1.5–2.6	1.1	16–18
		(−)-PEI			1.25	19–21
4	*R*-(−)-1-(1-Naphthyl)ethyl isocyanate	(−)-NEI	FP	1.5	1.1	22,23
5	2,3,4,6-Tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate	TAGIT/GITC	–	2.7	1.6	24–26
6	(+)-1-(9-Fluorenyl)ethyl chloroformate	FLEC	FP	1.2	1.06	27
7	(*R,R*)-O,O-Diacetyltartaric acid anhydride	DATAAN	–	5.0	2.7	10

Propranolol isomers have also been separated after derivatisation with (*R,R*)-O,O-diacetyltartaric acid anhydride (DATAAN) [252]. The advantage of this reagent is that, rather than binding to the secondary amino nitrogen, which is protected by ion-pair formation with, typically trichloroacetic acid, the reagent reacts with the secondary alcohol only. Keeping the two chiral centres in close proximity in this way accentuates the diastereoisomeric differences and enhances resolution of the enantiomers [253]. Fluorimetric detection following isocratic reversed-phase HPLC resulted in a reproducible and linear response for both enantiomers in the concentration range 4–100 ng/ml in human plasma and a total chromatography time of under 10 min Other enantiomeric separations of propranolol have reported derivatisation with (−)-menthylchloroformate (MCF) or *R*-(+)-α-methylbenzyl isocyanate to form respective diastereoisomers [254,255]. In both examples chromatography was performed on ODS-bonded silicas.

The preparative separation of metoprolol enantiomers after derivatisation with substituted tartaric acid anhydrides was described by Wilson *et al.* [253]. In this study the drug enantiomers were derivatised with (*R,R*)-O,O-di-*p*-toluoyltartaric acid anhydride and separated on a semi-preparative scale by normal-phase HPLC. To recover the free enantiomers the purified fractions were hydrolysed with ammonia, extracted at pH 4 to remove tartaric acid and chromatographed by reversed-phase HPLC as their 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) derivatives. The overall procedure gave 97% enantiomeric purity. Metoprolol was also used as a model compound for the application of (+)-1-(9-fluorenyl)ethyl chloroformate (FLEC) as an enantiomeric derivatising agent [256]. Resolution of diastereoisomers was virtually complete on reversed-phase HPLC and the derivatives exhibited intense fluorescence.

Derivatisation with GITC was applied to the resolution of metoprolol enantiomers from plasma samples [257]. The derivatives were separated on μBondapak C_{18}. With UV detection at 222 nm, the authors achieved a detection limit of 10 ng/ml for each isomer in plasma, adequate for pharmacokinetic studies of human drug dispostion. There was good correlation between their method and that of the total drug concentration of the racemic mixture. GITC has been applied to prepare derivatives of carvedilol and its desmethyl metabolite for resolution by C_{18} reversed phase [258]. Excellent resolution of all four diastereoisomers was obtained from plasma samples. Pharmacokinetic studies of pindolol enantiomers as GITC derivatives have also been reported [259].

The use of isocyanates for forming diastereoisomers has been widely reported. Atenolol enantiomers were separated after derivatisation with *S*-(−)-α-methylbenzyl isocyanate, separation on Ultrasphere ODS and fluorimetric detection [260]. Pindolol enantiomers, after extraction and derivatisation with the same reagent, were also separated on Ultrasphere ODS [261]. Betaxolol optical isomers were resolved after reaction with *R*-(−)-naphthylethylisocyanate (NEI), an agent which increases fluorescence intensity; chromatography was capable of complete enantiomeric resolution in under 15 min [244]. It was noted that derivatisation

was complete in approximately 60 min at room temperature: however, increasing the reaction temperature to 60°C decreased recovery Phenylethyl isocyanate (PEIC) reacted rapidly with metoprolol to form diastereoisomers, which were chromatographed on C_{18} silica using a simple methanol–water eluent [262]. With (fluorimetric) detection limits of approximately 2 ng/ml per peak, the procedure was suitable for human pharmacokinetic studies using blood or urine. Similar success was obtained with propranolol isomers; separation on C_8-reversed phase achieved an on-column sensitivity of 100 pg from as little as 100 μl of serum [263].

One of the problems associated with PEIC is that its optical purity is not 100%. (−)-MCF is readily available at 100% purity and its efficiency as a chiral derivatising agent was compared against that of PEIC Applying the technique to the analysis of flavodilol, MCF appeared to offer advantages over PEIC [264]. Mehvar [265] used the reagent to separate the enantiomers of several β-blockers In these applications of MCF, conditions were carefully controlled to ensure that only the secondary amino group was derivatised by the reagent; reaction with phenolic groups (*e.g* labetalol) must also be considered.

