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REVIEW

DRUG LEVEL MONITORING: CARDIOVASCULAR DRUGS

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1. INTRODUCTION

The development of new drugs within the cardiovascular field has been tremendous over the last twenty years, with several new principles as a result. As the understanding of the biochemical mechanism of action for some compounds has increased over the years, the introduction of more selective and efficient drugs has followed. Under the influence of this process many older drugs have been reevaluated and returned to clinical use, with a better understanding of the interactions between the drug and the human body.

In this process of development, measurements of concentrations of drugs in biological fluids have played an important role. In order to elucidate the fate in the body of a given compound in a particular formulation or to evaluate the effect of a dose to a group of patients, sensitive and selective assays are needed. Some cardiovascular drugs have narrow therapeutic ranges, which makes drug monitoring essential.

In this review various pharmacological groups of cardiovascular compounds will be dealt with from a bioanalytical point of view. The techniques available and their development will be discussed and evaluated. In the selection of compounds we have been influenced by our own experience in this field and by the therapeutic and clinical interest in Sweden for this important group of drugs reflected by the compilation of registered pharmaceutical specialities in Sweden, the FASS [1]. In addition, some compounds not mentioned there have been included merely because of observations of bioanalytical interest. It is our hope that this review will give the reader an idea of the major problems in the determination of cardiovascular drugs and the solutions thereof as presented in the literature and also as experienced in our own laboratories.

1.1. Abbreviations

In this review the following abbreviations are used: BSA = bis(trimethylsilyl-acetamide), BSTFA = bis(trimethylsilyltrifluoroacetamide), C.V. = coefficient of variation, ECD = electron-capture detection, EI = electron impact, EMIT® = enzyme-multiplied immunoassay technique, FID = flame ionization detection, FPIA = fluorescence-polarized immunoassay, GC = gas chromatography, HFB(A) = heptafluorobutyryl (anhydride), LC = (column) liquid chromatography, MS = mass spectrometry, NDA = new drug application, NICI = negative-ion chemical ionization, NPD = nitrogen—phosphorus detection (nitrogen-selective mode), PFB = pentafluorobenzoyl, PFP(A) = pentafluoropropionyl (anhydride), RIA = radioimmunoassay, RRA = radioreceptor assay, SIM = selected-ion monitoring, TFA(A) = trifluoroacetyl (anhydride), TLC = thin-layer chromatography, TMS = trimethylsilyl, UV = ultraviolet.

2. BIOMEDICAL IMPORTANCE OF THE PROCEDURES REVIEWED

Bioanalytical procedures for cardiovascular drugs are of importance in mainly three areas: (a) therapeutic drug monitoring, (b) pharmacokinetic studies, (c) drug formulation studies (biopharmaceutical evaluations).

The requirements on the bioanalytical methods differ considerably depending on the concentration range of interest. Therapeutic drug monitoring usually means that the determinations are performed at a comparatively high concentration and extreme sensitivity is not often needed. Higher demands regarding precision and sensitivity in the results may be required in pharmacokinetic and biopharmaceutical studies, especially if the terminal phase in the elimination process has to be accurately determined. It is obvious that a more sophisticated instrumentation is often required for studies of various pharmacokinetic parameters, while in therapeutic monitoring simple bed-side tests have been used.

Among certain classes of the cardiovascular drugs the availability of sufficiently sensitive methods has been of utmost importance for checking and adjusting the drug therapy as a means of counteracting observed side-effects. One relevant example is the digitalis glycosides. Other drugs that have narrow therapeutic ranges and frequently require monitoring are, for example, quinidine and lidocaine.

The therapeutic response might be the result of the action of both the drug

and its active metabolites, which sometimes can be monitored by pharmacological measurements. Non-chromatographic methods used in the early days of bioanalysis sometimes suffered from interferences from a codetermination of metabolites. However, the possibility to determine the drug and the metabolites simultaneously would offer more detailed and reliable information. This became possible with the advent of chromatographic methods, as structurally closely related compounds can easily be separated. Today it is also recognized that optical isomers in many instances behave differently in the body and therefore in principal require separate determinations. This is possible with enantioselective chromatographic methods and may also be performed with immunoassays.

The criteria of modern analytical methods are thus selectivity and sensitivity. In several reports the use of modern methods including efficient separation columns and highly selective detection devices has resulted in extensive re-evaluation of earlier data on the pharmacokinetic properties of older drugs.

In the development of new drugs the evaluation of their behaviour in experimental animals and in humans does require the analysis of large numbers of samples. The number depends, of course, on the properties of the particular drug, but seems to be increasing. As an example it can be mentioned that in the new drug application (NDA) for metoprolol in Sweden in 1975, analytical data from approximately 5000 samples were included. This number covered studies over at least four years. However, even after registration (approval by authorities), continuing studies of a new drug and its clinical effect (Phase IV) require bioanalytical service. For such reasons the number of metoprolol samples analysed in one year in our laboratory is now more than 5000, ten years after launching.

For the development of biopharmaceutically optimized formulations it may be necessary to follow the drug concentrations from a large number of experimental preparations. Thus the biopharmaceutical requirements of drug level monitoring can be as large as those for the monitoring of clinical studies.

The role of the bioanalytical chemist in possession of reliable and accurate methods has thus been to participate in both the development of new active principles and in the improvement of older drug formulations leading to better and safer treatment of patients and a more rational clinical use of the drugs.

3. GENERAL ASPECTS ON THE PROCEDURES USED FOR THE DETERMINATION OF CARDIOVASCULAR DRUGS

Problems encountered in the analysis of cardiovascular drugs are very much the same as for other physiologically active compounds. However, some points which are more or less typical for this particular group of drugs need to be discussed here, even if they are mentioned under the individual drug or type of compound.

3.1. Sampling

Many cardiovascular drugs of amine character have been shown to bind to one particular plasma protein, α_1-acid glycoprotein (orosomucoid). This protein binding can be displaced by one plasticizer, tris-butoxyethyl phosphate.

found in certain stoppers of the Vacutainer® brand, which will influence the distribution of the drug in whole blood resulting in lower plasma concentrations. As examples alprenolol and lidocaine can be mentioned. An evaluation of several brands of blood collection tubes has been made [2] as well as recommendations as to how to study this effect [3]. This source of interference should be evaluated for all analytes that are lipophilic amines when using this kind of sampling device.

The binding to α_1-acid glycoprotein also reflects another question of importance in the analysis of blood samples for content of cardiovascularly active amines, viz. the need to analyse for free drug concentrations. In some cases this information might be more relevant to use. A discussion on protein binding for mainly cardiovascular drugs has recently been reported [4]. The techniques used to separate the unbound fraction from the protein-bound one are usually ultrafiltration and equilibrium dialysis. The regular use of these steps is not generally accepted and will add a lot of complexity to the analytical procedure, mainly because of the separation step, but also owing to the lower concentrations of drug obtained in the free fraction of strongly protein-bound drugs.

3.2. Work-up procedures

Most work-up procedures involve solvent extraction. In general this isolation step is not severely complicated by the biological material. However, for some highly lipophilic compounds the extraction process from plasma samples might be so slow that special attention has to be paid to this in the methodological studies. In recent years liquid—solid extraction has gained a lot of interest as an alternative for hydrophilic compounds, as well as for strongly hydrophobic ones.

Extraction studies which describe in detail the distribution properties of the analyte are not that common. Our impression from the literature is that the extraction procedure would have gained a lot if more thorough studies were performed at the beginning of the method development. That would, in most cases, make one single extraction possible instead of two or sometimes three which are prescribed in some methods. With appropriate construction and calculation of the extraction conditions (e.g. type of solvent, phase volume ratio, base vs. ion pair, etc.) it should be possible to obtain extraction procedures with improved recovery leading to better accuracy and precision. A recent book by Schill et al. [5] describes an approach for extraction studies that would be most valuable to follow in the design of bioanalytical methods.

The extent of the work-up procedure is very much dependent on the selectivity in detection. With less selective detectors clean-up steps such as back-extraction or combined use of base and ion-pair extraction might be necessary. However, a tedious work-up procedure reduces the number of analyses in a laboratory considerably. Moreover, the transfer of extracts, evaporation steps and other manipulations can each give rise to adsorption or other losses. One way to circumvent that problem is to use a single extraction into a small volume of the organic phase without further treatment.

3.3. Quantification

Gas chromatographic (GC) procedures very often rely on the use of internal standards structurally related to the analyte. It has been demonstrated several times that the introduction of the internal standard at the earliest possible step in the method is beneficial for the precision of the assay. In that way the analyte and the internal standard follow each other in all steps in the integrated analytical procedure, viz. extraction, purification, derivatization, separation, and detection. The closely related internal standard has also to be studied carefully, and the extraction properties and the derivatization reactions have to be considered in particular [6]. In the extraction step recoveries ought to be high (usually $>90\%$) in order to obtain sufficient precision. Variations in the recovery are as a rule larger in the medium range than at the high recovery level and cannot be compensated for by internal standards except for those that are isotopically labelled. The use of structurally closely related compounds is, of course, most pronounced in mass fragmentographic procedures, where stable isotopes are introduced in the otherwise identical compounds. The possibility of the mass spectrometric technique to differentiate between several species of the analyte with different degrees of labelling has in the last years been utilized in bioavailability experiments where in addition to the oral dose of the analyte a stable isotope labelled compound is given intravenously [7].

The benefit of isotope labelling has also been pointed out for the double radioisotope derivative technique which included ^{14}C-labelled internal standard, acylation with [^{3}H]acetic anhydride, separation by two-dimensional thin-layer chromatography (TLC) and scintillation spectrometry. This has been exemplified for oxprenolol [8].

Liquid chromatographic (LC) procedures with more accurate sampling techniques than in GC do not rely on internal standards to the same extent. This is because LC does not usually depend on precolumn derivatization reactions, and internal standards are then only used to correct for minor volume or detector variations.

An important point in the quantitative methodology that is not considered very much, is the method of preparing standard solutions. It is our experience that the use of plain aqueous solutions for salts of hydrophobic amines and acids should be avoided. The dilution of such standard solutions may lead to adsorption losses. This can be counteracted by the use of 0.01 *M* hydrochloric acid (for amines) and buffer solutions (for acids). Examples from the literature verify that [9].

3.4. Validation

Different validation procedures occur in connection with new analytical methods for cardiovascular drugs and for other compounds. They should comprise data on precision and accuracy as well as selectivity, but it is also important that the conditions for sampling, transport, and storage are evaluated. In our view the discussions on potential interferences in papers on the determination of cardiovascular drugs are often biased. More attention is paid to the interference from various other drugs, the presence of which is more unlikely in conjunction with the analyte, while their metabolites are neglected.

It must then be essential to recognize the behaviour of potential metabolites

of the drug itself. In this respect it would be better to report on interference studies from samples taken from patients under treatment, not only blank samples from healthy individuals.

It must also be emphasized that it is of minor value to validate a new selective chromatographic method against a non-selective and non-chromatographic one, although this has been done for years.

4. DIGITALIS GLYCOSIDES

In this group digoxin and digitoxin are discussed.

4.1. Immunoassays

The radioimmunoassay (RIA) technique for digoxin and digitoxin was developed by Smith et al. [10, 11] at the end of the sixties. Digoxin is the most used of these two drugs and through the years this particular assay of digoxin is probably the single drug analysis most performed around the world. The relative easiness of the method and its high sensitivity make it well suited for the digitalis glycosides for which there definitely is a need for drug monitoring. The therapeutic index is narrow, 0.7–3 nmol/l (0.5—2.0 ng/ml) for digoxin; the concentration level is about ten times higher for digitoxin. Toxic effects are correlated to high plasma levels and absence of effect at subtherapeutic levels. In the last five to ten years other immunoassay techniques for digoxin have appeared, such as enzyme-multiplied immunoassay (marketed as EMIT®) [12] and fluorescence-polarized immunoassay (FPIA) [13].

Radioimmunoassays for digoxin and digitoxin are both sensitive and rapid, two properties of great value for the therapeutic monitoring of such potent drugs. However, there has been some concern through the years regarding accuracy and reproducibility of digoxin determinations.

Great variations, up to 100%, between results from the use of RIA kits from different manufacturers have been reported [14]. Along with the variation given by different batches of kits from the same manufacturer, there is a need for careful standardization within each laboratory. Interlaboratory studies have been performed to determine the quality of the analyses [15].

Cross-reactivity is another problem which to some extent is dependent on the properties of the antibody reagent. Digitoxin has been found to cross-react in the RIA procedure for digoxin, while there are divergent reports for spironolactone [16]. Metabolites of digoxin formed by hydrolysis are as reactive as the parent compound towards the antibody but are also regarded to be as potent. For the dihydro metabolite there are reports of 33% cross-reactivity but also lower figures.

In the EMIT methods the antigen competing with the analyte is enzyme-labelled digoxin and the measurement technique used is photometry. This corresponds to ^{3}H- or ^{125}I-labelled digoxin and liquid scintillation counting in radioimmunoassay. A number of comparative studies of EMIT versus RIA have been reported [17, 18] where the simplicity, cross-reactivity and precision have been focussed.

In the last few years FPIA has appeared as another alternative for the determination of digoxin. This method includes protein precipitation prior to

analysis which often is performed by an Abbot TDx analyzer. The antigen tracer here is a fluorescein compound and the intensity of polarized fluorescence is measured. A lot of studies have recently been published evaluating this technique in comparison to RIA [13, 19].

4.2. Chromatographic methods

There are few routine chromatographic methods for the determination of digoxin and digitoxin in plasma due to the low levels and lack of detection possibilities. Digoxin was measured by GC—ECD as its HFB derivative [20] in a method comprising solvent extraction, purification through a silica column, and two TLC separations prior to the GC procedure. The method seems quite tedious and 10 ml of plasma are required.

Digitoxin in human serum (2 ml) was determined by a TLC method employing fluorogenic detection after reaction with hydrogen chloride vapour [21]. This method with a limit of determination of 3 nmol/l (2 ng/ml) gave results for authentic samples with a ratio of 0.47 (range 0.09—0.95) compared to a RIA method.

TLC was also employed for the determination of digitoxin and two of its major metabolites [22]. After extraction with dichloromethane the digitoxigenin compounds were separated from the digoxin ones by reversed-phase TLC. This was followed by separation, within each group, of the parent compound and its metabolites. The concentration of digitoxin was measured by ^{125}I RIA and the recovery was obtained from a [^{3}H]digitoxin tracer present. Radiolabelled digoxin and its metabolites were assayed after column chromatographic extraction and TLC [23]. A couple of LC methods for selective determination of digoxin in plasma have been published. Collected fractions were measured with either radioimmunoassay [24—26] or directly by liquid scintillation counting after administration of radiolabelled digoxin [27, 28]. By including the LC process 16% lower digoxin values in one study [25] and 26% lower values in another [26] were obtained compared with results from a standard RIA method.

Chromatographic methods for digoxin are by nature tedious, since the quantification has to be made off-line with RIA, and they are mainly used for pharmacokinetic investigations.

5. ANTIARRHYTHMIC AGENTS

5.1. Membrane-stabilizing agents

In this group the following compounds are discussed: ajmaline, amiodarone, aprinidine, bepridil, disopyramide, encainide, flecainide, lidocaine, lorcainide, mexiletine, procainamide, propafenone, N-propylajmaline, quinidine, stirocainide, and tocainide.

5.1.1. Sampling and work-up procedures

Systematic extraction studies in combination with chromatographic methods are sparse, although recovery data are frequently reported. A more systematic approach is highly desirable as this can lead to improvements in the overall procedure. Back-extraction into an aqueous phase and reextraction into a new

organic phase have been presented as a way to obtain cleaner extracts for GC analysis, but with the advent of more selective detectors these tedious and material-requiring procedures can as a rule be omitted.

The most used antiarrhythmic drugs, disopyramide, lidocaine, procainamide, and quinidine, are tertiary amines with similar lipophilic and chromatographic properties. This is reflected in the access of analytical methods comprising simultaneous determination of some or all of these. The possibility of mutual interference in the chromatographic systems is also discussed since they may be administered together. The major metabolites of the mentioned drugs, above all disopyramide and procainamide, are almost as potent as the parent drugs and are usually included in the analysis. In the extraction processes non-polar organic solvents like pentane, hexane, and benzene, are used in some instances to give comparatively pure chromatograms. However, low extraction recoveries are then obtained probably affecting precision and accuracy in the analysis. Recoveries in the range of 40—80% have been reported with such extractants for quinidine [29, 30], mexiletine [31], lorcainide [32, 33], and flecainide [34], but also with diethyl ether for mexiletine [35, 36], procainamide [37], disopyramide [38], and quinidine [39]. Higher recoveries are generally obtained with ethyl acetate, chloroform and dichloromethane as organic solvent.

A study on the liquid—liquid distribution of lidocaine, some of its metabolites and tocainide between aqueous phase and dichloromethane or toluene, has been reported [40]. This paper could serve as a model for how distribution data should be generated.

The extraction from biological material has usually not been a problem from a kinetic point of view. Extremely short extraction times, 5—60 sec, are used in methods for procainamide without any reason or explanation given. For aprinidine, a lipophilic bis-tertiary amine, it has, however, been reported that it might be rather slowly extracted [41], particularly at pH above 9. Bepridil, another bis-tertiary amine, resembles aprinidine structurally. A recent GC procedure (NPD) presented observations, where initially somewhat low recoveries (~ 70%) were encountered [42]. In order to obtain consistent peak height ratios between bepridil and the internal standard (a homologue), incubation of the latter with plasma was prescribed for 30 min. Moreover, dilution of plasma and addition of strong phosphate buffer were used. This observation was interpreted as an effect of differences in protein binding. The explanation might also be that the extraction process is slow [43]. For amiodarone quantitative recovery was obtained at pH 6.0 but only 21% at pH 9.0 [44]. Ion-pair extraction has been the only choice for quaternary ammonium compounds like N-propylajmaline.

