Journal of Chromatography, 493 (1989) 105–115
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4806

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE SIMULTANEOUS DETERMINATION OF THE ENANTIOMERS OF CARVEDILOL AND ITS O-DESMETHYL METABOLITE IN HUMAN PLASMA AFTER CHIRAL DERIVATIZATION

EUGENE J. EISENBERG*, WESLIA R. PATTERSON and G. CLARE KAHN

Department of Drug Metabolism, SK&F Labs , P.O Box 1539, King of Prussia, PA 19406-0939 (U.S.A.)

(First received December 7th, 1988; revised manuscript received March 31st, 1989)

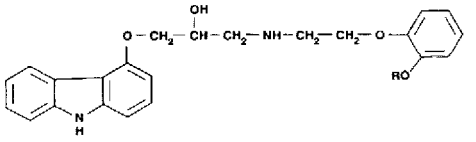
SUMMARY

Quantitative methodology for the simultaneous high-performance liquid chromatographic (HPLC) resolution and determination of the enantiomers of carvedilol, a new multiple-action antihypertensive agent exhibiting both vasodilator and β-blocking activity, and its active metabolite, O-desmethylcarvedilol, in human plasma is described. The method involves reversed-phase solid-phase extraction of the analytes, followed by derivatization of the extract with the chiral reagent, 2,3,4,6,-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate and injection of the resultant diastereoisomers onto a reversed-phase HPLC column coupled to a fluorescence detector. Both pairs of diastereoisomers formed are completely resolved within 12 min (resolution for the respective pairs is 2 26 and 3 32) and the baseline is clean and free from extraneous peaks. The assay is linear over the range 0.6-80 ng/ml of human plasma with a lower limit of detection of approximately 100 pg on-column for each of the enantiomers. The method can be adapted for a number of structural analogues of carvedilol and is currently applied in support of preclinical and clinical studies of the drug.

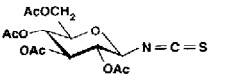
INTRODUCTION

Carvedilol {SK&F 105517, BM 14.190, (±)-1-(carbazol-4-yloxy)-3-[2-(2-methoxyphenoxy)]ethylamino-2-propanol} (Fig. 1) is a new multiple-action antihypertensive agent exhibiting both vasodilator and β-adrenergic blocking activity. It is being developed for the treatment of essential hypertension.

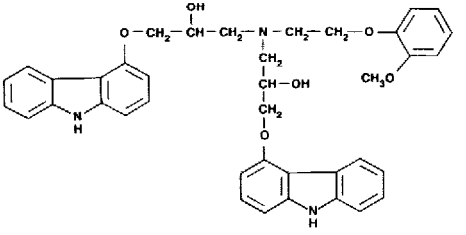
0378-4347/89/$03.50



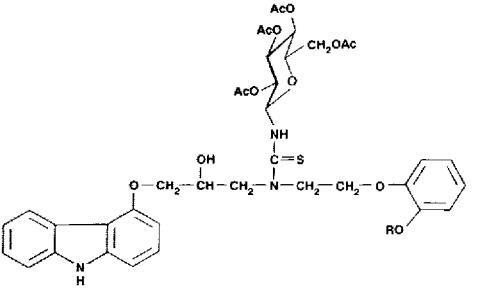
Carvedilol (R = CH_3)
O-Desmethylcarvedilol (R = H)



GITC



N,N-Bis-Carvedilol (Internal Standard)



Thiourea Derivative

Fig. 1. Structures of carvedilol, O-desmethylcarvedilol, GITC, the thiourea derivative and N,N-bis-carvedilol (the internal standard).

Pharmacological studies [1] indicate that β-blockade is limited to the $S(-)$ enantiomer whereas both enantiomers exhibit vasodilator activity. O-Desmethylcarvedilol {BM 14.242, (±)-1-(carbazol-4-yloxy)-3-[2-(2-hydroxyphenoxy)]ethylamino-2-propanol} (Fig. 1) is an active metabolite of carvedilol. Preliminary data suggest that it is a strong β-adrenergic antagonist but a weak vasodilator. The pharmacological activity of its individual enantiomers has yet to be characterized. In the case of several β-adrenergic receptor blocking agents, especially those that are highly metabolized, significant differences in the pharmacokinetics of the enantiomers have been described [2]. A simple, precise and sensitive stereospecific method for the separation and quantitation of the drug and its active metabolite(s) in body fluids is essential in order to determine the degree of stereospecificity exhibited in the metabolism and disposition of carvedilol.