8.4. Chiral mobile phases

Optical isomers are separated as diastereoisomers formed of the reaction of the solute material with chiral modifiers added to the mobile phase. This is a less frequently used approach, though is does obviate the requirement for the purchase of chiral columns and avoids the potential pitfalls of derivatisation. A general review on CMP additives was recently reported [266].

Cyclodextrins have been used widely for this purpose, however, as discussed above their potential is limited because they are considered to exhibit relatively low stereoselectivity for β-blockers [266], though an application in TLC was recently described (see Section 8 5). A modifier which was used successfully for chiral separation of β-blockers is D-10-camphorsulphonic acid (CSA). This compound has been used successfully over a number of years [227] and more recently Gupta *et al.* [267] preparatively separated propranolol isomers on a CN-bonded column eluted with a normal-phase solvent system composed of hexane–dichloromethane–acetonitrile (79.20.1), modified with *tert.*-butylamine (2.5 m*M*) and CSA (5 m*M*) In the absence of the competing base no optical resolution was obtained, nor if a C_{18} reversed-phase mode was selected, however, retention time for complete resolution was over 2 h under the conditions described. The authors also prepared optically active propranolol derivatives for use as stereoselective haptens for radioimmunoassay agents. To achieve complete resolution of isomers a switching valve was incorporated into the chromatographic system to recycle the column eluate back through the column, a process which took approximately 8 h for complete resolution. Native hapten isomers were recovered from their CSA ion pairs by disruption with sodium hydroxide and salting out the free bases with sodium chloride. Their chemical and optical purities were confirmed separately.

Preparative and analytical-scale separations of β-blocker enantiomers were reported and compared on Spherisorb S5W Silica (Phase Separations, Norwalk, CT, U.S.A.) and LiChrosorb Diol (E. Merck), using (+)-tartaric acid as the pairing ion [268]. Solutes were eluted from both columns in a hexane–isopropanol mobile phase modified with the ion-pair agent and competing base The contribution of the competing base to the separation was assessed (compare with observations of Gupta *et al.* above [267]) and it was concluded that solute–ion-pair complexes were subject to mixed retention mechanism. Native silica was shown to have a greater loading capacity and selectivity than the bonded diol phase and was chosen for preparative separations.

Another counter ion which has found successful application is N-benzoxycarbonyl-glycyl-L-proline (ZGP). This ion-pair agent offered clear improvements in selectivity over CSA [269] and retention could be regulated by adjustment of the concentration of competing base [270]. It was successfully applied to the resolution of enantiomers of a number of β-blockers extracted from plasma and urine on normal-phase bonded silica [269,271].

8.5. Thin-layer chromatography

The foregoing discussion holds true for TLC and other planar techniques. It is true that CSPs for TLC are limited, however, where good resolution of optical isomers is anticipated, TLC offers its usual advantage of potential for rapid sample throughput and preparative possibilities.

The application of TLC to chiral separations is limited and only three reports of the determination of β-blocker enantiomers were found in the recent literature. Pflugmann *et al.* [272] used *S*-(+)-benoxaprofen as a derivatisation agent for metoprolol, oxprenolol and propranolol extracted from urine Chromatography of diastereoisomers was undertaken in normal-phase mode on Kieselgel G plates (E. Merck) and quantitation was achieved by measurement of fluorescent intensity. The limit of sensitivity was 100 ng/ml drug in urine. Several β-blockers were separated as their diastereoisomeric *R*-(−)-1-(1-naphthyl)ethyl urea derivatives after treatment with *R*-(−)-NEIC. Separation was achieved on silica gel HPTLC plates eluted with methanol–water mixtures [273].

β-Cyclodextrin has been reported as mobile phase additive capable of achieving the partial separation of labetalol enantiomers in reversed-phase TLC [274].

8.6. Conclusions

Chiral chromatography is playing an increasingly important role in the separation of racemic drug substances, including β-blockers. It is likely that chromatographic methods may play a preparative as well as an analytical function. Some of the pitfalls of enantioselective analysis of chiral drugs were recently highlighted by Caldwell *et al.* [275]. These include a lack of optically pure standards (partic-

ularly for metabolic studies), differentiation of enantiomers during sample work-up and those problems already mentioned in the text. The requirement for rigorous method validation is emphasised. The future promises substantial technical development in this field to support potential advances in the requirements for chirally selective drugs.