A property in common for these amines seems to be their ability to adsorb to glass surfaces, etc., during the work-up procedure, especially when non-polar solvents are used, and in the evaporation process. This phenomenon has mostly been neglected, owing to the fact that therapeutic concentrations are rather high (μg/ml range), but for pharmacokinetic studies at the terminal phase the problem should be more severe (ng/ml). One paper describes the addition of triethylamine as an adsorption inhibitor prior to extraction [45], another trimethylamine [34]. Other additives used in the evaporation process are hydrochloric acid [31, 46] and butanol [47]. Silanization of pipettes and

centrifuge tubes and use of polypropylene tubes are other measures taken. To avoid adsorption losses in the evaporation process, back-extraction into a small acidic aqueous phase and injection of an aliquot of this onto a bonded-phase LC column is employed in many methods.

Direct injection of an aliquot of the organic solvent used by Holt et al. [48] and Hayler and Flanagan [49] in a miniaturized extraction procedure is a useful alternative. In the methods for lidocaine and mexiletine [48], and disopyramide [49] a volume of 200 μl of serum is extracted into only 50 μl of chloroform. No transfer or evaporation steps are used, thus reducing the possibilities for adsorption losses. An almost similar approach was used for aprinidine [50], as well as for disopyramide [51], procainamide [47], and for aprinidine and quinidine by Lagerström and Persson [41].

Liquid—solid extraction in small disposable columns has been used as an alternative to solvent extraction to isolate the drug compounds from the plasma sample. Low, and in some instances varying, recoveries are reported for lidocaine [52], flecainide [53], and disopyramide [54], probably because of a less straightforward design of the cartridge extraction procedures.

Protein precipitation prior to injection onto a bonded phase LC column is exemplified for procainamide [55—57], amiodarone [58], flecainide [59], and quinidine [39, 60—62]. Surprisingly high recoveries are reported in all cases but one [59], where a loss of 35% of flecainide in the precipitate is noted. Direct injection of plasma samples is suggested in a few papers for lidocaine [63], and procainamide [64].

The displacement of drugs from one particular plasma protein, α_1-acid glycoprotein, by a plasticizer (tris-2-butoxyethyl phosphate) in the rubber stoppers of Vacutainers has been observed for antiarrhythmic compounds such as lidocaine [65] and quinidine [66].

Quinidine, disopyramide, and lidocaine are bound to α_1-acid glycoprotein to such an extent that it is of great interest to monitor the free drug levels [67]. This adds somewhat to the complexity of the bioanalytical procedure, but in one paper an ultrafiltration kit is used in conjunction with an enzyme immunoassay and can thus be rather rapidly executed [67].

Other interactions with the biological sample have been observed for tocainide [68]. It was found that higher extraction yields from patient plasma were obtained as well as better precision in the final yields if, before the extraction into dichloromethane, the sample was treated for 30 min with hydroxylamine. This implies that tocainide, a primary amine, is present not only in free form, but also as a complex or condensation product with some plasma components. This complex can be broken in a competitive way with the addition of hydroxylamine [68].

5.1.2. Chromatographic methods.

5.1.2.1. Gas chromatography. Most compounds in this group are tertiary amines with additional functional groups such as amides and/or heterocyclic rings, which restricts the possibilities of quantifying trace amounts of the compounds by gas chromatography. Ten to fifteen years ago, before the advent of modern liquid chromatography, there was much interest in GC methods.

Liquid chromatography is preferred for most of the antiarrhythmics used today, especially those that cannot be masked to less polar entities.

(a) Direct methods. The determination of therapeutic levels of these drugs was early recognized as important. As the levels are usually in the low μg range, it has been possible to use gas chromatography with flame ionization detection, e.g. for lidocaine [69, 70] and quinidine after flash methylation [71]. Better selectivity and lower determination limits, from 80 nmol/l (20 ng/ml), were obtained with the introduction of the nitrogen-selective detector (NPD) ("alkali flame ionization"). The modern versions with electrically heated alkali metal containing beads have made the methods reliable and rugged for lidocaine [72—77] and for disopyramide [78—82]. Only one compound, lorcainide, is substituted with an electrophore group rendering it suitable for electron-capture detection (limit of determination ~30 nmol/l, 10 ng/ml) [83]. The major problem with direct analysis of these compounds by GC has been their polar nature. Low-level analysis is thus severely hampered by adsorption to various surfaces, not least those of the chromatographic system. The adsorption tendency among these compounds seems to increase in the series tertiary < secondary < primary amine. One example can be found in the standard curves from a SIM assay of lidocaine and its monodeethylated metabolite, where the latter was more prone to adsorption [84].

In the chromatographic column polar phases have been recommended frequently. For packed columns the combination of polyglycols with various amounts of potassium hydroxide has often been used [74], as for lidocaine for the quantification of levels from 400 nmol/l (100 ng/ml) upwards [71]. Phenyl-substituted polysiloxanes and polyester phases come next [72, 73]. One paper refers to the use of OV-101 and deactivation daily (or weekly depending on substance) with the injection of a γ-glycid-oxypropyltrimethoxy-silane. Lidocaine, disopyramide and tocainide have been assayed with this approach [48, 49, 85]. Mexiletine has been determined with the combination Apiezon L and potassium hydroxide [48].

For primary amines like mexiletine the combination of chloroform as solvent and polyglycol plus potassium hydroxide as stationary phase has been reported to result in on-column degradation to the corresponding isonitrile [86].

Procainamide has three nitrogen-containing functional groups, and rather high column temperatures are required to elute this agent and its N-acetylated metabolite. This excludes the use of polyglycols, alone or in combinations. A low-load OV-17 column (0.75%) was used at 270°C for procainamide and the metabolite in urine [87], but for plasma samples procainamide interfered with endogenous compounds. The limit of determination was 4 μmol/l (1 μg/ml). Procainamide has also been measured in a procedure where 0.2% OV-17 was applied on textured glass beads at 225°C [88]. It was claimed that this resulted in lower column adsorption.

In a paper on the determination of disopyramide (tertiary amine, primary amide, and pyridine) and its N-desisopropyl metabolite, the latter was acetylated [89]. Calibration graphs with *p*-chlorodisopyramide as internal standard were curved, a fact that was said to depend on incomplete acetylation at lower concentration. A more probable explanation is that the compound with the tertiary amine moiety adsorbs more than the acetamide derivative.

Surprisingly enough, only a few papers deal with the use of capillary columns for analysis of this group of compounds. Lidocaine and the trifluoroacetylated active metabolite were determined on a OV-17 capillary column at 190°C with excellent results [47]. A new antiarrhythmic compound, stirocainide, was quantified on a fused-silica column, with a limit of determination at 10 nmol/l (2 ng/ml) [90]. Recently a study was presented where a couple of antiarrhythmic compounds, lidocaine, disopyramide (derivatized), quinidine, and tocainide, were separated on a fused-silica column [81]. In recent times the development of inert and thermostable capillary columns has been strong [91], making it possible to handle smaller quantities than before, which would be favourable for this technique.

(b) Methods with derivatization. Two congeners of lidocaine, both active after oral administration, are the primary amines mexiletine and tocainide. For both of them direct GC analysis has been advocated as being simple. However, it is evident that such procedures are less reliable [92] and derivatization reactions are recommended for this particular group of compounds.

Acetylation and butyrylation reactions have been used for masking purposes in an assay of mexiletine [93]. When high selectivity and sensitivity in detection have also been the aim, perfluoroacylation and electron-capture detectors have been used. Mexiletine has been quantified with ECD only in a few studies [94—96]. One procedure determined one of the hydroxylated metabolites as well but was rather tedious [97]. As the levels are within the μg/ml range the nitrogen-selective detector seems to be as popular to use [93, 98—100]. No problems seem to be involved in derivatization of mexiletine.

The structural moiety found in tocainide with the amide group of lidocaine instead of the ether bond in mexiletine makes this compound more prone to side-reactions. This has been observed in connection with derivatization for ECD using HFBA. The reaction performed at 55°C in hexane was peculiar in that a fairly narrow maximum yield was observed between 45 and 55 min [101]. Later on Pillai et al. [102] reported slight modifications in the derivatization process, but still with the observation that longer reaction times should be avoided. The method had a limit of determination at 2 μmol/l (0.5 μg/ml). A reason for this behaviour upon treatment with HFBA was recently proposed [103]. It seems that the structural units of tocainide are sensitive to concentrations of the perfluorinated anhydrides higher than 0.01% when in toluene. Several products appeared, the major of which were dehydrated. It is important to note that this degradation does not appear with transacylating reagents such as HFB imidazole or bis-trifluoroacetamide. The former has been evaluated in comparison with HFBA but found to be less reactive and giving lower yields. The early acylation reactions with tocainide used HFB or TFA imidazole with FID [104]. The dehydrating effect of the anhydrides depends on the solvent and seems to be lowest in non-polar solvents like hexane. A recent study on TFAA-reacted antiarrhythmic compounds by GC on fused-silica columns included tocainide [81]. A dehydration cannot be excluded. Mexiletine does not undergo this reaction [105]. A reaction observed for tocainide is the formation of a hydantoin in a ring closure reaction with phosgene in dichloromethane [68]. This hydantoin compound has, moreover, been proposed to be a metabolite, but might also appear as a metabonate [106].

Another approach to improve the chromatographic properties of tocainide was demonstrated in a study by Johansson and Vessman [68] where Schiff base formation was exploited with nitrogen-selective detection. The reaction with methylisobutyl ketone was performed at 90°C for 10 min. The reaction mixture was cooled to room temperature to stop further reactions of the derivative. This quantitative procedure with a limit of determination of 0.5 μmol/l (0.1 μg/ml) correlated fairly well with an LC assay. It would also be possible to determine mexiletine using this approach.

Flecainide is a secondary amine that has been quantified with ECD after derivatization [53]. The choice of reagent, pentafluorobenzoyl chloride, permitted derivatization in the aqueous phase, which in this case occurred after prior extraction of the base. The high column temperature required for the PFB derivative would have made the PFP or HFB derivatives interesting to study. However, the temperature dependence of the ECD response could also be of importance to consider. The method compared fairly well with an LC assay in the range 400—3000 nmol/l (100—800 ng/ml).

Disopyramide is known to undergo degradation on-column. This has been postulated to depend on the primary amide moiety. Gal et al. [80] used the possibility to convert the primary amide to a nitrile by dehydration with TFAA, as has also been used for other drugs with that polar group [107, 108]. The resulting nitrile had much better chromatographic properties and required a lower column temperature. The limit of determination was set at 3 μmol/l (1 μg/ml). The problem of degradation is more pronounced for the N-desisopropyl metabolite with sometimes three peaks appearing [78]. Treatment with TFAA is important for the active metabolite as well, which was recently reported in an interesting study by Kapil et al. [81]. They used a fused-silica column for the separation of disopyramide, the N-desisopropyl metabolite and the *p*-chloro-substituted internal standard and obtained single symmetrical peaks for each compound.

(c) Separation of enantiomers. Recent publications on the determination of tocainide have used capillary columns, especially for the separation of the enantiomers. Although the first report by Gal et al. [109] made use of methoxytrifluoromethylphenacetyl chloride as chiral reagent with NPD and OV-17 in a packed column, a later modification described the use of more efficient capillary columns [110]. It was also demonstrated that this derivative has electrophore properties, making ECD possible. The structure of tocainide contributes to this as the derivative of amphetamine (primary amine only) gave no special response.

Recently, the use of chiral stationary phases for the separation of enantiomers of tocainide as derivatives with HFB has been described in two papers [105, 111]. Optimization of the HFB acylation was discussed including data on how the resolution depends on the type of perfluoroacylation agent (HFB = PFP > TFA) [105]. The dehydration effect was observed for all three anhydrides.

5.1.2.2. Selected-ion monitoring. For quantification of both lidocaine and the monodealkylated metabolite, SIM was used very early in the era of quadropole instruments by Strong and Atkinson [84]. The limit of determination was

in the same range as for other GC procedures, mainly owing to the column performance. An interesting and elegant approach, which included the two dealkylated metabolites of lidocaine, was described some years later [112]. To improve the chromatographic properties as well as the extraction behaviour, the metabolites were derivatized in a reductive alkylation reaction to form the mono- and dipropyl analogues of lidocaine. The relative standard deviation in the determination of the three compounds in that assay was better than 10% at the 200 nmol/l (50 ng/ml) level.

A stable isotope dilution assay has been reported for disopyramide and its $^{13}C,^{15}N$-labelled analogue in biological fluids with the aim to evaluate the bioavailability of the drug [113]. A $^{2}H_{14}$-labelled internal standard was used and the mass spectrometer was operated in the chemical ionization mode with ammonia as reagent gas. Similarly, Strong et al. [114] described a stable isotope method (methane chemical ionization) for the determination of absolute bioavailability of N-acetylprocainamide in man. The advantage of using simultaneous administration of ^{13}C-labelled drug (intravenous) and capsules (per os) for bioavailability studies was clearly demonstrated. A later paper reported on the simultaneous monitoring of procainamide and ^{13}C-labelled N-acetylprocainamide with the possibility to study the kinetics of both compounds in only three subjects [115].

Ajmaline, a bis-tertiary amine, and the pro-drug, 17-monochloroacetylajmaline, have been quantified with a GC—MS method using silylation to improve the GC properties [116]. The 17-ester was hydrolysed in the extraction procedure in a constant manner, making low levels of ajmaline less reliable to determine. The limit of determination was around 30 nmol/l (10 ng/ml) for both compounds.

Aprinidine, another bis-tertiary amine, has also been determined with GC—MS in the chemical ionization mode with ammonia as reagent gas [117]. The work-up included re-extraction, and amitriptyline was used as internal standard. The limit of determination was reported to be 30 nmol/l (10 ng/ml). The precision data varied between 4.4% and 11.2% in the range 300—1200 nmol/l (100—400 ng/ml), which seems somewhat high, possibly because of the non-structurally related internal standard.

5.1.2.3. Liquid column chromatography. A typical chromatographic system for the separation of antiarrhythmic drugs is composed of a stationary C_{18} bonded phase and a mobile phase of phosphate or acetate buffer of pH 2—6 with acetonitrile as organic modifier. Other bonded phases used are phenyl, C_8, nitrile, and cation-exchanger, and other organic modifiers are methanol and tetrahydrofuran. Control of the pH of the buffer solution should be of importance particularly for the divalent amines, disopyramide and quinidine, but is not considered very much. There are also examples of systems with very low buffer capacity which may contribute to a less satisfactory chromatographic performance. In a few instances ion-pairing sulphonates are added to the mobile phase [34]. Amine modifiers are not used to any great extent as means to improve the chromatographic behaviour of the compounds.

In quite a number of the LC methods for this group of drugs, bare silica is used as sorbent and stationary phase. A decent chromatographic performance is obtained by mobile phases containing either basic additives such as ammonia,

morpholine or ethanolamine, or acidic ones such as perchloric, acetic, camphersulphonic and hydrochloric acids. The stability of these systems is reported to be good even with those using alkaline mobile phases. With relatively non-polar mobile phases direct injection of the organic extract can be employed as shown by Flanagan et al. [44] and Lagerström and Persson [41].

As mentioned above, the major metabolites of some of the drugs in this group are almost as potent as the parent compound and must be separated and determined simultaneously; e.g. N-acetylprocainamide and the N-desalkyl derivatives of disopyramide, lidocaine, and lorcainide. For quinidine, the dihydro compound is always present as an impurity to the extent of 5—15% and must be separated. With the aim of developing general separation methods for all or most of these compounds, high resolution and column efficiency are needed. Besides, internal standards with closely related chemical structures also need space in the chromatograms. It is obvious that it is not possible to develop general methods. Interference between different compounds in this group has been reported and in some systems the separation of the internal standard from the parent drug is not complete enough to enable measurement of low concentrations.

Internal standards used are in most instances structurally closely related to the parent compound, such as cinchonidine and quinine for quinidine, the *p*-chloro derivative for disopyramide, the propionyl derivative and nitro or dipropyl analogue for procainamide, the methyl analogue for lidocaine, the positional isomer for mexiletene and the analogues for flecainide, tocainide and the amiodarone. For most of the antiarrhythmic drugs the therapeutic plasma level is in the μg/ml region. These comparatively high levels in combination with the high inherent UV absorbance of some of the most used compounds, disopyramide, procainamide, and quinidine, make the detection and sensitivity a minor problem. For these three compounds the eluent can be monitored at 254 or 280 nm or somewhere else in that region. Even for amiodarone, encainide, lorcainide and mexiletine, detection at 254 nm has been used although it is not optimal. Lidocaine and tocainide have to be monitored around 230 nm if a reasonable detector response is to be obtained.

Derivatization as a means of increasing the sensitivity has been used for mexiletine and tocainide, which have a primary aliphatic amino group useful for chromophore reagents. Dinitrofluorobenzene [35], Dns chloride [36, 118] and fluorescamine [119] have been used for photometric and fluorometric detection.

The inherent fluorescence of flecainide [53, 59, 120], mexiletine [36, 120], and quinidine [60, 62] has been utilized for selective and sensitive LC determinations (Table 1). The advantage of highly selective detection was demonstrated for the determination of quinidine in urine by direct injection of the sample into the liquid chromatograph [121]. Besides spectrophotometric detection in combination with liquid chromatography a recent paper demonstrated the use of electrochemical detection for lidocaine and its metabolites [52]. The electrode potential required for their oxidation was too high to mediate both ultimate sensitivity and good selectivity. As stated in the paper, these compounds are not ideal candidates for LC with electrochemical detectors. There has been a long tradition of monitoring plasma levels of some of the anti-

arrhythmic drugs, primarily procainamide and quinidine. The therapeutic indices have been known for some time and are rather narrow. For many years non-chromatographic methods based on fluorescence measurement have been available. In many papers presenting new or modified LC methods, these are validated against the non-selective fluorometric methods. Often the results from the two methods are linearly correlated with high regression coefficients. For quinidine some authors introduce a factor to correct the results from the fluorometric methods. However, looking at individual results there are divergencies of such a magnitude that it is not acceptable from an analytical chemical point of view. This approach to prove the value of new methods is not appropriate as pointed out by Guentert and Riegelman [122]. They have thoroughly investigated the influence of codetermination of metabolites on the results from fluorometric assay of quinidine [123] and also shown the effect on the measurement of pharmacokinetic parameters [124].