Indirect chiral resolution of the enantiomers via formation of diastereoisomer derivatives followed by reversed-phase high-performance liquid chromatographic (HPLC) separation is an attractive alternative to direct chromatographic resolution on chiral phases which are generally less efficient, less stable and more expensive. A number of stereoselective methods utilizing solvent extraction of a drug followed by on-column chiral derivatization has been reported for quantitation of β-adrenergic antagonists in biological fluids [3–8].

R(+)-Phenylethyl isocyanate, *S*(−)-N-trifluoroacetylprolyl chloride and *S*(+)-naphthylethyl isocyanate are among the most commonly used derivatizing reagents. All methods reported to date involve tedious solvent extraction procedure. Some require an additional clean-up step to remove an excess of the reagent while others utilize repeated reconstitutions in solvents suitable for the derivatization reaction and/or HPLC. Among the problems associated with these methods are, also, optical impurity of the enantiomeric derivatizing reagent, resulting in a mixture of four stereoisomers instead of two, and inadequate diastereoisomer resolution. A recently reported method for quantitation of the enantiomers of propranolol and its active metabolite, hydroxypropranolol, in human serum while utilizing derivatization with stereochemically pure chiral reagent, 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) (Fig. 1), still requires a time-consuming solvent extraction followed by reconstitution in the HPLC mobile phase [8].

In this paper we describe a new stereospecific HPLC method for the simultaneous determination of the enantiomers of carvedilol and its active metabolite, O-desmethylcarvedilol (ODMC) using solid-phase extraction (SPE) of racemic carvedilol and the metabolite from human plasma, followed by derivatization with GITC and direct injection of the reaction mixture into conventional reversed-phase HPLC column.

EXPERIMENTAL

Reagents and chemicals

Carvedilol, ODMC·HCl and the internal standard, N,N-bis-carvedilol (Fig. 1), were supplied by the Boehringer Mannheim (Mannheim, F.R.G.). A solution of guanidine hydrochloride (8M sequanal™ grade) was purchased from Pierce (Rockford, IL, U.S.A.), triethylamine from Sigma (St. Louis, MO, U.S.A.) and acetonitrile (HPLC grade) from J.T. Baker (Phillipsburg, NJ, U.S.A.). Derivatizing reagent GITC (2,3,4,6-tetra-O-acetyl-β-glucopyranosyl isothiocyanate) was purchased from Polysciences (Warrington, PA, U.S.A.). Phosphoric acid and extraction columns PrepSep™ R C_{18} were purchased from Fisher Scientific (Fair Lawn, NJ, U.S.A.).

Chromatographic system

The chromatographic system consisted of an HPLC pump (EM Science/Hitachi, Model 655A-12), an autosampler (EM Science/Hitachi, Model 655A-40) and a fluorescence detector (EM Science/Hitachi, Model F-1000) set at an excitation wavelength of 285 nm and emission wavelength of 360 nm. An Ultrasphere ODS analytical column (75 mm×4.6 mm, 3 μm, Beckman Instruments) was used at room temperature. The mobile phase consisted of a mixture of acetonitrile–methanol–water–1 *M* aqueous triethylamine, adjusted to pH 2.5 with phosphoric acid (29:29:41.5:0.5, v/v) delivered at flow-rate of 1.6 ml/min. The chromatographic data were collected in real time (CIS/CALS,

Beckman) with a chromato-integrator (EM Science/Hitachi, Model D-2000) as a back-up and further processed after examination of each chromatogram and the determination of a user-defined baseline.

Preparation of standards

Stock solutions of carvedilol, ODMC·HCl and N,N-bis-carvedilol were prepared in dimethylformamide, methanol and acetonitrile, respectively, and stored at −20°C. Diluted solutions in water [1 μg/ml carvedilol, 1.09 μg/ml ODMC·HCl (equivalent to 1 μg/ml ODMC as free base) and 1 μg/ml N,N-bis-carvedilol] were prepared weekly and stored at +4°C.

