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Analysis of metoprolol enantiomers in human serum by liquid chromatography on a cellulose-based chiral stationary phase

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ABSTRACT

Metoprolol is a lipophilic, cardioselective β-adrenergic blocking agent commercially available as a racemic compound. A normal phase high-performance liquid chromatographic method was developed to directly determine individual enantiomeric concentrations of metoprolol in human serum. Separation of the enantiomers was accomplished by a cellulose-tris(3,5-dimethylphenylcarbamate) chiral stationary phase. Metoprolol enantiomers were detected by means of fluorescence with excitation and emission wavelengths of 275 and 315 nm, respectively. Standard curves were linear over the concentration range 12.5–400 ng/ml for each enantiomer. Within-day coeffient of variation was $< 15\%$ at all concentrations and the between-day coefficient of variation ranged from 4.1 to 11.2%. The limit of detection was determined to be 5 ng/ml for each enantiomer and the stereoselective resolution (α) of *R*- and *S*-metoprolol was 3.08. The assay was employed to determine enantiomeric serum concentrations of metoprolol in healthy male volunteers.

INTRODUCTION

Metoprolol is a lipophilic, cardioselective β-adrenergic blocking agent which is marketed as a racemic mixture. The drug is approved by the United States Food and Drug Administration to treat such diseases as angina pectoris, hypertension, arrhythmias, and patients surviving the acute phase of myocardial infarction [1]. *S*-(−)-Metoprolol has been reported to be more potent than *R*-(+)-metoprolol

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in both animal and human models [2,3]. Furthermore, stereoselective drug disposition and genetically determined polymorphic drug metabolism have been cited as potential determinants of pharmacological response [4–6]. Since each of these processes apply to metoprolol, it is imperative to evaluate the disposition of each enantiomer when considering the pharmacokinetics and pharmacodynamics of this agent.

Although several methods have been used to separate the enantiomers of metoprolol, few of these methods have been employed to quantify enantiomeric concentrations of metoprolol in biological fluids. Separations of *R*- and *S*-metoprolol have been accomplished by high-performance liquid chromatography (HPLC) with 10-(+)-camphor sulfonate as an ion-pairing reagent [7], chiral derivatization and separation by non-chiral HPLC [8–13] or chiral stationary phases (CSP) [14–18].

Chiral derivatization has been used for the quantitation of metoprolol enantiomers in plasma or urine by several groups. Hermansson and Von Bahr [8] used *tert.*-butoxycarbonyl-L-leucine anhydride to prepare diastereomeric derivatives of metoprolol. The derivatization required 30 min to complete and was followed by deprotection of the primary amino group. This method requires precise timing and controlled temperatures. The derivatization procedure of Sedman and Gal [9] utilized the commercially available reagents 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) and 2,3,4-tri-O-acetyl-α-D-arabinopyranosyl isothiocyanate (AITC). A selectivity (α) value of 1.24 was reported for metoprolol. The derivatization with GITC has been used by Lennard *et al.* [14] for the determination of the *R*/*S* ratio of metoprolol in urine for a pharmacogenetic study. Schuster *et al.* [15] were able to measure the enantiomers of metoprolol in plasma by this method with a detection limit of 10 ng/ml for each enantiomer (α = 1.34). Recently, a quantitative thin-layer chromatographic method to determine the urinary excretion of *R*- and *S*-metoprolol and two other β-adrenergic blockers was developed [12]. This method used *S*-(+)-benoxaprofen chloride as the chiral derivatizing agent followed by chromatography on silica gel plates, and the limit of detection by fluorescent densitometry was found to be 0.5 ng. Pflugmann *et al.* [13] used (*S*)-(−)-phenylethyl isocyanate (PEIC) as a chiral derivatizing agent to determine the concentrations of *R*- and *S*-metoprolol in plasma and urine. The detection limit of this HPLC assay was 2 ng/ml with a coefficient of variation of 7–8% at 25 or 100 ng/ml and was based on a method developed by Thompson *et al.* [19] for propranolol enantiomers. The largest α value (α = 3.35) for the separation of metoprolol enantiomers by chiral derivatization was achieved by Lindner *et al.* [20] using (*R*,*R*)-O,O-dibenzoyltartaric acid anhydride as the derivatizing agent. While no quantitative method for metoprolol was reported, this group recently described [21] a method utilizing HPLC to quantitate propranolol enantiomers in human plasma by derivatization with the commercially available agent (*R*,*R*)-O,O-diacetyltartaric acid anhydride which produced an α value of 1.84. Direct separation of metoprolol enantiomers