TABLE 1

LC METHODS FOR MEMBRANE-STABILIZING AGENTS IN PLASMA

Drug	Metabolites determined	LC column	Detection	Work-up procedure	Recovery (%)	Limit of determination nmol/l (ng/ml)	Reference
Amiodarone	No	Silica	UV 240	Extr.—inj.	98	70 (50)	44
	Yes	Silica	UV 240	Evap.	?	7 (5)	125
	Yes	C_{18}	UV 242	Precip.	100	40 (25)	58
Aprinidine	No	Silica	UV 254	Extr.—inj.	100	100 (30)	41
	Yes	C_{18}	UV 259	Evap.	96	60 (20)	126
Disopyramide	Yes	C_{18}	UV 254	Back-extr.	97	150 (50)	127
	Yes	Silica	UV 265	Extr.—inj.	100	50 (15)	51
	Yes	C_{18}	UV 254	Back-extr.	73	150 (50)	38
	Yes	CN	UV 210	Sep-Pak silica	55	150 (50)	54
Encainide	Yes	Silica	UV 254	Evap.	?	30 (10)	128
Flecainide	No	C_3	UV 308	Evap.	70	70 (22)	34
	No	Silica	F 200 exc.	Extr.—inj.	?	60 (20)	120
	No	Phenyl	F 300/370	Vac-Elut	70	10 (3)	53
Lidocaine	Yes	Phenyl	UV 200	Back-extr.	87	200 (50)	129
	Yes	Silica	UV 225	Evap.	94	800 (200)	130
	Yes	Silica	ECD	Sep-Pak C_{18}	76	2200 (500)	52
Lorcainide	Yes	Phenyl	UV 196	Back-extr.	40	30 (10)	32
Mexiletine	Yes	C_8	F	Evap.—deriv.	80	20 (5)	36
	No	C_{18}	UV 210	Evap.	73	400 (100)	131
	No	Silica	F 200	Extr.—inj.	?	200 (50)	120
Procainimide	Yes	C_{18}	UV 275	Back-extr.	95	1200 (300)	132
	Yes	Silica	UV 280	Extr.—inj.	90	1000 (25)	47
	Yes	Silica	UV 274	Evap.	92	400 (100)	133
	Yes	Silica	UV 254	Back-extr.	98	2000 (500)	134
Propafenone	No	CN	UV 209	Back-extr.	78	15 (5)	135
N-Propylajmaline	No	Silica	F 242/> 320	Evap.	95	10 (4)	136
Quinidine	No	Silica	UV 254	Evap.	100	50 (15)	41
	Yes	C_{18}	UV 254	Evap.	98	?	137
	Yes	Silica	UV 235	Evap.	92	60 (20)	123
	Yes	Phenyl	F 320/418	Precip.	100	50 (15)	60
	Yes	C_{18}	F 340/418	Precip.	100	150 (50)	62
Tocainide	No	NH_2	F 360/409	Back-extr.—deriv.	?	400 (100)	118
	No	Phenyl	UV 230	Evap.	69	1500 (400)	138
	No	C_{18}	F 395/485	Precip.—deriv.	92	400 (100)	119

Analytical methods for the different antiarrhythmic drugs are given in Table 1. This is a selection of different approaches used and not a complete list, but it can be used as a guide to published LC methods. The extent of interest in the determination of plasma levels of this group of drugs is illustrated by the fact that in a recent paper on determination of quinidine in serum [30] 75 references were given.

5.1.2.4. Thin-layer chromatography. Quantitative TLC in connection with densitometric evaluation of the separated spots has found some use for therapeutic monitoring of antiarrhythmic drugs. As scanning can be made in the UV range as well as in the fluorescence mode, the flexibility is good. Conventional plates have been used for the determination of quinidine [30, 139, 140] and procainamide plus the N-acetyl metabolite [141] with results that in many cases are reported to be superior to GC data. In the paper by Christiansen [139] on quinidine determination the tiny serum sample (10 µl) is put directly on the plate loaded immediately before with 10 µl of ethanol. This precipitates the proteins, whereupon the plate is dried before development. Scanning is made in the fluorescence mode. Other applications use solvent extraction as for quinidine [140] and procainamide [141] and detection in the UV range. Fluorescence scanning has been used for ajmaline [142], quinidine [30], and disopyramide including its active N-dealkylated metabolite [143]. In some cases acidification of the layer to induce fluorescence was necessary before scanning.

Conventional TLC with fluorodensitometric detection of quinidine was compared with direct spectrofluorometry and after extraction, with EMIT, and with LC [30]. In many respects TLC was advantageous to use according to the author. With the advent of smaller plates with finer particles (as in LC) the performance has been improved. The term high-performance TLC has been coined. Applications have been described where antiarrhythmic drugs (lidocaine, disopyramide, propranolol, and procainamide) have been separated and quantified in a two-step development [144]. In a recent study instrumental TLC was used for the determination of disopyramide and the N-desisopropyl metabolite [145]. With an internal standard technique it was possible to determine between 2 and 30 µmol/l (0.5 and 10 µg/ml) of disopyramide with a precision comparable with GC. New chromatographic supports and new detectors make this technique interesting for therapeutic monitoring.

5.1.3. Non-chromatographic methods

5.1.3.1. Mass spectrometry. Lidocaine and quinidine were among the first drugs to be determined by direct inlet chemical ionization mass spectrometry and stable isotope labelling. In the procedure by Garland et al. [146] the plasma samples from lidocaine-treated patients were spiked with deuterated analogues of lidocaine and monoethylglycine xylidide, then made alkaline and extracted with benzene. The residues upon evaporation were placed on the probe and inserted. The drugs could readily be quantified from the spectrum within 5 min. This very powerful technique can thus determine several drugs and metabolites simultaneously. The drawback is, of course, the expensive stable

isotope-labelled internal standards as well as the possibility that isomeric compounds are codetermined. The dynamic range of detection was claimed to be from 0.02 to 16 μmol (5—4000 ng) for lidocaine, which is among the most sensitive approaches available. This work was extended later on to lidocaine plus six of the known metabolites [147]. Quinidine and dihydroquinidine were quantified in a similar way [146]. It was observed that the plasma quinidine values were lower than those of a fluorescence assay performed on the same sample.

5.1.3.2. Fluorometry. There are a number of compounds which have been determined fluorometrically, but where the results are biased because of metabolic interferences. Procainamide and N-acetylprocainamide have, however, been determined selectively at therapeutic levels with adjustment of excitation and emission wavelengths as well as pH value of the samples [148].

Flecainide has been determined fluorometrically with the limit of determination at 75 nmol/l (25 ng/ml) [149]. This fairly simple and rapid method (extraction and back-extraction) compared favourably with a GC assay. Fluorescent drugs such as propranolol and quinidine interfered, of course. For therapeutic monitoring in the non-research situation this was judged as sufficient.

5.1.3.3. Radioimmunoassay. In this group, aprinidine and encainide have been quantified with radioimmunological methods. A study of possible interferences with aprinidine revealed that two metabolites might displace the tritiated tracer from the antibody [150]. However, in the studies on plasma samples from dogs they did not interfere. The RIA method could determine 30 nmol/l (0.01 μg/ml) in plasma (without extraction), which is about ten times better than the GC method [6]. A comparison showed good agreement between the two approaches.

Encainide was analysed in a procedure where iodination of an encainide-tyramine derivative with the chloramine-T procedure was used [151]. This method was able to quantify 0.6 nmol/l (0.2 ng/ml) in plasma or urine. Plasma samples were extracted with ethyl ether at an alkaline pH which excluded two metabolites. The third one, an N-desmethylated compound, was usually not present in sufficient amounts to interfere, although the cross-reaction was 18%. The method compared very well with an LC method, which also detected the interfering metabolite. Its contribution in RIA was then estimated to be less than 5%, although the amount was about 20%.

5.1.3.4. Enzyme immunoassay. The interest in therapeutic drug monitoring of cardiovascular drugs, especially among antiarrhythmic ones, has been demonstrated in a number of so-called "bedside tests", intended to be used close to the patient with rapid delivery of the analytical result. Those based on a coupled enzymatic immunometric reaction in a homogeneous system have found widespread use and are marketed as EMIT®. In one reagent, antibodies towards the drug, and a substrate for the enzyme glucose-6-phosphate dehydrogenase are present. This is mixed with the drug-tagged enzyme in another

solution. The activity of the drug-labelled enzyme will be influenced depending on the amount of free drug present in the sample. Two absorbance measurements after 15 and 45 sec give a difference in reading which is inversely related to the amount of drug present as calculated from calibrator samples.

The procedure for lidocaine has been compared with GC procedures with good results [152, 153]. Several other cardioactive drugs have been studied for potential interference but none has given positive results nor did the monodeethylated metabolite. The EMIT kit is intended for use in the therapeutic range, but a slight modification has been described which will permit pharmacokinetic studies to be performed down to 200 nmol/l (50 ng/ml). This procedure was also validated with a GC method [154]. The specificity for intact lidocaine is, of course, an essential requirement. Yet, for patients on lidocaine therapy monitoring of the active metabolite is of interest, as it can appear in concentrations from one tenth to one sixth that of lidocaine. The lack of information on active metabolites points to a drawback for this elegant but expensive approach. The use of centrifugal analyser systems with smaller reagent volume requirements has been reported for quinidine [155]. This could reduce the costs.

Some other antiarrhythmic agents can at present be quantified with the enzyme immunoassay in methods which have been evaluated with chromatographic methods. The EMIT methods include procainamide and its N-acetylated metabolite [156], disopyramide [82], and quinidine [157]. In connection with these assays the interest can be mentioned in determining free drug levels of some compounds, like lidocaine, disopyramide, and quinidine [4]. An ultrafiltration device is then possible to use in conjunction with the sampling procedure [10].

5.1.3.5. Fluoroimmunoassay. A recent publication deals with the evaluation of a fluorescence immunoassay for total and unbound serum concentrations of disopyramide [158]. The method compared favourably with an LC method. The unbound fraction was obtained with equilibrium dialysis which is tedious. However, it has been reported that this approach compares well with ultrafiltration [159], thus making it possible to rapidly obtain the therapeutically more relevant concentration [158]. The fluoroimmunoassay did not distinguish between the enantiomeric forms of disopyramide.

5.2. Other agents

Bretylium and clofilium are two quaternary ammonium compounds that have been used because of their antiarrhythmic properties. Work-up procedures have to be based on their ionic character as both compounds always carry a positive charge. Initial isolation can be made with ion-exchange columns, but the method of choice for this particular type of compound is ion-pair extraction.

5.2.1. Chromatographic methods

Bretylium has been isolated as an ion pair with triiodide and dealkylated in the Jenden procedure with sodium thiophenolate [160]. The produced thio ether, an *o*-bromo-substituted compound, could easily be detected with GC—ECD with a limit of detection below 3 nmol/l (1 ng/ml).

Clofilium was transformed to a volatile derivative (a tertiary amine) in a Hofmann elimination reaction in the injector of the gas chromatograph [161]. The dealkylation took place in 0.01 *M* methanolic potassium hydroxide at a high injector temperature. Quantification was then made with GC—MS and had a limit of determination below 75 nmol/l (25 ng/ml).

6. β-ADRENOCEPTOR ANTAGONISTS

In this group the following drugs are discussed: acebutolol, alprenolol, atenolol, befumolol, betaxolol, bevantolol, bopindolol, bufuralol, butofilolol, carteolol, celiprolol, diacetolol, esmolol, mepindolol, metoprolol, moprolol, nadolol, oxprenolol, penbutolol, pindolol, practolol, propranolol, sotalol, talinolol and timolol.

6.1. Sampling and work-up procedures

More than twenty different β-adrenoceptor antagonists are included in this group. Even if they have structural features in common they differ more than 1000 times in polarity (distribution constant) going from the hydrophilic atenolol to the most lipophilic one, propranolol. From that reason it is not practical or optimal to use the same extraction procedure or solvent for all compounds. Attempts have been made by Walle [162] who ran into problems when extracting substances with low lipophilic character, like practolol and sotalol, and by Lefebvre et al. [163] who demonstrated a method for eight β-adrenoceptor antagonists.

The extraction procedure has to be adapted to the kind of detection device that is to be used. By using mass spectrometric detection one can allow for a more unspecific extraction method than when using electron-capture or nitrogen-selective GC detectors, or UV and fluorescence LC detectors. As all β-adrenoceptor antagonists are weak bases they are extracted from the biological sample into organic solvents generally at pH >11. In some cases where phenolic metabolites are to be coextracted, extractions are performed at pH ≈ 9.5. There seems to be no best choice of solvents, as all common extraction solvents are used either alone or in combination. Lo et al. [164] compared different organic solvents or solvent mixtures for the extraction of propranolol and 4-hydroxypropranolol and preferred diethyl ether, while Lefebvre et al. [163] for extraction of eight β-andrenoceptor antagonists ranging from propranolol to atenolol in lipophilic character chose chloroform—pentanol (3:1, v/v) as the best extractant. In LC methods back-extraction to an aqueous phase is often included, this provides better clean-up and adequate concentration. Lefebvre et al. [163] compared different acids and selected 0.1 mol/l sulphuric acid as the best in conjunction with chloroform—*n*-pentanol. However, they overlooked the fact that amines like β-blockers can be extracted as ion pairs with chloride, hydrogen sulphate and acetate ions from acidic media to organic solvents.

In order to obtain a quantitative back-extraction to the aqueous phase it is not suitable to use chloroform—*n*-pentanol (3:1) as extraction solvent for all the compounds studied. Even the moderately lipophilic metoprolol, oxprenolol and pindolol form extractable ion pairs too readily in the back-extraction,

giving low recoveries in that step (38—68%). It would be preferable to use a less polar medium such as dichloromethane or diethyl ether in the first extraction, alternatively add a large volume of hexane in the back-extraction step to decrease the ion-pair extraction. Pre-extraction of the acidified sample to remove neutral and acidic compounds [165] may for the same reasons give rise to losses of the analyte.

Recently work-up procedures including solid-phase extraction in small disposable reversed-phase columns have become popular both in LC and GC methods. Analytichem's Bond-Elut [166—168] and Waters Assoc. Sep-Pak are the most common. They are suitable for polar compounds by increasing the recovery compared with solvent extraction. Furthermore, formation of emulsions is avoided. Most often, however, an evaporation step is included, which means that the method is not too fast.

A simple work-up procedure in LC is protein precipitation. Lo and Riegelman [169] used addition of acetonitrile and evaporation of the supernatant to a small volume. Albani et al. [170] added acetonitrile and a mixture of sodium chloride and sodium carbonate to form a two-phase system, of which the acetonitrile-rich phase was injected. The methods may appear simple, but suffer from the fact that the injected samples are not very clean, leading to increased deterioration of the LC columns, unless small sample volumes are introduced. In this case the limit of determination may be too high. When possible, solvent extraction is preferred in order to increase the life-time of the columns.

A fully automated LC method has been presented by Lecaillon et al. [171], who used a column-switching technique with three different columns. Plasma was diluted with water and injected onto the first column (large particles) and rinsed with water. The sample was then back-eluted to another column where the first LC separation took place. Finally part of the eluate from this column (a heart-cut) was selected and loaded onto the second analytical column. The duration of one cycle was 30 min. The set-up seems promising and time-saving if the working conditions of three LC pumps and three LC columns can be maintained.

No stability problems for the β-adrenoceptor antagonists or their metabolites in biological samples have been reported except for esmolol [172], bopindolol [173] and 4-hydroxypropranolol. Esmolol is an ultra-short-acting β-blocker and contains an ester function, which is hydrolysed very rapidly in vivo.

For the accurate determination of the parent drug, freshly drawn blood was immediately extracted with dichloromethane containing a deuterated internal standard [172]. The metabolite in the remaining blood phase could be assayed as well. A problem similar to that for esmolol occurs with bopindolol [172], an inactive pro-drug of an active β-blocker. In order to ensure that bopindolol is not hydrolysed in the sample tube, the blood was collected in tubes on ice, immediately centrifuged at 4°C and the plasma phase separated and frozen. The assay for both parent compound and metabolite then took place at 4°C. An efficient way of stopping the enzymatic hydrolysis of esters as an alternative to immediate extraction or cooling is to add sodium dodecyl sulphate as an esterase inhibitor [174]. Oxidation of 4-hydroxypropranolol can be prevented by adding sodium metabisulphite [175] or ascorbic acid [169] to the biological sample.

It may be fruitful to take certain precautionary steps in the collection of samples, as mentioned in Section 3.1. Cotham and Shand [176] have shown that the distribution of propranolol between plasma and red blood cells could be markedly influenced when collecting blood samples in vacutainer tubes. The same effect has also been observed for alprenolol [177].

6.2. Chromatographic methods

6.2.1. Gas chromatography

The use of the GC technique to determine levels of β-adrenoceptor antagonists in biological samples was first reported in 1969 by Ervik [178] for alprenolol. The principle involved was to react the amino alcohol moiety in the molecule with trifluoroacetic anhydride to obtain a derivative with high electron-capture response to be selectively detected by GC—ECD. This principle has been adapted for a great number of β-adrenoceptor antagonists. Atenolol [179], befumolol [180], metoprolol [181], moprolol [182], oxprenolol [183], pindolol [184], practolol [185] and propranolol [186] have all been determined by this technique. A modification of the technique is to use pentafluoropropionyl or heptafluorobutyryl derivatives. This has been reported for alprenolol [187], atenolol [188], betaxolol [189], bevantolol [190], bufuralol [191], metoprolol [192], oxprenolol [193], propranolol [194] and timolol [195]. For bufuralol the derivative was a mixed derivative with O-TMS; N-PFP as pure TFA derivatives showed low stability.

The simplest way to produce these derivatives is to add the perfluorinated anhydride to the extracted solute, wait for a certain period of time, evaporate the excess reagent, and redissolve the residue in a suitable solvent.

When the reaction rate is low it can be speeded up by raising the temperature, by adding some kind of catalyst or by using a more reactive reagent. The increased temperature does not alter the general procedure, but using a catalyst adds a new step to the procedure. Walle and Ehrsson [196] used trimethylamine as catalyst and included a cleaning step by washing the reaction mixture with a phosphate buffer solution (pH 6). On the other hand, if evaporation is not needed as a concentration step, it can be excluded since this washing procedure also takes care of the excess anhydride. In a few cases where the heptafluorobutyryl derivative was highly sensitive to acid hydrolysis, heptafluorobutyric imidazole has been used. Besides high reactivity, imidazoles do not give acids as reaction product, but they are themselves extremely sensitive to moisture and are easily hydrolysed to imidazole and the corresponding perfluorinated acid.