Solid-phase extraction procedure

Extraction columns were placed in luer fittings in the top of Vac Elut SPS 24^{TM} vacuum manifold (Analytichem International, Harbor City, CA, U.S.A.) and activated with 10 ml of the elution solvent [acetonitrile–water–1 *M* aqueous triethylamine, adjusted to pH 2.5 with phosphoric acid (80:17:3, v/v)] followed by 2 ml of wash solvent [acetonitrile–water (35:65, v/v)]. A vacuum of 10–20 in·Hg was used to force solvents through the columns. Plasma samples (1 ml) were diluted with 1 ml of 8 *M* guanidine hydrochloride solution and applied to the columns. After plasma was applied, the columns were washed with 2 ml of the wash solvent and the analytes were eluted with two successive 200 μl aliquots of the elution solvent.

Chiral derivatization

A 50-μl volume of 100 m*M* triethylamine solution in acetonitrile and a 10-μl volume of 20 mg/ml GITC in acetonitrile were added to the extracts. After a brief vortexing, the samples were allowed to stand at room temperature for 30 min. A 150-μl volume of 0.5% phosphoric acid in water was added at the end of the incubation. Samples of the resulting mixture (50–100 μl) were injected onto the chromatographic column.

Validation procedures

A diluted stock solution of carvedilol and ODMC·HCl was added to drug-free human plasma to obtain concentrations of 160 ng/ml carvedilol and ODMC as free base (equivalent to 80 ng/ml individual enantiomers). This stock solution was diluted serially to provide a standard curve over the concentration range 0.625–80 ng/ml of the respective enantiomers. Each of the standard curves included eight standards and a blank plasma sample. A 100-μl aliquot of diluted stock solution of the internal standard was added to each of the plasma standards and samples. Peak-height ratios of each enantiomer to internal standard were plotted versus the enantiomer concentrations. The slopes and intercepts of the standard curves were estimated by least-squares linear regression. The method was validated by performing replicate analyses ($n=6$)

of spiked plasma pools (3.75, 16 and 60 ng/ml of both $S(-)$ and $R(+)$ enantiomers of carvedilol and ODMC) on three different days. Concentrations were determined by comparison with the corresponding standard curve prepared on the day of analysis. The analysis of variance (ANOVA) technique was applied to the data (one-way classification) to determine if the between-day variability had a significant effect on the precision of the method. The within-day and between-day precision as well as the overall accuracy of the method were estimated. The theoretical limits of detection (LOD) and quantitation (LOQ) were determined as described by Taylor [9]. The means of daily standard deviations were plotted at each of three concentrations (3.75, 16 and 60 ng/ml). After fitting a straight line to the data and extrapolating it to zero, the value of the standard deviation at zero concentration, S_0, was obtained. The LOD with 95% confidence was defined as $3S_0$ and the LOQ as $10S_0$. The recovery of ODMC and carvedilol in the extraction procedure was determined. Two pools of human plasma were spiked with ODMC and carvedilol standard solutions to obtain concentrations of 2 and 80 ng/ml. The replicate plasma samples were then extracted as previously described. Equal aliquots of the internal standard solution were then added to the extracted samples and to the corresponding non-extracted standards. The recoveries of ODMC and carvedilol were determined by comparison of peak-height ratios of the extracted plasma samples with those of the non-extracted standards. The recovery of N,N-bis-carvedilol was determined by comparison of peak heights of non-extracted standards in the elution solvent with those of the extracted plasma standards of the same concentration (100 ng/ml).

RESULTS

Selectivity

The GITC isothiocyanate group reacts rapidly and selectively with the secondary amino function of carvedilol and its metabolite in 30 min at room temperature generating the corresponding thiourea derivatives (Fig. 1). The resulting diastereoisomer pairs are separated after direct injection of the reaction mixture onto a reversed-phase column. Representative chromatograms for carvedilol and ODMC in human plasma are displayed in Fig. 2. All plasma samples were spiked with the internal standard. Chromatograms of derivatized extracts of drug-free plasma and plasma spiked with known amounts of carvedilol and ODMC are shown in Fig. 2A and B, respectively. A typical chromatogram of the derivatized extract of a plasma sample from a subject 1 h after oral administration of 25 mg carvedilol is shown in Fig. 2C. In all human plasma samples analyzed following oral administration of racemic carvedilol, the concentrations of the $R(+)$ enantiomer exceed those of the $S(-)$ enantiomer by two-to-five fold. The baseline is clean and free from interfering peaks. Chromatograms of derivatized plasma samples with no internal standard added