by HPLC on CSP has also been attempted. Armstrong *et al.* [22] reported a modest α value of 1.03 for metoprolol when two 25-cm long β-cyclodextrin columns were connected in series. Schill *et al.* [23] studied the resolution of metoprolol on an α_1-acid glycoprotein CSP silica column and found an α value between 1.44 and 1.64, depending on the mobile phase. Previously, a cellulose-tris (3,5-dimethylphenylcarbamate) CSP column was used to quantitate betaxolol enantiomers in rat hepatocytes [24]. The resolution factors (R_s) for several β-adrenergic blocking agents were reported, and the R_s value for metoprolol was 2.26. Straka *et al.* [25] used a similar cellulose-based CSP column for the quantification of propranolol enantiomers in human plasma. Recently, several HPLC assays to quantitate the enantiomers of metoprolol have been published using a cellulose-based CSP column [17,18] or a silica-bonded α_1-acid glycoprotein column [16].

This paper describes a direct normal-phase HPLC determination of metoprolol isomer concentrations after extraction from human serum. Separation of the enantiomers was accomplished on a cellulose-tris(3,5-dimethylphenylcarbamate) CSP. Eliminating the need to produce diastereomeric derivatives has greatly reduced the time and labor previously required to produce enantiomeric separation of metoprolol.

EXPERIMENTAL

Chemicals

R-Metoprolol hydrochloride and *S*-metoprolol hydrochloride were kindly donated by Ciba-Geigy (Basel, Switzerland), racemic metoprolol tartrate was purchased from Sigma (St. Louis, MO, U.S.A.) and the internal standard, racemic verapamil, was a gift from Knoll Pharmaceuticals (Whippany, NJ, U.S.A.). HPLC-grade hexane was purchased from Fisher Scientific (Fairlawn, NJ, U.S.A.) while HPLC-grade methanol and 2-propanol, as well as reagent-grade anhydrous diethyl ether were purchased from Mallinckrodt (Paris, KY, U.S.A.). Octylamine was purchased from Aldrich (Milwaukee, WI, U.S.A.). All other chemicals were reagent grade.

Instrumentation and conditions

All chromatography was performed with an LC-6A pump, RF-535 fluorescence detector and CR3A Chromatopac integrator (Shimadzu, Columbia, MD, U.S.A.). The samples were injected onto the column by means of a SIL-6A automatic injector (Shimadzu) with a 50-μl injection loop. Enantiomeric separation was carried out on a 25 cm $\times$ 0.46 cm I.D. stainless-steel analytical column packed with the CSP cellulose-tris(3,5-dimethylphenylcarbamate) polymer adsorbed onto macroporous silica (Chiralcel OD®) purchased from Diacel Chemical Industries (New York, NY, U.S.A.).

Metoprolol enantiomers were eluted from the column with a mobile phase consisting of hexane–2-propanol (90:10, v/v) which contained 10 m*M* octyla-

mine. The mobile phase flow-rate was maintained at 1.0 ml/min. The optimal excitation and emission wavelengths for fluorescence detection of the compounds of interest were determined to be 275 and 315 nm, respectively.

Calibration

A 50-μl aliquot of a standard racemic metoprolol solution in methanol (25, 60, 125, 250, 500 or 800 ng per 50 μl) was added to 1 ml of drug-free serum to prepare standard samples containing 12.5–400 ng/ml of each enantiomer. A 50-μl aliquot of a 1 μg/ml solution of racemic verapamil (internal standard) in methanol was then added to each standard.