The reasons for choosing between trifluoroacetyl, pentafluoropropionyl or heptafluorobutyryl derivatives are not always explained. In some instances when trifluoroacetyl derivatives were found to have low stability, this was improved by using heptafluorobutyryl derivatives. In other cases heptafluorobutyryl or pentafluoropropionyl derivatives are used because of claims of higher sensitivity.

Perfluoroacyl di-derivatives of the same type as those formed with β-adrenoceptor antagonists with the characteristic amino alcohol chain are of almost equal sensitivity irrespective of the character of the perfluoroacyl derivative formed [196]. As the monotrifluoroacetyl derivatives of coextracted

endogenous substances are between 100 and 1000 times less sensitive to ECD than their corresponding pentafluoropropionyl or heptafluorobutyryl derivatives [197], selectivity will be substantially increased by using trifluoroacetyl derivatives. This has been clearly demonstrated by Ervik et al. [179] in the determination of atenolol and can be visualized by comparison of the two chromatograms in Fig. 1.

Another derivative for ECD was proposed by Poole et al. [198] using 2,4-dichlorobenzeneboronic acid or 3,5-bis(trifluoromethyl)benzeneboronic acid to produce a transboronation reagent with 1,3-propanediamine. This reagent was then coinjected with the β-adrenoceptor antagonist (alprenolol) and a derivative with electrophore properties was formed by thermal reaction in the gas phase.

A more direct method of using boronic acids as reagents, forming cyclic boronates with β-adrenoceptor antagonists, was described by Yamaguchi et al. [199] for the determination of propranolol in plasma, using a nitrogen-selective detector. This detector has also been used by De Boer et al. [200] for determination of the acetyl derivative of propranolol, and by Gyllenhaal and Vessman [201] for the determination of metoprolol in plasma after reaction with phosgene, to a cyclic product, an oxazolidinone. This last method is very simple with extraction and reaction in one step followed by evaporation and reconstitution in ethyl acetate. This principle has recently also been applied for the determination of metoprolol and some of its more hydrophilic metabolites [202].

Before 1979 separation was performed entirely on packed columns with methyl silicone or methylphenyl silicone (OV-17) stationary phases with no claim of superiority for either of them. Sensitivity expressed as the limit

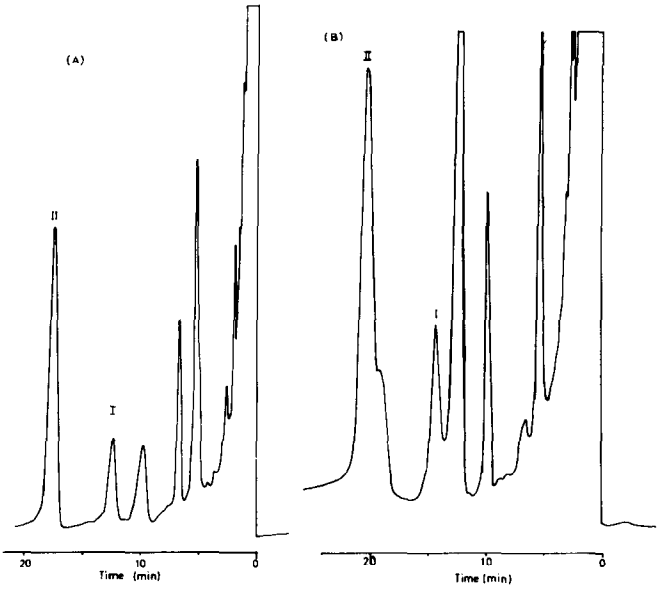

Fig. 1. Gas chromatograms obtained by analysing the same plasma sample by using (A) trifluoroacetic anhydride and (B) heptafluorobutyric anhydride as the derivatizing reagent. Peaks: I = atenolol (0.3 μmol/l) and II = internal standard.

of determination is reported to be 10—20 nmol/l (3—5 ng/ml) for both the electron-capture and the nitrogen-selective detector.

DeBruyne et al. [187] reported in their work of 1979 that switching from packed column to capillary column markedly increased the sensitivity of their method for the determination of alprenolol and oxprenolol in serum. The high resolving power of the capillary column increased the signal-to-noise ratio with a limit of determination of 4 pg, or in concentration unit according to the method 4 nmol/l (1 ng/ml).

The selection of the capillary column must be made carefully, however, especially when trifluoroacetyl derivatives are going to be chromatographed. Ahnoff et al. [203] have shown that certain capillary columns possess catalytic activity which will induce decomposition of the perfluoroacyl derivative. This activity could be measured according to a simple procedure [203]. The TMS and cyclic boronate derivatives of the β-adrenoceptor antagonists seem to be more stable under normal chromatographic conditions, but no thorough study on this matter has been reported. The stability of the oxazolidinone derivatives has been shown to be surprisingly good.

This property of the oxazolidinone derivatives has been of value also on chiral capillary columns as demonstrated by König et al. [204]. The major part of β-adrenoceptor antagonists are used as racemates but in recent years there has been interest in studying the fate of the enantiomers. Caccia et al. [205] reacted some enantiomeric β-adrenoceptor antagonists with trifluoroacetyl or heptafluorobutyryl-1-prolyl chloride and separated the derivatives on ordinary GC columns (packed or capillary). König and Ernst [206] could separate some enantiomeric β-adrenoceptor antagonists as HFB derivatives on a capillary column coated with XE-60-L-valine-(*R*)-α-phenylethylamide. This column was also used for the separation of the oxazolidinone derivatives, which in addition to excellent stability were also useful in separating the N-*tert*.-butyl-substituted compounds not previously resolved by GC [204].

6.2.2. Selected-ion monitoring

Problems often arise when the chromatographic separation has to be optimized for several analytes in a complex mixture. This is particularly valid when using a detector with only moderate selectivity. As SIM means improved selectivity compared with other detectors, chromatographic separation is not critical and a method for the drug can often be modified to include extractable metabolites [191, 207—209].

Another application of the SIM technique is to separate and determine isotopes. This has been used by Ehrsson [210] to study the metabolic elimination of the enantiomers of propranolol.

In an illustrative work by Carlin et al. [168] on the determination of timolol and [$^{13}C_3$]timolol in plasma for evaluation of bioavailability, the unique capability of the SIM technique is demonstrated. Timolol is active in low doses, which requires a sensitive assay. After administration of an oral dose of the drug and coadministration of an intravenous dose of the stable isotope labelled drug, the content of each form of the drug was determined with SIM after silylation. Each subject served as his or her own control and the number of subjects could be limited considerably compared to a corresponding study with an ECD method.

Methods using SIM are, except for the detector, much the same as those used with other detection devices. Separation is done by GC which means that derivatization is necessary. However, when an electron-capture response is not the aim, stability of the derivative and the fragmentation pattern become more important. By use of trimethylsilylated derivatives Funke et al. [211] made it possible to determine nadolol, a β-adrenoceptor antagonist containing two hydroxy groups in a tetrahydronaphthalene ring structure. Besides trimethylsilylated derivatives, trifluoroacetyl derivatives are frequent, partly because they are easily prepared and partly because they give very intense ions with sufficiently high masses (m/z = 266 and 308) upon electron impact. When Garteiz and Walle [212] published their results on the mass fragmentation patterns of trifluoroacetylated derivatives of β-adrenoceptor antagonists back in 1972, they postulated that this method "will permit measurement of picogram amounts of these compounds by mass fragmentography", as has been proved in several papers since.

Assay sensitivity is somewhat increased (five- to ten-fold) compared with ECD, but by using GC—MS—MS or high-resolution GC—MS and chemical ionization, Slayback et al. [213] reported a 100-fold increase in sensitivity compared with ECD for the determination of metipranolol.

6.2.3. Liquid column chromatography

Although GC methods using ECD have been successful in the analysis of β-adrenoceptor antagonists, they are relatively more complex for the less experienced analytical chemist and require derivatization before chromatography. Hence, in recent years LC methods have been increasingly popular for the determination of these drugs. The access of selective UV and/or fluorescence characteristics is a prerequisite for LC determinations, since assay of concentrations down to 10—100 nmol/l (3—30 ng/ml) may be required. Most of the β-blockers, e.g. acebutolol, alprenolol, atenolol, bopindolol, metoprolol, nadolol, pindolol and propranolol, contain a phenoxy or an aryloxy group (see Fig. 2), which gives a high fluorescence intensity with excitation maximum in the lower UV region (200—240 nm).

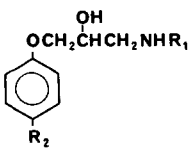

Fig. 2. General structure for most β-adrenoceptor antagonists.

Butofilolol, oxprenolol, practolol and talinolol contain phenoxy groups, which are substituted in the benzene ring in such a way that the fluorescence is greatly reduced or lacking, and timolol contains other rings which only exhibit UV absorption properties. From the methods published, it is obvious that it is necessary to detect the β-adrenoceptor antagonists by their fluorescence to obtain acceptable sensitivity.

The most common column packing material used for β-adrenoceptor antagonists, like most other compounds, is chemically bonded silica for

reversed-phase separations and among those materials the octadecylsilane column (C_{18}). Less frequently used materials are ethyl-, alkylphenyl-, octyl- and cyano-bonded phases. One interesting phase, PRP-1, polystyrene—divinylbenzene resin, which is stable over the entire pH range, was used for the separation of nadolol [214]. Separation on bare silica is also used (normal phase).

The separation of amines is mostly made in acidic mobile phases, e.g. phosphate or acetate buffers. While the development of new column packing materials over the last years may have led to increased stability, the problems with tailing peaks from amines have increased. Consequently it has almost become obligatory to add another aliphatic amine to the mobile phase, e.g.

TABLE 2

LC METHODS FOR β-ADRENOCEPTOR ANTAGONISTS IN PLASMA

Drug	Metabolites determined	LC column	Detector	Work-up procedure	Recovery (%)	Limit of determination nmol/l (ng/ml)	Reference
Acebutolol	Yes	C_{18}	F 254/460	Evap.	?	50 (20)	218
	Yes	C_{18}	UV 243	Back-extr.	?	50 (20)	222
	Yes	C_{18}	F 235/389	Back-extr.	80	25 (10)	163
Atenolol	No	CN	UV 224	Bond-Elut	106	100 (25)	167
	No	C_{18}	F 222/—	Back-extr.	84	80 (20)	163
	No	Silica	F 195/—	Extr. + inject.	?	80 (20)	223
	No	C_{18}	F 222/—	Back-extr.	?	40 (10)	166
Bopindolol	Yes	CN	F 220/360	Back-extr.	70	6 (2)	173
Bufuralol	Yes	C_{18}	F 250/300	Back-extr. + evap.	72	3 (1)	224
Butofilolol	No	C_{18}	UV 313	Evap.	67	125 (40)	225
Celiprolol	No	C_{18}	F 335/472	Back-extr.	?	10 (5)	226
Esmolol	Only	Phenyl	UV 280	Precipitation	97	3000 (1000)	227
Mepindolol	No	C_{18}	Electrochem.	Back-extr.	67	7.5 (2)	228
Metoprolol	No	C_{18}	F 222/320	Evap.	?	15 (5)	215
	No	C_{18}	F 275/300	Back-extr.	74	15 (5)	219
	Yes	C_8	F 225/320	Back-extr.	99	30 (10)	229
Nadolol	No	PRP-1	F 265/305	Bond-Elut	90	30 (10)	214
	No	C_{18}	UV 220	Evap.	96	30 (10)	230
Oxprenolol	No	C_{18}	UV 275	Back-extr.	83	30 (10)	231
Penbutolol	Yes	C_8	F 278/310	Evap.	92	15 (5)	232
	Yes	CN	F 275/324	Evap.	95	30 (10)	233
Pindolol	No	C_{18}	F 220/360	Back-extr.	96	20 (5)	234
Practolol	No	C_{18}	UV 245	Evap.	?	190 (50)	218
	No	C_{18}	UV 248	Evap.	75	110 (30)	235
Propranolol	Yes	Phenyl	F 205/340	Back-extr.	?	3 (1)	236
	Yes	C_8	F 290/> 340	Back-extr.	80	6 (2)	237
	Yes	CN	F 285/405	Precipitation	97	30 (10)	170
	Yes	C_{18}	F 216/> 340	Back-extr.	82	1.5 (0.5)	164
	No	C_8	F 230/340	Precipitation	?	12 (4)	169
	No	C_8	F 290/350	Evap.	?	6 (2)	238
	No	C_{18}	F 217/> 300	Back-extr.	80	6 (2)	239
	No	C_{18}	F 215/—	Back-extr.	84	3 (1)	163
Sotalol	No	C_{18}	F 235/—	Back-extr.	64	60 (20)	163
Talinolol	No	Silica	UV 245	Evap.	100	50 (20)	240
Timolol	No	C_{18}	Electrochem.	Back-extr.	80	15 (5)	241

triethylamine [166, 167, 215] or dimethyloctylamine, to improve the symmetry of the peaks [216, 217]. Addition of ion-pairing reagents (heptane sulphonate, octyl sulphate) can be used to change the selectivity in the separation. Mobile phases also contain one or two organic solvents of which acetonitrile and methanol are the most common, alone or in mixtures [164, 169, 218, 219]. Tetrahydrofuran, which unfortunately has a high absorbance in the far UV region, can be used in low concentrations to change the selectivity.

LC conditions for more than one β-blocker are sometimes given in papers, either to demonstrate the selectivity of the method or to give conditions for the determination of the other compounds. Patel et al. [220] reported retention times for eleven β-blockers on ethylsilane columns for pharmaceutical analysis. However, they used chloride-rich eluents, which will probably corrode the column and column packing. Verghese et al. [167] gave retention data for five β-blockers on a CN-bonded phase and Pautler and Jusko [165] data for six compounds on silica.

Table 2 presents data from selected LC methods for β-blocking drugs. Some compounds, e.g. acebutolol, metoprolol, penbutolol and propranolol, have active metabolites, thus methods have been included that can determine parent drug and metabolite simultaneously. Methods including lengthy work-up procedures have been omitted, as well as methods with poor recovery of the drug, since a high recovery is necessary to obtain a high degree of precision. According to our experience all β-blockers can be extracted with a recovery of at least 90%. For hydrophilic compounds such as atenolol [179], sotalol and practolol it may be favourable to saturate the aqueous phase with sodium chloride and then extract with dichloromethane. The distribution constant for many compounds is increased ten-fold after the addition of sodium chloride. Solid-phase extraction could also be used. Some papers have been included although nothing is mentioned about recovery, but a suitable extraction solvent has been selected. The paper by Lefebvre et al. [163] is the only paper that systematically investigates the extraction, back-extraction and detection conditions.

We use the limit of determination rather than detection limit and assume that such a concentration can be determined with a relative standard deviation better than 10—15%. This often roughly corresponds to a signal-to-noise ratio of 5—10, or twice the detection limit. These figures should, of course, be taken as relative guidelines only, as they might vary with the instrument and the performance of the column. (For a more complete list of LC methods, the excellent review by Mehta [221] can be consulted.)

6.2.4. Separation of optical isomers

Most of the commercially available β-adrenoceptor antagonists are racemic mixtures. A property in common for the analytical methods used is that they do not distinguish between the optical isomers of the drugs. The *S*-(−)-forms are considered to cause most of the pharmacological effects and for propranolol [242, 243], alprenolol and metoprolol [244], bufuralol [245] and moprolol [246] higher circulating concentrations of the *S*-(−)-enantiomer compared with the corresponding *R*-(+)-enantiomer are reported. Previous methods for

propranolol required sophisticated equipment and the use of isotopes [210, 247], immunological techniques of uncertain specificity [248] or GC techniques [249] not sensitive enough for studies of concentration—effect correlations in man.

Most often adequate separation of the isomers cannot be achieved by direct means, and it is necessary to derivatize the compounds. One method involves derivatization with an optically pure chiral reagent and the chromatographic separation of the resulting diastereomers. This technique, employing N-trifluoroacetyl-*S*-(—)-prolyl chloride has been used to measure each enantiomer of propranolol in plasma by GC [205] and LC [242, 243] following administration of the racemic drug and has also been used for acebutolol and its metabolite, diacetolol, in plasma [250]. Several investigators noted, however, that this reagent can racemize during synthesis or storage [242, 251, 252]. For LC separation *tert.*-butoxycarbonyl-L-alanine anhydride or *tert.*-butoxycarbonyl-L-alanine anhydride [244, 253] have also been used, but they have the disadvantage of not being commercially available, and the derivatization procedure is rather elaborate. These two disadvantages do not seem to be valid for derivatization with *R*-(+)- or *S*-(—)-phenylethylisocyanate [254] and 2,3,4,6-tetra-O-actyl-β-D-glucopyranosyl isothiocyanate [255]. The latter reagent was used, except for propranolol, to separate the enantiomers of hydroxypropranolol, alprenolol, atenolol, bupranolol, metoprolol, pindolol, practolol and sotalol. The last two chiral reagents were not used for determination in plasma, but only in pure aqueous solution.

6.2.5. Thin-layer chromatography

TLC has only been used for a couple of β-adrenergic agents. Two of the pioneers in this area seem to be Schäfer and Mutschler, who have published methods for atenolol [256], nadolol [257], oxprenolol [258] and propranolol [259]. In all methods fluorescence detection has been used, for oxprenolol after derivatization with 1-ethoxy-4-(dichloro-*s*-triazinyl)naphthalene to form a fluorophore. The methods described are relatively simple; however, they lack internal standards except for the method for oxprenolol [258], and the recoveries obtained are low (60—80%). The limits of determination are estimated at 15 nmol/l (5 ng/ml) for propranolol, 30 nmol/l (10 ng/ml) for atenolol and oxprenolol and 60 nmol/l (20 ng/ml) for nadolol, which makes the methods relatively comparable to the corresponding GC and LC methods.