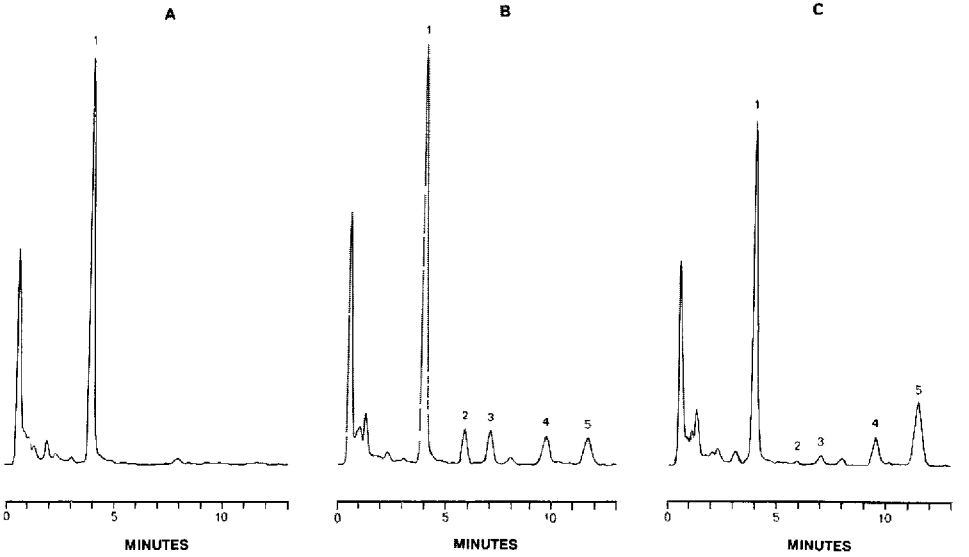


Fig. 2. Chromatograms of human plasma extracts spiked with 100 ng/ml internal standard after derivatization with GITC. Mobile phase: acetonitrile–methanol–water–1 *M* aqueous triethylamine, adjusted to pH 2.5 with phosphoric acid (29:29 41.5:0.5, v/v); flow-rate: 1.6 ml/min. (A) Drug-free plasma; (B) plasma spiked with racemic carvedilol and ODMC (10 ng/ml); (C) plasma from a human subject 1 h after oral administration of 25 mg of carvedilol. Peaks. 1 = internal standard; 2 = *S*(−)-ODMC; 3 = *R*(+)-ODMC; 4 = *S*(−)-carvedilol; 5 = *R*(+)-carvedilol.

TABLE I

SEPARATION CHARACTERISTICS OF DIASTEREOISOMERIC DERIVATIVES FORMED FROM THE ENANTIOMERS OF ODMC AND CARVEDILOL WITH GITC

Chromatographic, extraction and derivatization conditions are given in the Experimental section. t_R, k', α and R_s are defined in the text.

Compound	t_R (min)		k'		α	R_s
	S(−)	*R*(+)	*S*(−)	*R*(+)		
Carvedilol	9.9	11.9	10.0	12.2	1.22	3.32
ODMC	6.2	7.4	5.9	7.2	1.22	2.26

(not shown) produced no peaks that could interfere with the internal standard. Total chromatography time is 12 min, and peaks representing the internal standard and the diastereoisomer pairs derived from the *S*(−) and *R*(+) enantiomers of ODMC and carvedilol are symmetrical and well resolved. The retention time (t_R), capacity factor (k'), separation factor (α) and resolution (R_s) for respective pairs of diastereoisomers are given in Table I.

Linearity

Data on linearity and reproducibility of standard curves for the enantiomers of ODMC and carvedilol are shown in Table II. Linear regression parameters (correlation coefficients, slopes and y intercepts) of ODMC and carvedilol exhibited low coefficients of variation over the three-day validation period. Correlation coefficients for all linear regression plots exceeded 0.999.