Sample preparation

A 50-μl aliquot of a 1 μg/ml solution of racemic verapamil (internal standard) in methanol was added to each patient sample. A 500-μl volume of 0.5 *M* hydrochloric acid and 4 ml of anhydrous diethyl ether were then added, the samples were vortexed for 30 s and centrifuged at 2000 *g* for 5 min. The organic layer was removed and discarded. To the remaining aqueous layer, 100 μl of 2 *M* sodium hydroxide and 4 ml of diethyl ether were added, the tubes were vortexed for 60 s and centrifuged at 2000 *g* for 10 min. Finally, the organic layer was removed and placed into 12-ml centrifuge tubes, dried under a stream of helium gas in a water bath at 35°C and reconstituted with 150 μl of the mobile phase. A 25-μl aliquot of the final preparation was then injected onto the column.

Assay validation

Concentrations of *R*- and *S*-metoprolol were calculated from standard curves determined by the application of weighted least-squares regression analysis to peak-height ratios as a function of the enantiomeric concentration of standards [26]. The weight used at each concentration was the reciprocal of the observed sample variance, estimated by determining the variance of the peak-height ratios of the pooled data. Analysis of variance was performed on the cumulative data collected from nine duplicate standard curves run on nine separate days. The total variability (s_{total}), within-day component of variability (s_{wd}) and the between-day component of variability (s_{bd}) were determined as described by Amenta [27]. The s_{total} and s_{wd} were calculated by taking the square root of the corresponding sum of squares divided by their degrees of freedom. The s_{bd} was calculated by $(MS_{bd}/2)^{1/2}$ where MS_{bd} is the between-day mean squared error from the ANOVA table. An alternative determination of the within-day variability was performed from six replicates of each concentration on a single day. Extraction efficiency was determined by comparing the absolute peak heights of the extracted samples to the peak heights of the standard solutions (normalized for amount of drug) injected directly into the HPLC system.

RESULTS

R-Metoprolol, *S*-metoprolol and the internal standard were well separated with the hexane–2-propanol mobile phase. The calculated α value was 3.08 for the metoprolol enantiomers. Fig. 1 represents the chromatogram of blank human serum (A), an extracted sample spiked with racemic metoprolol (B) and an extracted patient serum sample collected 2 h after the ingestion of a 200-mg oral dose of metoprolol tartrate (C). Although the internal standard is a racemic compound, the enantiomers were not resolved on this column. Retention times for the *R*-and *S*-enantiomers were 6.13 and 14.34 min, respectively, and 9.80 min for the internal standard (Fig. 1B). The standard curves were linear throughout all standard concentrations. Mean (range) coefficients of determination (r^2) for *R*- and *S*-metoprolol were 0.990 (0.977–0.997) and 0.969 (0.928–0.995), respectively, for between-day curves. The corresponding r^2 values for *R*- and *S*-metoprolol within-day curves were 0.993 (0.988–0.997) and 0.986 (0.945–0.996), respectively. The extraction efficiency of this method of sample preparation was found to range between 63 and 79% for either the *R*- or *S*-enantiomer at the enantiomeric concentrations of 30, 125 and 250 ng/ml. The detection limit was determined to be 5 ng/ml (0.83 ng of each enantiomer on-column) based on a signal-to-noise ratio of 5:1.

Tables I and II summarize the accuracy and precision data. Metoprolol and

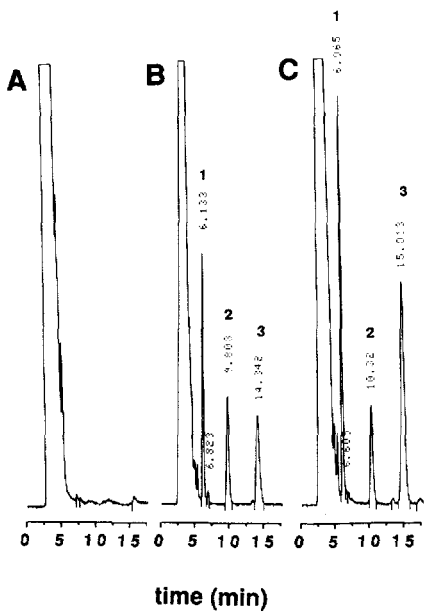

Fig. 1. Typical chromatograms of (A) blank human serum, (B) human serum spiked with 250 ng of racemic metoprolol and (C) human serum 2 h after oral administration of 200 mg racemic metoprolol. Peaks: 1 = *R*-metoprolol; 2 = internal standard; 3 = *S*-metoprolol.