6.3. Non-chromatographic methods

6.3.1. Spectrofluorometric methods

These methods have lower sensitivity and much lower selectivity than chromatographic methods and cannot be recommended for β-adrenoceptor antagonists in biological samples. There may be occasions, however, when their use can be justified. Nadolol is a β-adrenoceptor antagonist known to excrete no detectable metabolites and a spectrofluorometric method described by Ivashkiv [260] was later confirmed by GC—MS [191] to give representative nadolol level data.

6.3.2. Radioimmunoassay

This technique has been adapted to the determination of acebutolol [261], carteolol [262], diacetolol [263] and propranolol [248, 264, 265]. Although the immunization procedure for producing antisera is time-consuming, this assay is a straightforward, very rapid and sensitive method which requires only 50—100 µl of sample. This would be an advantage when thousands of samples are to be monitored. However, so far no commercial source is available for the antisera used in radioimmunoassays for β-adrenoceptor antagonists.

Propranolol presents an intricate problem owing to its extensive metabolism and lack of appropriate functional groups amenable to conjugation to a protein carrier to produce antibodies. Three papers have been published, two of which [248, 265] show good specificity to propranolol compared with ring-oxidized metabolites such as 4-hydroxypropranolol, but demonstrate significant cross-reactivity with inactive side-chain metabolites. The third method [264] exhibits the reverse characteristics. In this case an extraction step eliminated the interfering compounds (conjugates) and then the method compared favourably with GC—MS. The prerequisites for conjugation of the immunogen to bovine serum albumin were discussed. The limit of determination for propranolol in unextracted plasma is usually 3—6 nmol/l (1—2 ng/ml) using 50 µl of sample. The optical isomers of propranolol have been selectively determined by Kawashima et al. [248], who used antisera for both *R*-(+)-*S*-(−)-propranolol and *S*-(−)-propranolol.

6.3.3. Fluoroimmunoassay

Al-Hakiem et al. [266] developed a fluoroimmunoassay method for propranolol in plasma. They employed antibodies to propranolol, covalently coupled to magnetizable solid-phase particles, and fluorescein-labelled propranolol as tracer. The principle is similar to radioimmunoassay and has the same problems with specificity and cross-reaction. This method seems to be worse in these respects than the previously mentioned radioimmunoassays [248, 264, 265]. The limit of determination seems to be 30 nmol/l (10 ng/ml) with 100 µl of sample.

6.3.4. Radiochemical assays

A method using the double radioisotope derivative technique was first described by Riess [8] for the assay of oxprenolol in blood. This method has also been used to determine metoprolol levels in plasma [267]. The limit of determination is claimed to be 60 nmol/l (20 ng/ml) using 1-ml samples. The method is based on the formation of a ^{3}H-labelled derivative of the isolated drug followed by TLC separation of the derivative and radiometric measurement. The internal standard used is the ^{14}C-labelled drug. This method puts strong demands on the equipment and the experience of handling radioactive reagents without contamination.

6.3.5. Receptor assays

The radioreceptor assay used for propranolol [268] is interesting because the method measures the biological activity of the compound. This procedure

is based upon competition between the radioligand ([^{125}I]iodohydroxybenzylpindolol) and propranolol for binding sites in turkey erythrocyte plasma membranes. The assay will give total activity of compounds competing for the sites, unless precautions are taken. One sample may be stabilized by adding sodium bisulphite and in one sample 4-hydroxypropranolol is quantitatively oxidized by addition of hydrogen peroxide. These two analyses then give the proportion between propranolol and 4-hydroxypropranolol. The assay can also be used to determine free drug and metabolite levels. The limit of determination was 1.5 nmol/l (0.5 ng/ml).

7. VASODILATING AGENTS

7.1. α-Receptor blocking agents

Phentolamine is discussed in this category.

7.1.1. Chromatographic methods

7.1.1.1. Gas chromatography. Phentolamine is an amino phenol and due care has to be taken in the extraction which has been performed from an aqueous phase at pH 10. One paper deals with the quantification of phentolamine in biological fluids [269]. The drug is structurally a phenol and an imidazoline, and both functional groups could be used for derivatization purposes. Sioufi et al. [269] used HFB-imidazole as an electrophore reagent with a monoderivative on the phenol as result. No attempts were reported to introduce a second HFB group. The proposed method did not suffice for plasma levels of the drug (< 5 ng/ml), but only 3 μl of the extract containing the derivative (~ 2 ml) were utilized.

7.1.1.2. Liquid column chromatography. A reversed-phase ion-pair LC method was reported by De Bros and Wolshin [270]. The drug was extracted with diethyl ether in the presence of structurally related internal standards and back-extracted to sulphuric acid, from which an aliquot was introduced onto the C_{18} column. Octane sulphonic acid was used as ion-pairing agent in the mobile phase. The method could measure 50 nmol/l (15 ng/ml) which was sufficient to trace the compound for 1 h after intravenous administration.

7.1.2. Non-chromatographic methods

A platelet aggregation-inhibition test based on the pharmacological effect of phentolamine has been reported [271]. The effect corresponded to phentolamine levels of ~34 ng/ml for a 40-mg dose. A comparison of these results with those from the GC method described above, indicates that some active metabolite might contribute in the aggregation test.

7.2. β-Receptor stimulating agents

Isoxsuprine belongs to this group. It is a phenolic propanolamine derivative.

7.2.1. Chromatographic methods

7.2.1.1. Gas chromatography. A sensitive and selective method using ECD has recently been described, where isoxsuprine was quantified in cord plasma samples from newborns [272]. The method comprised solvent extraction at a

pH between 9 and 10 (amino phenol) and derivatization with TFA-anhydride to form a tris-TFA derivative. As outlined in the section on β-blocking agents (Section 6), this reagent gives a much better selectivity in comparison with HFBA and hence cleaner chromatograms. This was fully confirmed in this study, where the excellent properties of the derivative gave a limit of determination of 4 nmol/l (1 ng/ml) plasma. It is mentioned that this tris derivative is stable for at least 24 h in the dry state.

7.2.2. Non-chromatographic methods

An application of a radioimmunological assay has been reported [273]. The ethyl ether extracted drug could be quantified at the 4 nmol/l (1 ng/ml) level with a precision of ± 12%, which is competitive with the GC method.

7.3. Organic nitrate esters

Nitroglycerine, isosorbide dinitrate and isosorbide-5-mononitrate are discussed here. Isosorbide dinitrate and nitroglycerine, containing two and three nitrate ester groups, respectively, are rapidly metabolized in the human body to monohydroxy nitrate esters. The two mononitrate metabolites of isosorbide dinitrate contribute significantly to the therapeutic effect, and are normally included in recent drug monitoring assays. The role of dinitrate metabolites of nitroglycerine is less well elucidated and they are less frequently included in assays for nitroglycerine. Active plasma concentrations of nitrate esters are strongly dependent on the number of nitrate groups: nitroglycerine is probably active below 0.5 nmol/l (0.1 ng/ml), isosorbide dinitrate at 5 nmol/l (1 ng/ml) and isosorbide-5-mononitrate above 200 nmol/l (40 ng/ml).

7.3.1. Sampling and work-up procedures

Nitroglycerine and isosorbide dinitrate degrade in whole blood and, at lower rates, in plasma [274, 275]. Yap et al. [276] used silver nitrate to inhibit nitroglycerine degradation in rat plasma. For human plasma, rapid cooling has been found sufficient [277]. Lutz et al. [278] found that low levels of isomannide mononitrate and isoidide mononitrate occur in human plasma after administration of isosorbide dinitrate, making these substances less suited as internal standards for the isosorbide mononitrates.

The nitrate esters have relatively high vapour pressures. The volatility of nitroglycerine may cause problems with losses of the analyte as well as with contamination of low-concentration samples [279]. Problems may also arise due to nitroglycerine adsorbing onto plastic surfaces.

Different procedures, constituting different compromises between analytical recovery, sample purity and procedural simplicity, have been used for the isolation of nitrate esters from plasma, ranging from simple extractions without subsequent solvent removal [280] to elaborate purification [281].

Ethyl acetate permits quantitative extraction of nitroglycerine and isosorbide dinitrate [282], and is a rather efficient extraction solvent for the glyceryl dinitrates [277] and the isosorbide mononitrates. However, co-extracted plasma components disturb the chromatographic determination, sometimes in an unpredictable manner [277]. Nitroglycerine and isosorbide dinitrate are sufficiently non-polar to distribute into alkane solvents which

yield much cleaner plasma extracts but lower recoveries. Yap et al. [276] observed that the distribution constant for nitroglycerine was lower between hexane and plasma than between hexane and water. They used twelve consecutive extractions to obtain 92% recovery. Single extractions with larger volumes of petroleum spirit or *n*-pentane have been used for nitroglycerine and isosorbide dinitrate [283—285]. The glyceryl dinitrates and isosorbide mononitrates appear to have a very low distribution constant in alkane solvents. This has been used to eliminate metabolites in assays for nitroglycerine or isosorbide dinitrate. It has also been used for purification of extracts for the determination of the isosorbide mononitrates [286, 287], or for obtaining the parent drug and the metabolites in two different fractions [281, 288].

The high demands on assay sensitivity and selectivity for nitroglycerine make it less attractive to use a common work-up procedure for this drug and its active metabolites. On the other hand, common procedures for isosorbide dinitrate and its metabolites have been reported, based on extraction with diethyl ether [289], extraction with ethyl acetate and purification with activated charcoal [290], extraction with dichloromethane [278, 291], with chloroform [275] or with dichloromethane—ethyl acetate [292].

Some investigators have found concentration-dependent recovery of nitrate esters. Gerardin et al. [293] observed strongly concentration-dependent losses of nitroglycerine during extraction of human plasma with pentane—methyl acetate and during a subsequent purification procedure. The losses were suppressed and compensated for by using [^{15}N]nitroglycerine as internal standard. Morrison and Fung [275] observed concentration-dependent recovery of isosorbide dinitrate from rat plasma.

7.3.2. Chromatographic methods

7.3.2.1. Gas chromatography. During the seventies, GC with packed columns and ECD totally dominated as the instrumental technique. While the electron-capture detector exhibits excellent sensitivity for nitrates at detector cell temperatures near 200°C or lower, chromatography has been troublesome, and the selectivity of packed-column GC—ECD has often not been sufficient for the determination of low concentrations of analytes.

Nitrate esters are thermally labile at temperatures above 150—200°C. Contact with catalytically active surfaces may cause decomposition at lower temperatures [294]. Adsorption losses may cause low response, non-linear calibration curves and even total loss of analytes. Packed columns have often had to be "primed" with high loads of the analyte to improve response, limiting the reliability of such chromatographic methods.

The application of open tubular capillary columns [285, 290, 291, 295] has led to strongly improved separations. Problems with adsorption seem to be overcome more easily [281] although great care still has to be taken in the selection of the column [291].

When using vaporizing injectors, deactivation and regular cleaning or replacement of the injector insert is necessary for a constant and linear response [290, 291]. Cold on-column injection has been used to improve the precision of the instrumental analysis. In this case, the column had to be rinsed at regular intervals [295].

In addition to the better separation offered by capillary column systems, higher sensitivity has been achieved. This has made it possible to inject smaller samples, thus saving the instrument from heavy contamination by the biological samples, and making the method suitable for use with autosamplers on a routine basis [278, 291].

Isosorbide-2-mononitrate, isosorbide-5-mononitrate and the 1,2- and 1,3-glyceryl dinitrates are monohydroxy compounds which may be derivatized to improve their chromatographic properties. Isosorbide-5-mononitrate is especially difficult to elute as a symmetrical peak with the same detector response as the other isosorbide nitrates. Trimethylsilyl derivatives of nitroglycerine metabolites were prepared using bis(trimethylsilylacetamide) (BSA); however, no figures on accuracy or precision of the method were given [296]. Smith and Besic [286] used *tert.*-butyldimethylchlorosilane to silylate the two isosorbide mononitrates. Miyazaki et al. [281] silylated the glyceryl dinitrates with bis-(trimethylsilyltrifluoroacetamide) (BSTFA).

7.3.2.2. Selected-ion monitoring. The classical approach for determination of nitroglycerine in plasma, using packed columns and ECD, has been replaced in recent works by GC—MS techniques. Reported attempts to reach the desired selectivity and sensitivity by high-resolution chromatography and ECD are few.

Electron-impact ionization of nitrate esters produces $(NO_2)^+$ ions at *m/z* 46. They have been used for determination of nitroglycerine down to 200 pmol/l (50 pg/ml) [293]. It was necessary, however, to purify extracts before injection. Chemical ionization does not produce positive ions useful for monitoring. However, in the negative-ion mode, methane has been used as the reactant gas, producing $(NO_2)^-$ and $(NO_3)^-$ at *m/z* 46 and *m/z* 62. As little as 100—200 fg of pure nitroglycerine injected onto a capillary column could be detected by single-ion monitoring at *m/z* 62 [285]. The lower limit of determination for nitroglycerine, 200 pmol/l (50 pg/ml), was set by the background of nitroglycerine in blanks, 40—80 pmol/l (10—20 pg/ml). An even higher sensitivity has been reported with the same mass spectrometric technique [295]. Linearity of response was achieved between 6 pg/ml and 6 ng/ml. High demands were placed on column deactivation.

In another approach, chemical ionization with dichloromethane and monitoring of $[M + Cl]^-$ ions were used to determine nitroglycerine [297]. Plasma samples were extracted and purified using silica gel and Sephadex LH-20. The method was developed further by including the glyceryl dinitrates which were determined as their trimethylsilyl derivatives [281]. As internal standards were used $[^2H_5, ^{15}N_3]$nitroglycerine and $[^2H_5, ^{15}N_2]$glyceryl dinitrate. The reported limit of determination for nitroglycerine, 0.4 nmol/l (0.1 ng/ml), was not as low as that reported for the methods using chemical ionization with methane mentioned above [285, 295]. This may, however, be due to differences in instrumentation rather than different ionization techniques. For the glyceryl dinitrates the limit of determination was 4 nmol/l (1.0 ng/ml).

7.3.2.3. Liquid column chromatography. A thermal energy analyser has been used as a nitrosyl-specific detector for the determination of nitrate esters in plasma [292, 298]. By extracting 3 ml of plasma with dichloromethane—

ethyl acetate and injecting the whole concentrated extract on a Zorbax NH_2 column, isosorbide dinitrate, isosorbide-2-mononitrate and isosorbide-5-mononitrate were determined with limits of determination of 2.5, 3.5 and 8 nmol/l (0.6, 0.9 and 1.7 ng/ml), respectively. The sensitivity of the detector seems adequate for isosorbide nitrates but is probably too low for nitroglycerine determinations. It should be mentioned that the thermal energy analyser is a gas phase detector which gives better signal-to-noise ratios when used as a GC detector [299].

7.4. Calcium antagonists

7.4.1. Dihydropyridine derivatives

Nifedipine, nitrendipine, nimodipine, felodipine and nicardipine are discussed here.

7.4.1.1. Sampling and work-up procedures. The dihydropyridines nifedipine, nitrendipine, nimodipine and felodipine are neutral dihydropyridine diesters with pronounced hydrophobicity. Nicardipine is a tertiary amine. The dihydropyridine structure is amenable to oxidation to the corresponding pyridine. The pyridine analogues to nifedipine, nicardipine, nimodipine and felodipine have been found in human plasma [300—303]. There are only a few examples of metabolites that are dihydropyridines [304]. The nitrophenyl-substituted dihydropyridines are quite sensitive to daylight and form the corresponding nitrosophenyl pyridines. Nifedipine with its nitro group in the 2-position is more sensitive than nimodipine and nitrendipine with their nitro group in the 3-position.

Chromatographic methods include extraction of the dihydropyridine from plasma or serum. Due to strong plasma protein binding [305], stronger solvents and longer extraction times than for protein-free samples may be required [303]. The extraction efficiency for the neutral dihydropyridines is not pH-dependent. Alkaline conditions are often used for extraction in order to minimize coextraction of interfering plasma components, although such an effect has not been well documented. The solubility in water is low (except for nicardipine) making plain water unsuitable as a solvent even for dilute standard solutions. Bach [306] used gelatine and boric acid to stabilize aqueous nifedipine solutions. Bonded-phase column extraction has been used for isolation of nifedipine from plasma [306, 307] but did not offer substantial advantage over liquid—liquid extraction.

7.4.1.2. Chromatographic methods. (a) Gas phase techniques. GC methods dominate because of the high sensitivity or selectivity that can be achieved with ECD [300, 302, 303, 308], mass-selective detection [301, 309] or nitrogen-selective detection [310].

The dihydropyridines may undergo partial oxidation to the pyridine under the influence of active surfaces and elevated temperatures. To avoid uncontrolled oxidation, several methods include quantitative oxidation and subsequent determination of the corresponding pyridine [302, 308]. To make such a method selective towards the pyridine metabolite, preseparation by TLC or liquid column chromatography is necessary [301]. Other methods are based on detection of the unchanged dihydropyridine [300, 303, 310]. In this case, oxidation in the GC injector and column must be minimized [303].

Most methods employ GC with packed columns. However, increased selectivity and to some extent increased sensitivity is achieved with capillary columns [303, 310]. Undesired oxidation was minimized by the use of high-temperature silylated injector liners and columns [303] and by cold on-column injection [310].

Nitrophenyl- or dichlorophenyl-substituted dihydropyridines are detected with high sensitivity using ECD. SIM and nitrogen-selective detection are less sensitive but this is compensated for by higher selectivity against plasma components, making preconcentration of extracts useful. For the electron-capture detector, detection limits are below 1 pg injected [303]. Reported limits of determination in plasma are 1.5—25 nmol/l (0.6—10 ng/ml). With the mass spectrometer as detector nifedipine and nicardipine have been determined as their pyridine analogues down to 15 nmol/l (5 ng/ml) using EI ionization [301, 309]. No published EI—MS methods are based on the detection of the unchanged dihydropyridine, which has a different, and for nifedipine less favourable, fragmentation pattern. On the other hand, unchanged felodipine can be determined in plasma down to 0.5 nmol/l (0.2 ng/ml) using capillary chromatography, EI and mass-selective detection at *m/z* 238 [311]. NICI—MS has recently been used for the determination of dihydropyridines with very high sensitivity [312].