9 NEW DEVELOPMENTS

Two new areas of separation are likely to make some impact on pharmaceutical analysis in the next decade, capillary zone electrophoresis (CZE) and supercritical fluid chromatography (SFC). The merits of these techniques have been discussed elsewhere, however, some relevant applications have already been reported.

SFC has had some limited application to pharmaceutical analysis [276–278]. One of the advantages that the technique has over HPLC is that its more favourable kinetic conditions may allow greater resolution at higher flow-rates. The improvement of separating efficiency is of particular importance in the resolution of enantiomeric compounds. Steuer *et al.* [279] applied packed-column SFC to the separation of β-blocker enantiomers by the addition of a chiral ion-pairing agent (Fig. 6), N-benzoxycarbonylglycyl-L-proline and triethylamine (competing base), to the carbon dioxide–acetonitrile mobile phase. Niessen *et al.* [280] recently summarised potential applications of SFC in bioanalysis.

Although not applied to β-blockers, CZE has been described for the sep-

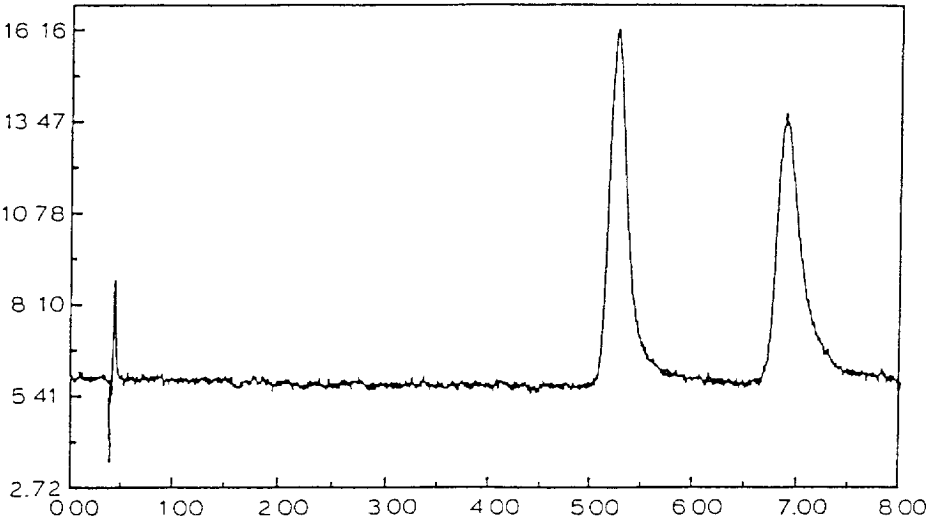

Fig 6 Resolution of propranolol enantiomers by supercritical fluid chromatography Conditions carbon dioxide, subcritical 21°C, 250 bar, 35 m*M* ZGP, 5 m*M* triethylamine, column, 100 mm x 4 6 mm Type CS-GU (Brownlee Labs , Santa Clara, CA, U S A) (Reproduced from ref 279 with permission)

aration of optical isomers of some sympathomimetic drugs [281]. Various cyclodextrins were assessed as chiral modifiers to the background electrolyte; high efficiencies and short run times (< 5 min) were achieved by the use of a relatively short capillary with small I.D. (20 cm x 0.025 mm). The technique, which has already been applied to a limited range of pharmaceuticals [282], has potential application to β-blockers.

10 SUMMARY

The determination of β-blockers has posed pharmaceutical analysts with a variety of problems arising from the essential characteristics of these compounds as bases and the variability of physicochemical properties of individual drugs.

Liquid chromatography has become the favoured method of analysis and to a certain extent there is a standardised approach to analysis based on either solvent or solid-phase extraction and reversed-phase high-performance liquid chromatography coupled to fluorescence detection The analyst must be aware of interactions occurring during extraction stages. All manipulations should be fully evaluated for individual drugs and metabolites prior to use. Other analytical options are chosen for specific or more demanding applications. The use of unmodified silicas for the liquid chromatography of β-blockers (and other basic drugs) is an example of a potential alternative mode of chromatography

The stereoselectivity of the pharmacology of β-blockers has spawned a great deal of literature describing the resolution of enantiomers by chromatographic methods. It is envisaged that this area will achieve greater prominence in the future as drug development pursues optical purity. The demand for the availability of enantiomerically pure pharmaceutical preparations will certainly see developments for preparative-scale separations as well as analytical methods and will surely promote developments in new and established methods of chromatography.

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