TABLE I

ACCURACY AND PRECISION DATA FOR *R*- AND *S*-METOPROLOL

s_{total} represents the total variability (standard deviation), s_{wd} represents the within-day component of variability and s_{bd} represents the between-day component of variability. C.V. is coefficient of variation.

Concentration added (ng/ml)	Concentration measured (ng/ml)	n	s_{total}	C.V.$_{total}$ (%)	s_{wd}	C.V.$_{wd}$ (%)	s_{bd}	C.V.$_{bd}$ (%)
R-Metoprolol								
12.5	12.5	17	0.85	6.80	0.76	6.08	0.66	5.28
30	30.0	15	1.97	6.57	2.15	7.17	1.26	4.20
62.5	63.9	16	5.95	9.31	5.07	7.93	4.82	7.54
125	123.2	16	8.99	7.30	5.40	4.38	7.94	6.44
250	254.6	17	12.10	4.75	8.42	3.31	10.48	4.12
400	412.4	18	31.58	7.66	27.60	6.69	25.14	6.10
S-Metoprolol								
12.5	11.7	15	1.47	12.56	1.51	12.91	1.01	8.63
30	28.8	15	3.42	11.88	3.68	12.78	2.22	7.71
62.5	65.2	16	8.81	13.51	7.19	11.03	7.32	11.23
125	129.0	16	11.26	8.73	5.58	4.33	10.25	7.95
250	263.8	17	24.64	9.34	24.30	9.21	17.66	6.69
400	403.7	18	51.25	12.70	59.90	14.84	27.82	6.89

TABLE II

ACCURACY AND PRECISION DATA FOR *R*- AND *S*-METOPROLOL

From an alternative method of determination of within-day variability.

Concentration added (ng/ml)	Concentration measured (ng/ml)	*s*	C.V. (%)
R-Metoprolol			
12.50	12.43	0.45	3.64
30.00	28.97	1.45	4.99
62.50	59.1	5.24	8.86
125.00	127.65	3.75	2.94
250.00	260.61	15.78	6.06
400.00	431.02	24.01	5.57
S-Metoprolol			
12.50	12.84	1.19	9.27
30.00	29.81	1.84	6.17
62.50	66.2	4.53	6.85
125.00	124.03	11.48	9.25
250.00	260.01	20.30	7.81
400.00	374.95	36.56	9.75

the internal standard appear to be relatively stable chemical entities, as no appreciable degradation of the compounds was observed while they were stored in methanol at 4°C for 90 days.

DISCUSSION

The assay described in this paper provides a method by which enantiomeric concentrations of metoprolol may be determined by means of normal-phase HPLC with a cellulose-tris(3,5-dimethylphenylcarbamate) CSP. This method offers significant advantages over earlier methods. The α value of 3.08 achieved by this column was much higher than those reported for other assays, with the exception of the method reported by Persson *et al.* [16] who used an α_1-acid glycoprotein column. The R_s value was found to be 4.11, and the peaks of interest were essentially symmetrical with symmetry values for *R*- and *S*-metoprolol of 1.0 and 1.19, respectively. Sample preparation was relatively simple and required less time per sample compared to procedures requiring derivatization of the enantiomers. Furthermore, retention times were shorter when compared to previous reports of CSP assays [16–18]. While the extraction procedure described herein is somewhat more time-consuming than that reported by Rutledge and Garrick [17], our experience is consistent with that of Ching *et al.* [18], who found a single liquid–liquid ether extraction step to be insufficient in removing sub-

stantial interfering endogenous peaks found in the blank serum. Unlike Ching *et al.* [18], our method used an internal standard that appeared as a single peak and avoids the somewhat costly use of solid-phase extraction columns. Extraction efficiency did not appear to be concentration-dependent and was determined to be in the order of those seen with similar liquid–liquid extractions of this compound. Fig. 1A demonstrates the extraction of blank human serum with diethyl ether after alkalinization of the serum by sodium hydroxide. The endogenous potentially interfering substances observed at 6.60, 6.92 and 13.66 min were substantially removed by acidification of the serum with hydrochloric acid and extraction by diethyl ether prior to the alkalinization step.