(b) Liquid column chromatography. Methods have been designed for nifedipine [306, 313, 314] and the structurally similar nitrendipine [315] and nimodipine [320]. One method involves oxidation of the pyridine to its pyridine analogue which is detected with UV at 280 nm [313] while the other methods selectively determine the unchanged drug, detected at 238 or 235 nm. Separation is in most cases carried out on reversed-phase systems. The use of two different LC systems has been reported for the elimination of false positives from blank sera [306]. The methods can be used for concentrations down to 5—25 nmol/l (2—10 ng/ml) and are thus somewhat less sensitive than GC methods. Krol et al. [302] found that their LC method correlated well with a GC method.

7.4.1.3. Non-chromatographic methods. The binding of dihydropyridines and other calcium antagonists to cell membranes from rat heart or cerebral cortex has been exploited for the design of radioreceptor assays. [^{3}H]-nitrendipine is displaced by the drug and the remaining radiolabelled nitrendipine is measured by liquid scintillation spectrophotometry. The sensitivity of the assay is similar to that of LC and GC procedures, but interference from other drugs and from plasma or serum proteins has to be considered [315—317].

7.4.2. Other structures

Verapamil, diltiazem, flunarizine, and prenylamine are discussed here.

7.4.2.1. Sampling and work-up procedures. Verapamil is a rather lipophilic compound. A few papers include data for absolute recovery. They show that the recovery using heptane [318] or pentane—1-pentanol (19:1) [319] is only about 55—75%, which is not adequate to obtain good precision and accuracy. However, both diethyl ether [320] and methyl-*tert.*-butyl ether [321] give recoveries exceeding 95% and should be preferred. For LC determinations it is also suggested to add methanol to the plasma sample to precipitate the proteins [322].

After the extraction from plasma, verapamil can either be injected directly onto an LC column [321], or be back-extracted into dilute acid before the injection [323], or be evaporated and redissolved in mobile phase [320]. For GC determination a second transfer to an organic solvent after back-extraction was used [318].

A study of the distribution properties would have been useful for the extraction procedure of flunarizine, a piperazine derivative [324], which in addition showed adsorption losses. The same is valid for the isolation of diltiazem, where repeated extraction with hexane was used [325]. An alternative approach was demonstrated for diltiazem, where the plasma samples were lyophilized until analysis, reconstituted and extracted once with hexane [326]. This procedure avoided the gelification otherwise observed.

A better way to extract diltiazem, as used in an LC method, seems to be extraction with methyl-*tert.*-butyl ether followed by back-extraction into acid [327]. The recovery was >95%. In a GC—NPD assay old plasma samples were reported to give rise to interferences not present in fresh samples [325]. This was not observed in an ECD-based assay [328].

7.4.2.2. Chromatographic methods. (a) Gas chromatography. The fluorescence properties of verapamil were first exploited, but the results from effect studies correlated, not surprisingly, badly with plasma concentrations. However, a GC assay revealed the presence of at least two metabolites, which represented up to 80% of the "fluorescent verapamil" concentration [318]. The assay was based on extraction to heptane, reextraction and concentration, silylation (only for metabolite), and analysis by FID. The tertiary amine appears to behave fairly well on the column presented, Dexsil 300, at 270°C. The lowest concentration in the recovery study was 75 nmol/l (25 ng/ml) which could be measured with a C.V. of 11%.

An approach with NPD was presented, demonstrating the advantage of the selectivity in detection [329]. The limit of determination was now 12 nmol/l (4 ng/ml) (C.V. of about 5%). The use of a homologous internal standard was claimed to contribute to this improvement in the assay of verapamil [223] and flunarizine [218]. The high column temperature (300—310°C) with verapamil caused depletion of stationary phase at the beginning of the column resulting in adsorption losses after periods without analysis [329]. This was counteracted by regularly introducing up to 5 µl of a blank plasma extract concentrated about 25 times, and the column could, interestingly enough, be in continuous use for several weeks.

Prenylamine, which is a secondary amine, has been determined in serum (10 ml) after extraction to heptane, back-extraction to aqueous acetic acid, evaporation and dissolution before analysis by FID. The limit of determination was 150 nmol/l (50 ng/ml) [330], which would be improved by derivatization with selective detection (ECD or NPD).

Diltiazem represents a somewhat different structure with a heterocyclic ring system and a tertiary amine in the side-chain. GC procedures have relied on NPD and made quantification of 1 ng/ml possible when homologous internal standards were used [325, 326]. A high column temperature (270°C) was required for the OV-17 column.

A recent study made use of ECD for the determination of diltiazem and its

desacetyl metabolite in human plasma [328]. The electrophore properties of the compound are not anticipated but nevertheless enabled quantification of 6 nmol/l (2 ng/ml).

(b) Selected-ion monitoring. An early gas phase method for verapamil by Spiegelhalder and Eichelbaum [331] used SIM and stable isotope labelled internal standards. The work-up procedure was similar to the gas chromatographic ones, but the limit of determination reduced to 3 nmol/l (1 ng/ml). The stable isotope labelled internal standard ($^{13}C + {}^{2}H$) had isotopic impurity to a level of 9.2%, which had to be compensated for and reduced the possibilities to go further down in concentration.

This method with [$^{2}H_7$]verapamil as internal standard has been used in biopharmaceutical studies by Eichelbaum and co-workers [332, 333], where unlabelled verapamil (intravenous) and [$^{2}H_3$]verapamil (oral) were simultaneously administered to estimate bioavailability data. For a drug which, like verapamil, has an extensive first-pass metabolism, this technique is very well suited. Other assays cannot give the same amount of information without many more subjects participating in the study.

(c) Liquid column chromatography. Verapamil has three main metabolites of which norverapamil is the most potent and important. In some of the methods verapamil and the metabolites can be separated [319, 321, 322, 334]. A normal-phase system with potassium bromide and perchloric acid in methanol as mobile phase [321] and a reversed-phase system with methanol and ammonium acetate solution as mobile phase [322] seem to be preferable. However, these two methods contain either no internal standard [322] or an unsuitable one [321]. In one paper verapamil and seven metabolites (not norverapamil) are separated [320].

As mentioned earlier, the fluorescence properties of verapamil are excellent and can be used for detection after LC separation. A limit of determination of 3—15 nmol/l (1—5 ng/ml) can then be obtained, which is sufficient for most purposes. In two papers [321, 322] only 100 µl of sample were required.

Diltiazem is a new compound and only two papers on LC methods have been published [335, 336]. One report [335] deals with reversed-phase separation and UV detection of the parent drug and its desacetyl metabolite. The limit of determination is 30 nmol/l (10 ng/ml) (C.V. $<$15%) which seems to be sufficient. The other paper [336] describes the chromatographic separation of the enantiomers of diltiazem requiring another chiral species to be introduced. As diltiazem lacks an active group, it first had to be hydrolysed to the desacetyl compound, which was reacted with *d*-2-(2-naphthyl)propionyl chloride to form separable diastereomers.

(d) Thin-layer chromatography. Diltiazem together with the desacetyl metabolite were extracted three times with diethyl ether and assayed by TLC with densitometric evaluation at 237 nm [337]. In bioavailability studies the limit of determination was 60 nmol/l (20 ng/ml).

7.4.2.3. Non-chromatographic methods. (a) Spectrometric techniques. An early method described the use of a fluorometric assay for verapamil where, however, other fluorescent derivatives accounted for up to 80% of the content measured [338]. Some were later separated and identified in a GC assay from the same laboratory [318].

(b) Receptor assays. Both verapamil and prenylamine can be detected in a radioreceptor assay for calcium channel antagonists, where they affect the binding of [^{3}H]nitrendipine to the receptors [317]. As they do not compete directly for the binding sites their limit of determination (~ 0.05—1 μg/ml) is not as low as for the dihydropyridine derivatives. Diltiazem could also be quantified with a slight modification of the procedure. In all these assays the concentrations obtained reflect not only the parent drug but also pharmacologically active metabolites.

7.5. Various compounds

Under this heading terolidine and bencyclane will be discussed.

7.5.1. Sampling and work-up procedures

The first method for the determination of terodiline by Vessman and Strömberg [339] used a degradative derivatization reaction, which for selectivity reasons required an extraction and clean-up procedure. This was based on distribution studies and included base extraction from serum, back-extraction to perchloric acid and finally isolation of the drug and the internal standard as perchlorate ion pairs from dichloromethane.

In a second study, when ^{14}C-labelled material was available, it was revealed that the extraction of this lipophilic compound with heptane from a serum sample was very slow [340]. The samples were therefore diluted with water prior to extraction giving an extraction recovery close to 100% after 30 min. This effect has been attributed to a slow transfer from the lipoproteins (containing the drug) to the organic solvent [43].

7.5.2. Chromatographic methods

7.5.2.1. Gas chromatography. Two methods using ECD have been described. The "benzophenone method" utilized the oxidation of the diphenylmethane moiety of the drug to benzophenone, which enabled the assay of 15 nmol/l (5 ng/ml) [339]. Interferences from metabolites were counteracted by the extraction conditions and by the oxidation procedure which destroyed compounds hydroxylated in the benzene rings. Yet the influence from metabolites with a hydroxylated side-chain could not be fully ruled out.

Therefore, the possibility to introduce an HFB group onto the strongly sterically hindered secondary nitrogen was explored in a second paper [340]. By use of extreme reaction conditions (amount of anhydride and catalyst), it was possible to prepare the HFB-terodiline derivative. The choice of internal standard was critical. The second ECD method was able to determine 10 nmol/l (3 ng/ml) and gave results which agreed fairly well with the benzophenone method.

From a sensitivity point of view an NPD procedure could give about the same information, either direct or after acylation.

7.5.2.2. Selected-ion monitoring. Terodiline has been determined in a pharmacokinetic study by GC—MS as a TFA derivative with a decadeuterated internal standard [341]. The results indicated that aromatic hydroxylation is a major metabolic route explaining the good agreement with the earlier procedures.

Bencyclane is a fairly old drug. A recent study discussed analytical data obtained with a capillary GC chemical ionization mass spectrometric method [342]. Compared with earlier methods lower results were obtained, indicating that previous approaches (TLC, GC, LC) had not been without interferences or sensitive enough. The use of ammonia as selective reagent gas was emphasized. The procedure was able to quantify 2 pmol (0.5 ng) with a precision better than 8%.

8. BLOOD PRESSURE RAISING AND HEART STIMULATING AGENTS

In this group the following compounds are discussed: prenalterol, xamoterol, dobutamine and dopamine.

Dopamine, besides being a potent drug used for acute situations, is also an endogenous compound, one of the catecholamines. Accordingly there are a large number of methods and papers dealing with the determination of endogenous levels of dopamine in plasma, urine and various tissues. It is beyond the scope of this review to go into detail of the various methods. Several reviews have been published; for example, Baker and Coutts [343] edited a book on all available methods, Allenmark [344] and Kissinger et al. [345] wrote papers on LC with electrochemical detection, Andersson and Young [346] on LC with fluorometric detection and Baker et al. [347] reviewed the use of GC—ECD.

8.1. Sampling and work-up procedures

Prenalterol and xamoterol are β-adrenoceptor agonists and structurally related to β-adrenoceptor antagonists, the main difference being the presence of a phenolic substituent. Sampling and work-up procedures are therefore much the same as for the phenolic metabolites of β-adrenoceptor antagonists. In a method for prenalterol the plasma samples are saturated with sodium chloride in order to improve the extraction recovery. This procedure increases the recovery from 70% [348] to >95% [349]. For LC methods back-extraction into dilute acid is added. Xamoterol [350] (with prenalterol as internal standard) is extracted into a small ion-exchange column. The two compounds are eluted with ammonium hydroxide.

Both dobutamine and dopamine are amino phenols with catechol structure and special care must be taken to minimize oxidation in the plasma samples. Oxidation can be hindered by collecting the blood in EDTA tubes, quick centrifugation and freezing in a dry ice—acetone bath. For dobutamine two LC methods have been published, one that uses repeated extraction to ethyl acetate, evaporation, washing and reconstitution in mobile phase [351] and the other extraction to small disposable C_{18} columns, washing and elution [352]. The recoveries obtained were 77% [351] and 93% [352].

For LC determination of dopamine the sample work-up procedure is often based upon some kind of selective extraction. Catecholamines can form cyclic complexes with, for example, alumina [353] or boric acid [354]. Smedes et al. [355] formed a complex between dopamine and diphenylborate followed by ion-pair extraction with tetraoctylammonium into hexane with 1% 1-octanol. The recovery was 93%. A boronic acid substituted silica adsorbent has been synthesized by Mosbach et al. [356]. A cation-exchanger, either in column or

batch mode, has also been used, but it is not as selective as the other techniques.

8.2. Chromatographic methods

8.2.1. Gas chromatography

The first published method for the determination of prenalterol in plasma was briefly outlined in a paper by Rönn et al. [357]. The method which included derivatization with heptafluorobutyric anhydride and ECD was later optimized by Degen and Ervik [358]. A drawback of the method is the necessity to use reextraction in order to have sufficient selectivity at low concentrations.

Gas chromatography with ECD may be used to determine dopamine levels, if the derivatization is selective enough. Bock and Waser [359] described a method using acylation with pentafluorobenzoyl chloride. The detection limit was reported to be 0.1 pmol (20 pg) per injection, for a pure solution of the derivative. They reported problems with the chromatographic system and used priming with ephedrine to avoid memory effects. In combination with a higher background signal when analysing biological samples, this placed the limit of determination at a much higher level. The only application demonstrated was the determination of the dopamine content of corpus striatum of a rat at a level of 50 nmol/g (10 μg/g).

8.2.2. Selected-ion monitoring

SIM has successfully been used for the determination of dopamine since the early seventies [360] owing to the high assay sensitivity achieved with the mass spectrometric detector. Ehrhardt and Schwartz [361] measured concentration levels of dopamine in plasma at 2.5 nmol/l (0.5 ng/ml) with a C.V. of 2%. The limit of determination was 10 fmol (2 pg) of dopamine per injection. Prenalterol has also been assayed with the SIM technique [349] by a simpler procedure than with the GC—ECD technique as it only involves a single extraction step. The limit of determination is at the same level, or 5 nmol/l (2 ng/ml).

8.2.3. Liquid column chromatography

Like many β-adrenoceptor antagonists, prenalterol and xamoterol have fluorescent properties [348, 350]. In addition, the phenolic group with a *p*-alkoxi group offers great possibilities for electrochemical detection [362]. Prenalterol and xamoterol can easily be chromatographed on a reversed-phase column (C_8 or C_{18}) with either methanol [362] or acetonitrile [348, 350] in the mobile phase. In one method [362] propylamine was added as a modifier to improve the performance of the packing material. The limit of determination for prenalterol is lower for the electrochemical method [362], 2 nmol/l (0.5 ng/ml) with 1 ml of plasma, than for the fluorescence method [348], 4 nmol/l (1 ng/ml) with 2 ml of plasma. However, the electrochemical detector is subject to more disturbances such as problems with electrode condition and more interferences from endogenous compounds than is the fluorescence detector.

Dobutamine is also an amino phenol, with catechol structure, which

enhances the possibilities to use electrochemical detection. Dobutamine is chromatographed on C_{18} columns with mobile phases containing acetonitrile, EDTA and buffers at pH 2 or 3. The catechol structure of dobutamine readily undergoes oxidation at a glassy carbon electrode (+ 0.55 V) with an amperometric detector [352]. The limit of determination is as low as 300 pmol/l (100 pg/ml). The use of internal standards with a catechol structure is necessary to compensate for variation in the extraction step. The fluorescence method [351] is not at all as sensitive; the limit of determination is 30 nmol/l (10 ng/ml). The internal standard used in that case was not as well selected as it lacked the catechol structure and has too long a retention time.

The LC systems used for dopamine are in most cases based on cation-exchange or bonded-phase separation. Cation-exchange LC seems to represent the most obvious and straightforward method since acidic and neutral compounds may elute in the void volume. However, it is often difficult to obtain high column efficiency, and the batch-to-batch variation in the ion-exchange properties is too large. Other LC systems use reversed phase with either only phosphate buffers in the mobile phase or some ion-pairing agent, e.g. sodium dodecyl sulphate, and an organic modifier.

For the detection of dopamine either electrochemical or fluorescence detectors can be used. The most commonly used is the amperometric detector, with a thin-layer flow-cell first developed by Kissinger et al. [363] and further developed by Kissinger et al. [345]. A comparison of the performance of various electrochemical detectors for the determination of catecholamines and related compounds has been published by Humphrey et al. [364].

The native fluorescence of dopamine is not very high, but for fluorometric detection a higher sensitivity can be obtained with derivatization by precolumn or postcolumn technique. The main disadvantage with all precolumn methods is the laborious pretreatment of the sample. The most commonly used reagents for dopamine are *o*-phthaldialdehyde, Dns chloride and fluorescamine. Postcolumn derivatization in a reactor can be made either to form the trihydroxyindole by oxidation of the catechol ring or reaction with *o*-phthaldialdehyde. The last method takes only 20 sec as compared to 4 min for the trihydroxyindole method under optimal conditions [365]. By comparing amperometric and fluorometric detection [366] it seems as though the trihydroxyindole method is as sensitive as the electrochemical methods, 25—125 pmol/l (5—25 pg/ml) can be detected in 1—2 ml of plasma, and as sensitive as the radioenzymatic methods. However, the low sample volume required in the latter method ($\leqslant 100\,\mu l$) is difficult to match.

8.3. Non-chromatographic methods

The most sensitive method for dopamine is still the radioenzymatic one [367—369] which requires only 100 µl of sample. In short, the methods comprise the transfer of a ^3H- or ^{14}C-methyl group from [^3H]- or [^{14}C]-methyl-S-adenosyl-L-methionine to the 3-position of the catecholamine. This is catalysed by the presence of the enzyme catechol-O-methyltransferase, forming labelled 3-methoxytyramine from dopamine. After solvent extraction and TLC separation on aluminium foil [370] the methoxytyramine is extracted from the silica gel with ammonia, and subjected to liquid scintillation counting.