Precision

The within-day precision expressed as the mean of the daily coefficients of variation (C.V., %) at each concentration was determined (Table III). For the $S(-)$ and $R(+)$ enantiomers of carvedilol and ODMC, the within-day precision ranged from 5.0 to 11.4% at 3.75 ng/ml, from 6.7 to 7.6% at 16 ng/ml and from 4.5 to 5.1% at 60 ng/ml of each enantiomer. ANOVA procedures (at a significance level of 0.05) indicated that there were no significant differences for the within-day and between-day variances in assay results for any of the enantiomers (Table III), and so data for all three days were pooled ($n=18$). For the $S(-)$ and $R(+)$ enantiomers of carvedilol and ODMC, the between-day precision, expressed as the C.V. of the pooled data at each concentration, ranged from 6.7 to 11.7% at 3.75 ng/ml, from 8.9 to 9.9% at 16 ng/ml and from 8.5 to 11.4% at 60 ng/ml (Table III).

Accuracy

The accuracy of the method was expressed as the ratio of predicted to actual concentration (C ratio). The means of daily accuracies for each of the enantiomers of carvedilol and ODMC were found to be in the range 0.92–0.95 for $S(-)$-carvedilol, 0.92–0.98 for $R(+)$-carvedilol, 0.96–1.02 for $S(-)$-ODMC and 1.00–1.06 for $R(+)$-ODMC (Table III).

TABLE II

LINEAR REGRESSION PARAMETERS FOR THE STANDARD CURVES OF THE ENANTIOMERS OF CARVEDILOL AND ODMC OVER THE THREE-DAY STUDY PERIOD

Plasma standard concentrations ranged from 0.625 to 80 ng/ml for the enantiomers of both carvedilol and the metabolite.

Compound	Slope (mean ± S.D., $n=3$) ($\times 10^{-3}$)	y Intercept (mean ± S.D., $n=3$) ($\times 10^{-3}$)	R^2 (mean ± S.D., $n=3$)
$S(-)$-Carvedilol	1.01 ± 0.15	4.01 ± 0.11	0.9996 ± 0.0001
$R(+)$-Carvedilol	1.05 ± 0.42	3.87 ± 0.12	0.9995 ± 0.0002
$S(-)$-ODMC	3.43 ± 0.17	3.04 ± 0.13	0.9999 ± 0.0000
$R(+)$-ODMC	2.41 ± 0.05	2.96 ± 0.13	0.9999 ± 0.0001

TABLE III

ACCURACY AND PRECISION DATA FOR THE DETERMINATION OF THE ENANTIOMERS OF ODMC AND CARVEDILOL IN HUMAN PLASMA

The F ratio, within-day and between-day precision and accuracy are defined in the text.

Compound	Plasma concentration (ng/ml)	Assay concentration (mean ± S.D.) (ng/ml)	F ratio[a]	Within-day precision ($n=6$) (%)	Between-day precision ($n=18$) (%)	Accuracy (mean ± S.D.) ($n=3$)
$S(-)$-Carvedilol	3.75	3.56 ± 0.41	2.02	11.39	10.74	0.95 ± 0.05
	16 00	15.52 ± 1.04	3.01	6.71	9.47	0.97 ± 0.06
	60.00	55.16 ± 2.50	2.99	4.54	11.32	0.92 ± 0.04
$R(+)$-Carvedilol	3 75	3.68 ± 0.16	1.91	3.37	6.72	0.98 ± 0 07
	16.00	15.70 ± 1.13	3.44	7.18	9.86	0.98 ± 0 04
	60.00	54.99 ± 2.56	3 00	4 66	11 20	0.92 ± 0.06
$S(-)$-ODMC	3.75	3.61 ± 0.22	2.25	5.99	11.70	0.96 ± 0 05
	16.00	16.96 ± 1.24	1.63	7.29	8.97	1.06 ± 0 03
	60.00	61.21 ± 3.06	2.82	5 00	8 48	1.02 ± 0 04
$R(+)$-ODMC	3.75	3.76 ± 0.23	1.94	6.06	11.10	1.00 ± 0.02
	16.00	16.99 ± 1.28	1.30	7.56	8.92	1.06 ± 0 04
	60.00	60.40 ± 3.07	2.94	5.08	11.40	1 01 ± 0.02

[a]The critical F value for the between-day variance was 3.59 (degrees of freedom = 2) at a significance level of 0.05

Method LOD and LOQ

For $S(-)$-ODMC the LOD was found to be 1.1 ng/ml and the LOQ 3.5 ng/ml. For $R(+)$-ODMC, $S(-)$- and $R(+)$-carvedilol the LOD was found to be 0.6 ng/ml and the LOQ 2.0 ng/ml.