The accuracy and precision estimates of the data were found to be acceptable for each enantiomer for the purpose of clinical pharmacokinetic studies. The detection limit of this assay was determined to be 5 ng/ml (for *R*- or *S*-metoprolol). The lower limit of reliable estimate may lie between this value and the lowest concentration of the standard curve (12.5 ng/ml) given the reasonable estimates of precision at that concentration (Tables I and II).

The maximum coefficient of variation can be seen from Table I to be less than 15%. The use of weighted regression dramatically improved the precision of the assay at the lower concentration ranges. This was evident by the inclusion of zero within the 95% confidence interval for each estimate of the intercept of each standard curve subsequent to weighted analysis. Prior to the application of weighting analysis, zero was not uniformly included within the 95% confidence intervals. The assay was linear over the concentration range used. The coefficient of determination exceeded 0.924 for all standard curves. Of note were the relatively larger coefficients of variation observed for the *S*-metoprolol compared to *R*-metoprolol. This may be a function of the longer retention time of the relatively broad *S*-enantiomer peak. Although the addition of an amine modifier improved the peak shape, the interaction of the CSP column with *S*-metoprolol appears to result in a less than optimal peak shape.

Several substances were evaluated for their potential to interfere with the quantification of metoprolol enantiomers. Table III summarizes the respective k' values of these compounds. No interference with any of the peaks of interest was observed with any of the listed compounds. Overall, the CSP was relatively stable and performed well under the conditions of this assay. Of note was an apparent difference in the inter-column performance. Experience with two different columns from the same manufacturer revealed substantial differences in retention times with the same flow-rate and mobile phase. The α values were found to be 2.73 and 3.08 for the metoprolol enantiomers on the two columns tested. This apparent lack of reproducibility between columns may be important to other applications of this methodology where α values approach 1.00. The column life is not yet known, but each column may be used for at least 1000 injections, in contrast to the relatively less stable α_1-acid glycoprotein column used by Persson *et al.* [16].

TABLE III

HPLC CAPACITY FACTORS (k') FOR *R*- AND *S*-METOPROLOL, VERAPAMIL AND OTHER COMPOUNDS OF POTENTIAL CONCOMITANT USE

Compound	k'
(*R*)-Metoprolol	0.78
(*S*)-Metoprolol	4.78
Verapamil	2.11
Procainamide	3.67
Quinidine	–[a]
Diazepam	–
Digoxin	–
Furosemide	–
Lidocaine	–
Prochlorperazine	–

[a] Not detectable under present conditions.

This assay was employed to evaluate the pharmacodynamics of metoprolol enantiomers in healthy male volunteers [28]. The logarithm of concentration *versus* time plots are illustrated in Fig. 2 for two subjects identified as one extensive metabolizer (EM) and one poor metabolizer (PM). The total (*R*- + *S*-metoprolol) area under the concentration–time curve (AUC) was determined to be 757 and 6937 ng·h/ml for the EM *versus* PM respectively. Other investigators [5] evaluating the two metabolic groups reported mean ± S.D. AUC values of 1246 ± 796 and 7250 ± 1220 for EMs and PMs, respectively. The AUC values calculated for each subject evaluated by this procedure compare favorably with these results.

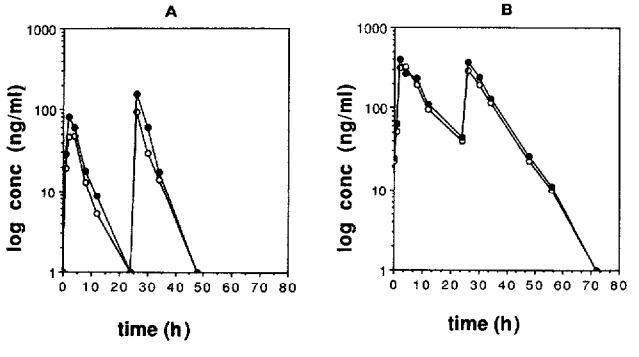

Fig. 2. Serum concentration–time profile for *R*-metoprolol (o) and *S*-metoprolol (●) in extensive (A) and poor (B) metabolizers after a 200-mg dose of racemic metoprolol at 0 and 24 h.