9. ANTIHYPERTENSIVE AGENTS

9.1. Antisympathetic drugs

9.1.1. Guanidine derivatives

Of the drugs treated in this part several contain guanidino moieties, i.e. betanidine, debrisoquine, guabenzodioxane, guanethidine, guanfacine, and guanoxan.

9.1.1.1. Sampling and work-up procedures. The distribution of four guanidino-containing drugs in various organic solvents was reported by Hengstmann et al. [371]. Rather high pH values were required due to the pK_a values of the guanidino group. Pre-extraction with less effective solvents, like toluene, and back-extraction were needed for successful quantification. This relied on hydrolysis of the guanidino compound to the corresponding primary amine.

An extractive derivatization procedure was introduced by Erdtmansky and Goehl [372]. This utilized cyclization of the guanidino group with an acetylacetone reagent in the biological sample itself. The work-up procedure gained much in simplicity with this approach.

9.1.1.2. Chromatographic methods. (a) Gas chromatography. The first attempts to derivatize the guanidino-containing drugs with TFAA or PFPA were not successful as the derivatives were very unstable [371]. The hydrolysis approach was then tried. For most of the compounds this process was performed in vials with 8% potassium hydroxide solution for 4 h at 110°C. The resulting primary amines were then perfluoroacylated with an attempt to use ECD. This means of detection, surprisingly, did not give any increase in response compared with FID, which is why GC—MS was chosen.

A later paper by Pellizzari and Seltzman [373] assayed guanethidine in plasma with the HFB derivative of the hydrolysis product. In order to simplify the work-up procedure a two-dimensional procedure was introduced. Clean-up was obtained in the first column (a polyester phase) and only two plugs or "windows" were transferred to the second column (OV-225). In this way 3 nmol/l (0.7 ng/ml) in plasma could be determined. With only one column the limit of determination was 50 nmol/l (10 ng/ml), which indeed shows the tremendous power of the two-dimensional approach.

The hydrolysis method seems to be selective for the original drugs as the guanidino group is not metabolically transformed [374]. Yet it is of interest to be able to determine the intact drugs. The transformation of the guanidino-substituted substances to the corresponding pyrimidines in a reaction with hexafluoroacetylacetone presented such a possibility, as first described by Erdtmansky and Goehl [372]. With ECD they were able to quantify 100 nmol/l (25 ng/ml) of three typical drugs, among them debrisoquine. Guanfacine was quantified down to 2 nmol/l (0.5 ng/ml) with ECD [375]. Later on this method was extended to cover also the 3-hydroxy metabolite [376]. In this procedure the phenol and the amide groups were methylated (extractive alkylation) before separation on capillary columns. This method was claimed to be less sensitive for guanfacine than the previous one using a packed column.

Using a similar derivatization approach (acetylacetone but not two-phase)

with NPD, Lennard et al. [377] determined debrisoquine and its 4-hydroxylated metabolite in body fluids. In blood and serum only debrisoquine could be determined. The hydrolysis procedure cannot be used for the metabolite due to degradation [378]. For debrisoquine there is now less of an interest in its cardiovascular properties than in its suitability as a model compound for the detection of genetic polymorphism in the oxidative metabolism.

(b) Selected-ion monitoring. Debrisoquine and its 4-hydroxy metabolite were determined as the pyrimidino compounds using GC—MS. Malcolm and Marten [378] could from very clean chromatograms quantify 3 nmol/l (1 ng/ml) and 15 nmol/l (5 ng/ml) of the drug and metabolite, respectively. This approach has been applied to guabenzodioxane and guanethidine as well [379]. ECD was reported as a less sensitive approach.

A recent study by Murray and Waddell [380] on the electron-capture negative-ion chemical ionization mass spectra of the bis(trifluoromethyl-pyrimidinyl) derivatives of debrisoquine and the 4-hydroxylated metabolite revealed a strong response. It was thus possible that femtogram amounts could be traced. Whereas 0.3 fmol (50 fg) of the debrisoquine derivative gave a signal-to-noise ratio of 3, the TMS derivative of the metabolite required more than 3 fmol (500 fg) for the same response. This study is a good example of how the sensitivity can be increased from ECD (30 fmol, 5 pg, in ref. 372) to NICI—MS (0.3 fmol, 50 fg). An application on the determination of the 4-hydroxylated metabolite in in vitro studies with human liver microsomes was recently reported [381].

9.1.2. Clonidine and tiamenidine

Clonidine and tiamenidine are potent antihypertensive drugs dosed in amounts as low as 100 μg. This has created real problems from a bioanalytical point of view. Structurally they are related with the previous group in that the guanidino group can be seen partly in the imidazole or the pyrimidine rings.

9.1.2.1. Sampling and work-up procedures. Usually base extraction and clean-up extraction are utilized to improve the situation from a chromatographic point of view. Extraction of plasma with silica columns also facilitated the process, as shown by Edlund [382].

9.1.2.2. Chromatographic methods. (a) Gas chromatography. Clonidine has electrophore properties in itself but the performance on column requires a derivatization reaction. Chu et al. [383] prepared a bis-HFB derivative of clonidine without deducing their position. Silica gel columns were used here to clean up the derivatization mixture. The limit of determination was 100 pmol/l (25 pg/ml) from a 4-ml sample.

Edlund [382] improved a packed column method by using capillary columns. The derivatization with pentafluorobenzyl bromide in acetone (carbonate catalysis) resulted in a cyclized product. The derivative was very stable and exhibited an excellent ECD response with a strong dependence on detector temperature. With a 5-ml sample 400 pmol/l (100 pg/ml) could be quantified but the procedure required extensive treatment and cleaning of glassware to keep the contamination level low. A non-chlorinated analogous compound acted favourably as carrier in the work-up and chromatographic steps. This derivative was evaluated on some other capillary columns and detectors. Silar 10 in combination with ECD was most suitable [384].

(b) Selected-ion monitoring. Early GC—MS data were based on electron-impact ionization [385]. A substantial improvement in selectivity for clonidine was the introduction of chemical ionization with ammonia as reagent gas [386]. Clonidine and the tetradeuterated internal standard were methylated on-column with trimethylanilinium hydroxide. The limit of determination was 400 pmol/l (100 pg/ml) clonidine (4 ml of plasma). It would be of interest to explore the use of the pentafluorobenzylated derivative prepared by Edlund [382] in the electron-capture negative-ion chemical ionization mode.

The on-column methylation reaction resulted for tiamenidine in strong interferences in the EI mode, why a bis-HFB derivative was prepared instead [387]. The derivative gave an intense molecular ion at *m/z* 607, which allowed quantification down to about 2 nmol/l (0.5 ng/ml) (5 ml sample). The drawback is that the derivative is unstable without an excess of HFBA, adsorbs to column material even if glass beads are used, and that the interface must be regularly deactivated with large amounts of hexamethyldisilazane. An alternative method reported on stable dibenzyl derivatives, which were prepared in a crown-ether catalysed reaction with benzyl bromide in the presence of potassium *tert.*-butoxide [388]. The formation of an isomeric form could be supressed as well as the two mono derivatives. This bis derivative is less volatile than the cyclization product formed by Edlund [382]. It was possible to quantify 8 nmol/l (0.2 ng/ml) with a precision better than ± 20%.

9.1.2.3. Non-chromatographic methods. (a) Immunological assays. The low concentrations of clonidine in plasma samples have focused interest on immunoassays as a means of quantification. Arndts et al. [389] developed an assay in which the tracer ligand was labelled with tritium. The limit of determination was 400 pmol/l (100 pg/ml), but some interfering components reduced the specificity considerably. A later version by Arndts et al. [390] introduced ^{125}I labelling of the ligand with a 25-fold increase in specific activity. This resulted in lower requirements on both the sample volume (50 µl) and the amount of antiserum. This assay could quantify down to 40 pmol/l (10 pg/ml) without interference. This modified radioimmunoassay with high sample capacity was compared with GC—MS methods and older radioimmunoassays.

With the prerequisites that interferences have been minimized over the entire range of sample concentration, there seems to be no method that can compete with RIA with respect to both sensitivity and capacity. The costs for production of antibodies and the evaluation of its suitability are therefore soon outweighed if the number of samples in the development of new drugs is considered.

Tiamenidine is a structurally related compound, the determination of which has preferably been made with RIA [319]. Tritium-labelled tracer was used with a limit of determination at 80 pmol/l (20 pg/ml) (100 µl sample). This method was also evaluated for its specificity with a GC—MS assay.

9.1.3. Methyldopa

α-Methyldopa has a catechol structure, is easily oxidized and amenable to electrochemical detection as used in recent LC methods. Separation is performed on a bonded strong cation-exchanger [392, 393] or on alkyl-bonded

phases such as C_{18} [394], C_8 [395] and phenyl [396]. With the alkyl-bonded phases, heptane or octane sulphonate has been added to the mobile phase probably as ion-pairing agent for methyldopa, although the choice of pH does not promote this. As high a pH as 6.4 is used for the separation and determination of adrenaline, noradrenaline and α-methylnoradrenaline in the same run as methyldopa [395]. The amines are then retained as ion pairs, but their chromatographic performance is not that good. Generally EDTA is present in the mobile phases used for separation of methyldopa.

Isolation of methyldopa from plasma is achieved by adsorption to alumina [394, 395]. In the other papers protein precipitation with perchloric acid has been employed. This treatment may give a contribution of methyldopa by hydrolysis of its conjugated metabolites [396]. In some papers the assay includes also conjugated forms of methyldopa which are acid-hydrolysed [392, 393].

The recoveries reported for the two different work-up procedures are above 85%. Therapeutic plasma concentrations are in the range 1—10 μmol/l (0.2—2.0 μg/ml) and are well covered by the sensitivity of the methods discussed.

9.2. Hydrazine derivatives

In this group the following compounds are discussed: hydralazine, cadralazine, dihydralazine, endralazine and propildazine.

9.2.1. Sampling and work-up procedures

The hydrazine derivatives hydralazine, cadralazine, dihydralazine, endralazine and propildazine are not chemically stable in biological systems. They readily react with endogenous pyruvic acid in plasma at 37°C to form the corresponding hydrazones, 50% of hydralazine being transformed in 15 min [397]. Earlier procedures for hydralazine with derivatization under acidic conditions [398—400] are not selective for the unmetabolized drug. Ludden et al. [401] suggest that derivatization is made in whole blood within 30 sec after collection, to minimize the risk of unwanted reaction. If hydralazine in plasma is to be determined, separation of the red blood cells should be performed within 5 min, immediately followed by derivatization with a suitable reagent [397, 402]. If hydralazine has to be stored underivatized, ascorbic acid is added [400]. Hydralazine, dihydralazine and propildazine are rapidly oxidized at alkaline pH and cannot be extracted as bases.

Derivatization procedures will be discussed later but as regards standardization the following has to be considered. In plasma standards prepared by adding the drug to blank plasma, hydrazones are easily formed in vitro as shown by Haegele et al. [403]. Unless these are hydrolysed in the derivatization procedure the content of free drug in the standard sample will be too low.

9.2.2. Chromatographic methods

9.2.2.1. Gas chromatography. The derivatives formed directly in the biological sample, or those formed on the isolated drug separated from plasma by means of a cation-exchange resin [404], have excellent GC properties.

The first published work using this technique was a method on the determination of hydralazine [399]. Hydralazine and the internal standard, 4-

methylhydralazine, were reacted with sodium nitrite at an acidic pH to form tetrazolophthalazine and 6-methyltetrazolophthalazine, respectively, directly in the biological sample. The derivatives were then extracted into organic solvent and gas-chromatographed. Detection was performed with an electron-capture detector. Sufficient sensitivity could also be achieved by using a nitrogen-selective detector as has been shown by Riis Angelo et al. [400] using formic acid to form triazolo derivatives.

Hydralazine, dihydralazine and propildazine react with aldehydes and ketones to form hydrazones which can be hydrolysed at low pH to regenerate the drug. The above-mentioned methods thus measure the apparent drug level and not the level of the unmetabolized drug.

The deviation between these two levels is considerable. Degen [405] reacted hydralazine with 2,4-pentanedione at pH 6.4 to selectively determine the unmetabolized hydralazine. He found that in rat plasma after treatment with an oral dose of hydralazine only about 10% of the apparent hydralazine was unchanged hydralazine.

Another way to avoid codetermination of hydrazone metabolites is to isolate the drug from plasma by cation-exchange chromatography and to derivatize with heptafluorobutyric anhydride. This method has been applied to propildazine in rat plasma [404]. The limit of determination for these methods is in the range 20—50 nmol/l (5—10 ng/ml).

9.2.2.2. Selected-ion monitoring. Only two methods using SIM have been published so far. Both deal with the determination of hydralazine and its metabolites.

The first method [406] prescribes derivatization at acidic pH with sodium nitrate, and will in accordance with the previous GC methods codetermine hydrolysed hydrazones as hydralazine. This has been dealt with in the second method [407] where derivatization is performed with cyclohexanone at neutral pH. No information referring to the sensitivity of this technique is given in either method.

9.2.2.3. Liquid column chromatography. As in GC, all LC methods include derivatization. Ludden and co-workers [401, 402] used *p*-anisaldehyde as reagent at pH 7.4 to form a hydrazone from hydralazine. This was extracted into hexane, which was evaporated and the residue dissolved in mobile phase. The derivatization recovery was 103% and the extraction recovery 70%. The LC separation was performed with a cyano-bonded phase and the derivative was detected by UV. Using whole blood [401], which seems to be the safest way to avoid incorrect hydralazine levels, the limit of determination was as low as 5 nmol/l (1 ng/ml) with 3 ml of blood. The use of whole blood also decreased the total blood volume required per determination. The pyruvic acid hydrazone yielded negligible concentrations of hydralazine during the analytical process. One method [397] used one of the original derivatization procedures (cf. ref. 398) for hydralazine, i.e. reaction with sodium nitrite, in this case at a much higher pH (pH 5.5). As the determination is performed in plasma and at a lower pH, it seems to be more uncertain, owing to a greater risk of hydrolysis and co-determination of metabolites. This method used fluorescence detection and the limit of determination was 5 nmol/l (1 ng/ml).

Another method [408] used formaldehyde as a reagent to form *s*-triazolo-

[3,4a]phthalazine. This compound is reported to be a metabolite, but only in trace amounts. The reaction is performed in plasma at acidic pH and after a suitable time an aliquot of the plasma is injected onto the reversed-phase column. The limit of determination is 15 nmol/l (3 ng/ml) and can be lowered if the product is extracted and concentrated before the LC separation. Fluorescence detection was used.

Dihydralazine is assayed in plasma [409] using the same derivatization with sodium nitrite at very low pH as in the method described in ref. 398. As nothing is known about the presence of hydrolysable dihydralazine metabolites forming dihydralazine at low pH, this has to be considered [397, 401]. The limit of determination was estimated at 30 nmol/l (10 ng/ml).

Endralazine, a new drug with a chemical and pharmacological resemblance to hydralazine and dihydralazine, has been assayed together with two metabolites using formic acid as reagent [410]. As endralazine is much more stable in plasma and much more lipophilic than hydralazine, it could be extracted in underivatized form from plasma into chloroform, and reacted with formic acid at high temperature. Three internal standards were used, one for each of endralazine and the two metabolites. The products obtained had a high fluorescence response and the limit of determination for endralazine was estimated at 2 nmol/l (0.5 ng/ml). The absolute recovery was 84%, the losses probably occurring in the extraction step.

Finally, cadralazine, a hydrazine ester (carbazate), has been determined without derivatization, simply by extraction at neutral pH, back-extraction into an acidic phase and injection onto an octyl-bonded reversed-phase column [411]. The recovery was only 57%. The compound was detected by UV at 254 nm and had a limit of determination of 50 nmol/l (15 ng/ml). Since cadralazine, unlike the other compounds in this group, is not a hydrazine but a hydrazine ester, its reactivity towards endogenous compounds is not as great, making it easier to obtain a correct measure of the plasma level than for the other compounds.

9.3. Other agents

In this group the following compounds are discussed: captopril, prazosin, tiodazosin, labetalol, medroxalol and diazoxide.

9.3.1. Sampling and work-up procedures

Captopril is a substituted proline compound containing a thiol group. It is not stable in whole blood or plasma. One way to avoid losses of captopril after sampling is immediate derivatization with N-ethylmaleimide, which has been used in GC and radioimmunoassays [412–415]. Addition of ascorbic acid and disodium edetate [416] and plasma protein precipitation with sulphosalicylic acid [417] have also been used to protect captopril from oxidation. Disulphide metabolites of captopril are of interest since interconversions between them and captopril have been reported. Kawahara et al. [418] added tributylphosphine to plasma to reduce disulphide metabolites to captopril including them in the determination.

For GC analysis the N-methylmaleimide adduct of captopril has been isolated from blood or plasma by adsorption onto XAD-2 resin after precipitation

of proteins [412, 413] or by solvent extraction with ethyl acetate [419, 420] or benzene [421]. Several additional purification steps have been added to the procedures, making them quite laborious [412, 413, 419]. A simpler procedure designed by Drummer et al. [420] consisted of extraction of excess N-methylmaleimide reagent followed by extraction of captopril with ethyl acetate from the acidified sample. Prior to gas-phase analysis, the carboxylic groups of the N-methylmaleimide—captopril adduct and of the symmetrical disulphide have to be protected. Funke et al. [412] used methylation, while alkylation with hexafluoroisopropanol has been used by others [413, 419—421].

In LC methods derivatization has also been used, both to protect the mercapto group and to make the compound more prone to detection by UV [418], fluorescence [417], or electrochemical detectors [422]. These methods are also rather complex and tedious and include both a derivatization step, several extractions, washings and evaporations before injection onto the column. The reagents used are *p*-bromphenacyl bromide (UV [418]), N-(1-pyrene)maleimide (fluorescence [417]) and N-(4-dimethylaminophenyl)-maleimide (electrochemical [422]). Recoveries are about 70% [417, 422].

One LC method [416] makes use of an original property of captopril, namely the possibility to oxidize the mercapto group to the corresponding disulphide at a Hg/Au electrode. The sample work-up is then very simple. Plasma proteins are immediately precipitated by the addition of sulphosalicylic acid. Part of the supernatant is then injected onto the LC column. The recovery was 89%.