Recovery

The recoveries of carvedilol and the metabolite in the extraction procedure were determined as follows:

$$R = A_{ext}/A_{std} \times 100\%$$

where R = recovery (the mean of five determinations) and A_{ext} and A_{std} = peak-height ratio of the analyte to the internal standard for the extracted samples and non-extracted standards, respectively. The recoveries of carvedilol and ODMC were found to be 94.2% (C.V. = 4.2%) and 87.4% (C.V. = 5.1%) at 2 ng/ml and 92.6% (C.V. = 3.7%) and 91.3% (C.V. = 6.4%) at 80 ng/ml. The recovery of N,N-bis-carvedilol was found to be 96.7% (C.V. = 6.7%, $n=5$).

Cross-validation

The method was extensively cross-validated using the non-enantioselective SPE assay [10] as a comparative method. Plasma samples from twelve sub-

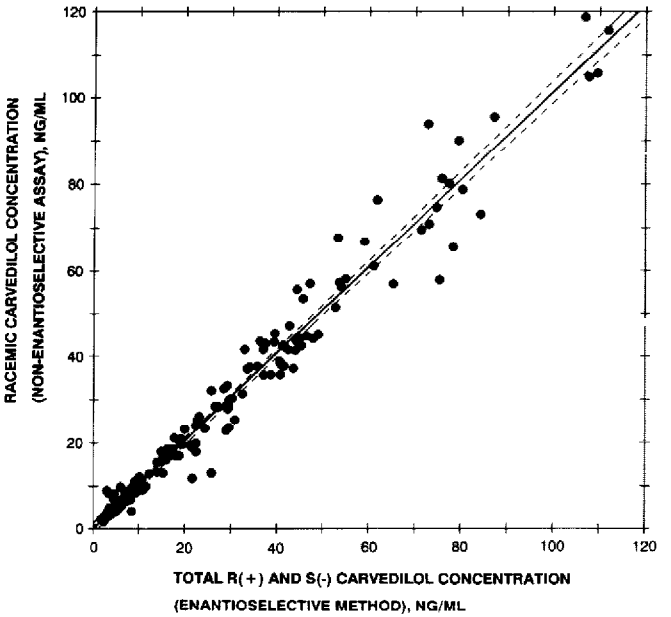


Fig. 3. Cross-validation of the enantioselective method against the non-enantioselective SPE assay. Plasma concentrations of carvedilol in 174 clinical samples were determined by using the enantioselective method (concentrations of carvedilol represented by sums of the individual enantiomers) (x axis) and the non-enantioselective SPE assay (concentrations of racemic carvedilol) (y axis). The solid line represents the linear regression line fitted to the data (slope = 1.01, intercept = 0.121, correlation coefficient = 0.971) and the dotted line a 95% confidence interval around the fitted line.

jects taken at 0.167–24 h following oral administration of 25 mg carvedilol (total of 174 samples) were analyzed using both methods. The sums of the concentrations of $S(-)$ and $R(+)$ enantiomers of carvedilol determined by the enantioselective method were plotted versus concentrations of racemic carvedilol determined in respective plasma samples (Fig. 3). The least-squares linear regression fit exhibited excellent correlation between two sets of data (correlation coefficient of 0.971). The line of unity lies well within the 95% confidence interval around the fitted line indicating the absence of proportional error with respect to the comparative method. The low concentrations of the $S(-)$ enantiomer of ODMC relative to the $R(+)$ enantiomer in the human plasma samples used did not allow the corresponding cross-validation to be made. However, validation samples for ODMC showed an excellent correspondence of the two methods (data not shown).

DISCUSSION

The chiral reagent GITC has been shown to react readily with primary and secondary amines under mild conditions forming thiourea derivatives [11].