In conclusion, the assay described in this paper is capable of measuring clinically significant enantiomeric metoprolol concentrations in human serum with reasonable accuracy and precision. Although the initial cost of purchasing the chiral column is relatively large, the simplicity and efficiency of this assay provide a novel and important method for the quantification of metoprolol enantiomers in human serum.

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REFERENCES

1 G. K. McEnvoy (Editor), *American Hospital Formulary Service Drug Information*, ASHP, Bethesda, MD, 1987, p. 821.
2 N. Toda, S. Hayashi, Y. Hatano, H. Okunishi and M. Miyazaki, *J. Pharmacol. Exp. Ther.*, 207 (1978) 311.
3 M. S. Lennard, G. T. Tucker, J. H. Silas, S. Freestone, L. E. Ramsey and H. F. Woods, *Clin. Pharmacol. Ther.*, 34 (1983) 732.
4 J. R. Idle, A. Mahgoub, R. Lancaster and R. L. Smith, *Life Sci.*, 22 (1978) 979.
5 M. S. Lennard, J. H. Silas, S. Freestone, L. E. Ramsey, G. T. Tucker and H. F. Woods, *N. Engl. J. Med.*, 307 (1982) 1558.
6 D. M. Roden, S. B. Reele, S. B. Higgins, R. F. Mayol, R. E. Gammans, J. A. Oates and R. L. Woosley, *N. Engl. J. Med.*, 302 (1980) 877.
7 C. Pettersson and G. Schill, *J. Chromatogr.*, 204 (1981) 179.
8 J. Hermansson and C. Von Bahr, *J. Chromatogr.*, 227 (1982) 113.
9 A. J. Sedman and J. Gal, *J. Chromatogr.*, 278 (1983) 199.
10 W. A. Konig, K. Ernst and J. Vessman, *J. Chromatogr.*, 294 (1984) 423.
11 G. Gubitz and S. Mihellyes, *J. Chromatogr.*, 314 (1984) 462.
12 G. Pflugmann, H. Spahn and E. Mutschler, *J. Chromatogr.*, 416 (1987) 331.
13 G. Pflugmann, H. Spahn and E. Mutschler, *J. Chromatogr.*, 421 (1987) 161.
14 M. S. Lennard, G. T. Tucker, H. F. Woods, J. H. Silas and A. O. Iyun, *Br. J. Clin Pharmacol.*, 27 (1989) 613.
15 D. Schuster. M. W. Modi, D. Lalka and F. M. Gengo, *J. Chromatogr.*, 433 (1988) 318.
16 B. A. Persson, K. Balmer and P. O. Lagerstrom, *J. Chromatogr.*, 500 (1990) 629.
17 D. R. Rutledge and C. Garrick, *J. Chromatogr.*, 497 (1989) 181.
18 M. S. Ching, M. S. Lennard, A. Gregory and G. T. Tucker, *J. Chromatogr.*, 497 (1989) 313.
19 J. A. Thompson, J. L. Holtzman, M. Tsuru, C. L. Lerman and J. L. Holtzman, *J. Chromatogr.*, 239 (1982) 470.
20 W. Lindner, C. Leitner and G. Uray, *J. Chromatogr.* 316 (1984) 605.
21 W. Lindner, M. Rath, K. Stoschitzky and G. Uray, *J. Chromatogr.*, 487 (1989) 375.
22 D. W. Armstrong, T. J. Ward, R. D. Armstrong and T. E. Beesley, *Science*, 232 (1986) 1132.

23 G. Schill, I. W. Wainer and S. A. Barkan, *J. Liq. Chromatogr.*, 9 (1986) 641.
24 A. M. Krstulovic, M. H. Fouchet, J. T. Burke, G. Gillet and A. Durand, *J. Chromatogr.*, 452 (1988) 477.
25 R. J. Straka, R. L. Lalonde and I. W. Wainer, *Pharm. Res.*, 5 (1988) 187.
26 L. Oppenheimer, T. P. Capizzi, R. M. Weppelman and H. Mehta, *Anal. Chem.*, 55 (1983) 638.
27 J. S. Amenta, *Am. J. Clin. Pathol.*, 49 (1968) 842.
28 R. J. Straka, K. A. Johnson and J. W. McBride, in preparation.