Prazosin, an antihypertensive agent of the quinazole family, lowers blood pressure in humans at doses as low as 0.5 mg. The determination of plasma drug concentrations after such low doses requires an assay with a limit of determination below 3 nmol/l (1 ng/ml). Of the methods published three LC methods seem to be the most useful. Two of them use extraction of prazosin from plasma, to either diethyl ether [423] or chloroform [424], followed by back-extraction into acid [423] or evaporation and redissolution in mobile phase [424]. The absolute recovery is 100% with chloroform and probably with diethyl ether too. The third method [425] utilizes precipitation of plasma or whole blood with a double volume of acetonitrile, evaporation to a small volume and injection. Recovery is 100%. Tiodazosin is an analogue of prazosin. One LC method has been published [426] which strongly resembles one of the methods for prazosin [425].

Labetalol [427—430] and medroxalol [431] are in most cases determined by LC methods. They are amino phenols, which implies that the pH optimum for the extraction is around 10. They exhibit a somewhat polar character and a rather polar extraction medium has to be used. Most papers have used six to eight times as large a volume of diethyl ether as that of plasma and the absolute recovery for labetalol ranges from 80% [428] up to 96% [427].

In another method [429] a stronger extraction solvent was used, namely ethylene chloride—diethyl ether—isopropanol (9:9:2), which gave a recovery of 95%. Most methods have included evaporation before dissolution in mobile phase [428, 429]; one method used a back-extraction step instead [430], and one method used first back-extraction and then reextraction into chloroform followed by evaporation [427]. The length of the work-up procedure is of

course mostly dependent on the sensitivity required and the detector available.

Diazoxide is a benzothiazine with weak proteolytic properties. In the LC method published [432] the sample work-up procedure is very simple: 100 µl of plasma are mixed with 400 µl of perchloric acid, and 100 µl of the supernatant are injected on to the column. The recovery from plasma is only 83%.

9.3.2. Chromatographic methods

9.3.2.1. Gas chromatography. Little work has been done to optimize the GC of the derivatives of captopril and related substances. Recent work [420, 421] shows that good linearity of signal response at low concentrations can be obtained with common-type methylsilicone-packed columns. Split peaks were observed for the derivatives of captopril and its disulphide but this did not impair quantification.

Flame photometric detection has been used but the sensitivity seems not sufficient for low concentrations [419]. Adequate sensitivity has been obtained with the electron-capture detector [421] which makes use of the electron affinity of hexafluoroisopropyl derivatives. The limit of determination for captopril in blood was 60 nmol/l (20 ng/ml), corresponding to ca. 10 pg injected.

9.3.2.2. Selected-ion monitoring. Positive-ion electron-impact ionization mass spectrometry has been used to detect the derivatives of captopril [412, 413, 420] and of its disulphide [420]. In the early work by Funke et al. [412], the limit for quantification of captopril in 5 ml of blood was 50 nmol/l (15 ng/ml). Higher sensitivity was reached with 1-ml blood samples in a more recent work by Drummer et al. [420]. The limit of determination was 6 nmol/l (2 ng/ml) but no data on precision were given for concentrations below 270 ng/ml.

9.3.2.3. Liquid column chromatography. Prazosin is separated using reversed-phase columns, C_{18} [423, 425] or phenyl [424], and mobile phases with methanol [423, 425] or acetonitrile [424] at an acidic pH. In one case pentane sulphonate [423] was added as ion-pairing agent, which does not seem to be necessary.

Prazosin fortunately has a very high fluorescence response, which is necessary in order to obtain a low limit of determination. In all the three methods it is possible to measure concentrations down to 0.25—0.5 nmol/l (0.1—0.2 ng/ml). In one case only 0.2 ml of sample was required [425] compared with 1—2 ml in the other cases. Structurally similar internal standards have been used except in ref. 425, where carbamazepine was used, which necessitated the use of a UV detector coupled in series with the fluorescence detector.

Labetalol and medroxalol are also separated by use of reversed-phase LC, on either C_{18} or C_8 columns. Mobile phases contained acetonitrile [427, 429], methanol [429] or methanol—tetrahydrofuran [428, 431]. In three cases ion-pairing agents have been added: octyl sulphonate [428, 431] and perchlorate [429]. Labetalol was detected either by UV at around 210 nm [427, 430] or by fluorescence [428, 430] and medroxalol by fluorescence [431]. The limit of determination for labetalol is 30 nmol/l (10 ng/ml) for the UV methods [427, 430] and 30 nmol/l [428] and 10 nmol/l [429] for the fluorescence methods. Medroxalol has a similar limit of determination [431]. In addition to these

figures, it must be noted that ref. 427 contains a lengthy work-up procedure and ref. 429 a postcolumn alkalinization process to optimize the fluorescence response, limiting the use of the method. Generally the fluorescence methods give much cleaner chromatograms. A variety of internal standards have been used, none of them similar to labetalol, which is a common disadvantage.

Non-derivatized captopril does not chromatograph very well [416]. C_{18}-columns are preferred and the pH should be below 3 if a single peak is to be obtained. The limit of determination is around 50 nmol/l (10 ng/ml) for three of the methods [416, 418, 422], which is sufficient.

Diazoxide, being a very weak protolyte, can be separated on a reversed-phase system with a mobile phase only containing methanol and water [423]. UV detection is used and the limit of determination is 400 nmol/l (100 ng/ml).

9.3.3. Non-chromatographic methods

Two methods for determination of captopril with RIA have been published [414, 415]. One of them [414] measures captopril alone and the other [415] total captopril, i.e. unchanged captopril, reduced disulphide and reduced mixed disulphide forms. While most other methods for captopril are complex and laborious, the RIA methods have facilitated the determination of captopril in biological fluids. Because of the reactive nature of the captopril thio-group, captopril was measured as the N-ethylmaleimide complex, total captopril after reduction with tributyl phosphine. The limit of determination of the assay is 25 nmol/l (5 ng/ml) [414] and 100 nmol/l [415], respectively.

10. DIURETICS

10.1. Thiazides and benzene sulphonamide derivatives

The following substances are treated here: bumetanide, chlorthalidone, furosemide, hydrochlorothiazide, mefruside, methyclothiazide and metolazone.

10.1.1. Sampling and work-up procedures

Chlorthalidone and mefruside exhibit strong affinity to red blood cells, a fact that has to be considered for proper treatment of collected blood samples when plasma or erythrocytes are to be analysed. The concentration of chlorthalidone found in erythrocytes is 50—100 times higher than in plasma [433]. The partitioning process is slow so that chlorthalidone in blood samples drawn during the first few hours after dose has not reached the equilibrium distribution. Further, the distribution is temperature-dependent and more than 50% too low plasma concentrations may be found compared to in vivo values if the plasma is not separated immediately. Mefruside occurs in ca. 30 times higher concentrations in erythrocytes than in plasma [434]. In this case, the distribution process is very rapid, and blood samples, although centrifuged immediately after they are drawn, exhibit lower plasma concentrations than in vivo, due to a decrease in sample temperature. Still, Fleuren et al. [434] found that the decrease after direct centrifugation was more reproducible than when the blood samples were allowed to stand during a certain period before centrifugation. Hydrochlorothiazide has a less pronounced affinity to red blood cells [435]. For some of the drugs within this group precautions during collection

of blood samples must be taken if relevant results are to be obtained. However, in most of the procedures given this has not been considered.

The work-up procedure prior to the chromatographic separation and determination either comprises extraction with proton-accepting solvents such as ethyl acetate, diethyl ether and methyl isobutyl ketone or in some LC methods protein precipitation. Low extraction recoveries, 70—80%, were reported for hydrochlorothiazide [436], chlorthalidone [437], bumetanide [438] and for metolazone [439], while furosemide seems to be more easily extracted [440—442]. Column extraction on to Sephadex G-15 as an alternative to solvent extraction gave comparable recovery for hydrochlorothiazide [443]. In a recent paper on chlorthalidone in whole blood [444], extraction to Bond-Elut C_{18} was chosen. The suggested procedure seems neither to be rapid nor to give precise or accurate results.

A fully automated LC system is demonstrated for hydrochlorothiazide in blood plasma using the Technicon FAST-LC® apparatus [445]. Extraction is made by a mixture of ethyl acetate, chloroform and isopropanol, which is then evaporated and the extract reconstituted in an aqueous phase and injected into the liquid chromatograph.

Protein precipitation was employed for analysis in plasma for bumetanide [446], chlorthalidone [444] and furosemide [447—450], generally with high recoveries. In furosemide samples 4-chloro-5-sulphamoyl anthranilic acid is reported to be present as a metabolite or decomposition product [441, 451].

10.1.2. Chromatographic methods

10.1.2.1. Gas chromatography. GC methods are based on alkylation of the sulphonamide group and other groups containing active O- or N-bonded hydrogen atoms. Successful derivatization has been achieved using extractive methylation [452], on-column methylation [453] and alkylation with dimethylformamide dimethylacetal [454]. Extractive methylation is the most common technique [433—435, 452, 455, 456]. The drug is extracted with a suitable solvent, such as methyl isobutyl ketone for chlorthalidone [433, 452, 456] and diethyl ether for furosemide [455] and mefruside [434], and back-extracted to alkaline aqueous solution in order to eliminate sample components that would disturb the derivatization reaction. The drug anion is then extracted as an ion pair with tetrahexylammonium into dichloromethane where it reacts with methyl iodide under mild conditions.

The methyl derivatives of chlorthalidone, mefruside, furosemide and hydrochlorothiazide have been separated on packed column using ECD or nitrogen-selective detection. We have found no GC methods published after 1980, and no applications of capillary GC.

The methyl derivatives elute at fairly high temperatures, around 250°C, while the dimethylaminomethylene derivative of mefruside elutes at temperatures above 300°C.

High assay sensitivity has been obtained with both ECD and nitrogen-selective detection, the most sensitive assays having a lower limit around 7 nmol/l (2 ng/ml) with both detector types.

10.1.2.2. Liquid column chromatography. The chromatographic systems for the diuretic drugs are as a rule composed of an octadecyl-bonded stationary phase and a mobile phase of acetate or phosphate buffer, pH 2—5, and aceto-

nitrile or methanol as organic modifier. In a few cases [436, 445] tetrabutylammonium was added to the mobile phase, and at the pH used by Weinberger and Pietrantonio [445] it slightly increased the retention of hydrochlorothiazide. With few exceptions the chromatographic behaviour of the diuretics is not adequate in the separation systems suggested. A mixture of water and methanol without control of pH [443, 457, 458] seems not to be appropriate, neither is addition of sodium acetate to acetonitrile—water [437] for the chromatographic performance of chlorthalidone.

Detection of the diuretic drugs in the eluent is made at 226 nm or 272 nm depending on what compound is monitored. Furosemide is strongly fluorescent, which is utilized for selective detection [440, 442, 449, 459]; this detection principle is also used for bumetanide [438, 446] and metolazone [458]. The limit of determination is most often in the range 30—150 nmol/l (10—50 ng/ml), which in most instances is sufficient for control of therapeutic levels. For pharmacokinetic studies it is doubtful in many cases whether the methods are sensitive enough for a complete evaluation of the elimination of the drugs.

10.1.2.3. Thin-layer chromatography. Two fluorometric methods were developed for hydrochlorothiazide in plasma [460]. In the first method the substance is hydrolysed and the liberated amino group is diazotized and coupled to a fluorescent group prior to TLC separation. In the second method the inherent fluorescence of hydrochlorothiazide is utilized for detection. The two methods give about the same sensitivity of about 70 nmol/l (20 ng/ml). Furosemide and its metabolite or decomposition product, 4-chloro-5-sulphamoyl anthranilic acid, were determined in plasma by TLC and fluorescence detection after either protein precipitation [447] or extraction with diethyl ether [441]; 30 nmol/l (10 ng/ml) furosemide in plasma could be measured.

10.2. Potassium-saving diuretics

In this group spironolactone, canrenoate and amiloride will be discussed.

Spironolactone and canrenoate are competitive mineralocorticoid antagonists which can both form canrenone. Dethioacetylation of spironolactone gives this metabolite which for a long time was considered to account for the major activity of spironolactone. Canrenone is also formed by lactonization of the γ-hydroxycarboxylic acid group of canrenoic acid, the corresponding acid to canrenoate.

Almost all studies on these two drugs, evaluation of results and conclusions were, besides pharmacological measurements, based on a fluorometric method [461] which as such or after modification was supposed to be selective for canrenone. However, after the introduction of LC methods [462, 463] it became evident that previous comparative studies on spironolactone and canrenoate and found correlations between "canrenone" concentration and pharmacological effect had to be reevaluated. Canrenone was responsible for only 10—25% of the effect [464]. The three LC methods developed were based on solvent extraction and separation on silica with UV detection. Down to 30 nmol/l (10 ng/ml) could be determined. Later on LC methods employing C_{18}-bonded phases were presented [465, 466].

Amiloride is a guanidine derivative with inherent fluorescent properties which have been utilized for detection in LC and TLC methods. It is a hydrophilic compound and extraction with butanol—diisopropyl ether prior to TLC separation gave only 62% recovery [467]. High sensitivity was achieved and less than 1 ng/ml (5 nmol/l) could be determined. In an LC method [468] ethyl acetate was the best of ten tested solvents and gave a recovery of 66% from plasma compared to 71% from water. LC separation on a C_{18}-column and fluorometric detection completed the method [468]. Less than 40 nmol/l (10 ng/ml) could be determined. Protein precipitation with zinc sulphate and barium hydroxide was combined with a similar LC system for amiloride and other drugs [469].

11. CHROMATOGRAPHIC PROCEDURES IN RELATION TO OTHER ASSAY TECHNIQUES

The demand for drug assay in a biological fluid can be different in a research-orientated situation as compared with a test for compliance or follow-up of a given therapy. This is in some instances the borderline between the two different approaches to be discussed — chromatographic techniques and non-chromatographic techniques.

In the clinical situation the aim is to decrease the need for instrumental sophistication and methodological complexity, as discussed in an interesting paper on clinical monitoring of therapeutic drugs [470]. The rapid delivery of results might therefore be more essential than the precision. In many instances there is more interest in observing the pharmacological response of a treatment than relying on concentration measurements as for some cardiovascular drugs. The pharmacological effect is often the sum of action of the parent drug and its active metabolites. Before any chromatographic method can give information on active metabolites, these have to be isolated and evaluated pharmacologically. A new bioanalytical technique to obtain a measure of the total pharmacological activity (in terms of the parent drug) is radioreceptor assay. This has been discussed briefly in two recent review papers [471, 472]. Among the cardiovascular drugs this possibility seems to be realistic, but differences in the affinity of various receptors have to be considered [471].

In pharmacokinetic, biopharmaceutical, or clinical pharmacological studies, it is generally agreed that the results should be precise in order to reveal differences between compounds, formulations or patients. The best approach to obtain this accurate information is obviously via chromatographic methods, which can distinguish between closely related compounds, and make interference from metabolites and other sample components unlikely. The change in physicochemical properties brought into the metabolite compared with the drug itself, is often large and no real challenge for the chromatographic separation procedure.

Even though the financial investment for GC—MS is considerable, the accuracy of the results can sometimes not be guaranteed with other methods [473, 474]. Less accurate methods can lead to totally wrong conclusions, which from the economical and ethical points of view must be carefully considered [475]. For substances which lend themselves to MS detection but where introduction by GC is impractical, LC—MS may come to play an im-

portant role. Eckers et al. [476] used microbore LC—MS for determination of reserpine.

In a limited number of cases the only conceivable way to quantify a drug in biological fluids from a sensitivity point of view is radioimmunoassay. The selectivity has to be established for a given antibody, before a new drug assay can be introduced and accepted for general use. The high sample capacity of RIA is impressive and is probably outstanding compared to other techniques, which explains the great interest shown. The work involved in raising antibodies is time-consuming and requires a thorough know-how to evoke the most selective response. The combination of chromatographic separation and RIA can give the desired selective information but is an off-line technique with low sample capacity.

An interesting paper on ion-selective electrodes for some β-adrenergic and calcium blockers was recently published [477]. It points out the possibility for the measurement of amines as ion pairs with dinonylnaphthalene sulphonic acid and a response time for concentrations above 10^{-5} *M* of a few seconds. This paper demonstrated the determination of acebutolol, diltiazem, nicardipine, and verapamil and illustrates the great interest in cardiovascular drug analysis. There will continue to appear interesting approaches, of both a chromatographic and a non-chromatographic nature.

12. CONCLUDING REMARKS

The development of instrumental techniques has resulted in improved tools for the bioanalytical chemist. In GC, capillary columns offer improved separations, compared with packed columns, as well as better performance when small amounts of analyte are to be separated. Much work still remains to be done to exploit fully the possibilities of modern GC in the determination of different classes of cardiovascular drugs. The development of mass spectrometers leads, on one hand, towards sophisticated instruments equipped for different ionization techniques, including NICI. On the other hand, new instruments appear on the market which are suitable as mass-selective GC detectors. This makes methods using SIM accessible for laboratories without highly qualified MS specialists.

In LC, the miniaturization of columns has led to lowered detection limits, as has the development of better fluorometric, photometric and electrochemical LC detectors. While mass fragmentography with a GC inlet has gained great importance in quantitative bioanalysis during the last decade, the corresponding LC—MS technique is still at an early stage of development.

The analytical chemist will thus rely on instruments that are more efficient in terms of selective detection. As a consequence, sample work-up procedures can be made simpler. This can, however, only partially replace knowledge of the chemical behaviour of the analyte. A close insight into the chemistry involved in the sampling process and in different steps of sample treatment is necessary to secure the required accuracy of an analytical method.

13. SUMMARY

Methods for the determination of cardiovascular drugs in blood and plasma

are critically reviewed with emphasis on gas and liquid chromatographic techniques. The importance of the various procedures is discussed, in particular sample work-up where the conditions for isolation and derivatization of the compounds are decisive for the accuracy and precision of the methods. Compared with other assay techniques chromatographic methods are generally to be preferred owing to their better selectivity.

In the review the following groups are discussed: digitalis glycosides, antiarrhythmic agents, β-adrenoceptor antagonists, vasodilating agents, antihypertensive compounds, and diuretics.

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