The presence of triethylamine or other tertiary nitrogen bases apparently facilitates the reaction, possibly, via a transition state [12]. Derivatization of carvedilol and ODMC is easily achieved in chloroform, hexane, diethyl ether and acetonitrile, as well as in aqueous solutions of the latter (up to 30% water). The derivatives did not show signs of degradation after two weeks at room temperature. The glucoside isothiocyanate reagent is stereochemically pure and stable. It is, also, chemically stable for at least a year when stored in a desiccator at room temperature. GITC has been succesfully applied to HPLC resolution of the enantiomers of such bifunctional compounds as amino acids, catecholamines and β-adrenergic antagonists [8,11,13–16]. Hydrogen bonding between acetyl groups of the rigidly fixed acetyl glucosyl residue and a hydroxy group in the β position relative to the derivatized amino function apparently facilitates the formation of hydrophobic surfaces which are reportedly responsible for the separation of the diastereoisomer pairs formed in the reaction [13]. The sterically unhindered hydroxypropylamino moiety of both carvedilol and ODMC satisfies this precondition and good resolution of their respective derivatives is easily achieved on a conventional reversed-phase HPLC column. An SPE method for the isolation of carvedilol and ODMC from human plasma, recently developed in our laboratory [10], is very suitable for the direct derivatization with GITC. The elution solvent of the SPE (acetonitrile–water–tertiary nitrogen base) is used directly as a reaction medium. The addition of diluted phosphoric acid to terminate the reaction serves two purposes. First, it significantly improves the stability of the derivatives. Secondly, diluting the acetonitrile-rich reaction mixture to a resulting solvent that is weaker than the HPLC mobile phase allows improvement in the detection limits of the method as larger volumes may be injected onto the short HPLC column without distortion of early-eluting bands.

The internal standard, N,N-bis-carvedilol, exhibited consistently high recovery from the SPE procedure. It is unreactive with GITC and therefore does not compensate for the incomplete derivatization. However, evidently, the derivatization reaction was essentially quantitative. Under the conditions being used there was no evidence of underivatized material. (Non-derivatized carvedilol and ODMC are easily detected as peaks eluted at the retention times of 1.5–2 min.)

The method we have described is suitable for the determination of the enantiomers of carvedilol and its metabolite following rapid extraction from plasma on SPE cartridges and, as shown, is sufficiently sensitive to allow quantitation of the individual enantiomers after the administration of therapeutic doses of racemic carvedilol. Currently, the method is being successfully utilized in pharmacokinetic studies in man. The accuracy and reliability of the method has been well established. Preliminary results, also, demonstrate that the method may be applicable to chiral resolution of other racemic lipophilic β-

blocking drugs sharing structural features susceptible to the described extraction and derivatization procedures.

ACKNOWLEDGEMENTS

The clinical samples were kindly supplied by Dr. C. deMey, SK&F Institute for Applied Clinical Pharmacology, Goettingen, F.R.G., and the SPE analysis of total carvedilol was performed by Mr. M. Cryonak and Mr. M. diCristo.

REFERENCES

1 G. Sponer and B. Muller-Beckmann, Naunyn-Schmiedeberg's Arch. Pharmacol., 322 (Suppl.) (1983) R46.
2 J. Hermansson and C. Von Bahr, J. Chromatogr , 227 (1982) 113.
3 W. Henke, H. Spahn and E Mutschler, ISSX Meeting, Frankfurt, 1987, Abstract P42.
4 M.J. Wilson and T. Walle, J. Chromatogr., 310 (1984) 424.
5 P -H. Hsyu and K.M. Giacomini, J. Pharm. Sci., 75 (1986) 601.
6 A. Rouchouse, M. Lienard, J.T Burke and J.P. Thénot, The 1st International Symposium on Separation of Chiral Molecules, Paris, 1988, Abstract VI.21.
7 M.G Sankey, A. Gulaid and C.M. Kaye, J Pharm. Pharmacol., 36 (1984) 276.
8 A M J.A. Duchateau, C.C Pijnenburg and J.M.H. Conemans, The 1st International Symposium on Separation of Chiral Molecules, Paris, 1988, Abstract VI 3.
9 J.K. Taylor, in Quality Assurance of Chemical Measurements, Lewis Publishers, 1987, p. 79.
10 E.J Eisenberg, M.J. Cryonak, M.B. diCristo and G.C. Kahn, J. Pharm. Sci., submitted for publication.
11 H. Takahashi, N. Nimura and H. Ogura. Chem. Pharm. Bull., 27 (1979) 1130.
12 D.P.N Satchell and R.S. Satchell, Chem Soc. Rev., 4 (1975) 231.
13 T. Kinoshita, Y. Kasahara and N. Nimura, J. Chromatogr., 210 (1981) 77.
14 N. Nimura, H. Ogura and T. Kinoshita, J. Chromatogr , 202 (1980) 375.
15 J. Gal, J. Chromatogr., 307 (1984) 220.
16 A.J. Sedman and J. Gal, J. Chromatogr., 278 (1983) 199.