Journal of Chromatography, 497 (1989) 181–190
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4990

RAPID HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE MEASUREMENT OF THE ENANTIOMERS OF METOPROLOL IN SERUM USING A CHIRAL STATIONARY PHASE

DAVID R. RUTLEDGE* and CINDY GARRICK

Department of Pharmacy Practice, Wayne State University, College of Pharmacy and Allied Health Professions, Detroit, MI 48202 (U.S.A.)

(First received May 3rd, 1989; revised manuscript received August 11th, 1989)

SUMMARY

Metoprolol, a β-adrenergic blocker, is only available as a racemic mixture for clinical use. A high-performance liquid chromatographic method for the separation of the optical isomers (enantiomers) in human serum is described utilizing a commercially available column with a cellulose tris(3,5-dimethylcarbamate) chiral stationary phase. Separation of the enantiomers is accomplished without precolumn derivatization using a mobile phase of hexane–2-propranol–diethylamine (91:8:1, v/v). The method can be successfully applied to pharmacokinetic studies in man.

INTRODUCTION

Any molecule that contains at least one carbon atom with four different ligands attached to it is said to be chiral or asymmetric. Metoprolol is such a compound available as a racemic mixture for clinical and basic use. This chirality results in the production of two mirror images that cannot be superimposed on each other. Stereoselectivity in hepatic metabolism of drugs is documented from both in vitro and in vivo studies [1]. This is particularly true of metoprolol and other β-adrenergic receptor antagonists [2–4]. In fact, stereoselective oxidation of metoprolol was described in subjects phenotyped as

0378-4347/89/$03.50

'extensive' metabolizers, resulting in 1.5 times greater bioavailability for the *S*(−)-isomer when compared to the *R*-(+)-isomer [2]. Total body clearance and bioavailability of the two enantiomers were approximately the same in the subjects phenotyped as 'poor' metabolizers. Therefore when racemic metoprolol is used clinically it is administered as two different drugs with different pharmacokinetic and potentially pharmacodynamic properties in select patient populations. Timolol, commonly used topically in the treatment of glaucoma, is another example of a β-blocker with clinically significant differences in enantiomer disposition [5]. The *R*-enantiomer is considerably less potent at extraocular sites compared to the *S*-enantiomer (1/13 to 1/80), yet it provides a useful decrease in intraocular pressure (1/4 potency of the *S*-enantiomer) without the high risk of undesired effects.

Metoprolol, like the majority of commercially available drugs, is marketed as a racemic mixture [6]. The importance of drug enantiomers has been extensively reviewed elsewhere [7–9]. Several differences between enantiomers of a compound may potentially be clinically significant, i.e., the inactive form may potentate or oppose the desired and undesired effects of the active form, they may act at same or different sites, they may be eliminated by different metabolic pathways and they may produce metabolites with different activity or toxicity. Stereoselective receptor affinity, clearance, protein and tissue binding, metabolism and even drug interaction have been demonstrated for many agents [7,10–14]. In addition, age-associated stereoselective alterations in hexobarbital metabolism have been reported [15].

The lack of availability of simple analyses for enantiomers has slowed the development of our understanding of this important aspect of drug disposition and effect. Previous assay methods for the determination of metoprolol enantiomers in biological fluids involve derivatization steps [16–18] which many chromatographers would rather not use due to lack of experience. The development of an analysis that does not require this additional procedure is essential to facilitate further investigations in the important area of enantiomer pharmacokinetics and pharmacodynamics. Therefore a rapid and simple analytical procedure is presented based on the direct high-performance liquid chromatographic (HPLC) analysis of an extract of a relatively small sample volume using a cellulose tris(3,5-dimethylphenylcarbamate) chiral stationary phase (CSP). This procedure can successfully be applied to pharmacokinetic studies in man.

The biomedical application of the proposed methodology follows the recognition that the resolution of the enantiomers of metoprolol on the OD-CSP has been previously reported in a technical brochure issued by Daicel Chemical Industries (Fort Lee, NJ, U.S.A.) [19]. In addition, the development and use of the OD-CSP has been published [20,21]. The former paper reports the enantiomeric resolution of five β-blockers, e.g., alprenolol, oxyprenolol, propranolol, pindolol and atenolol by HPLC on thirteen CSPs composing of cellulose

triphenylcarbamate derivatives. All β-blockers were completely resolved on a cellulose tris(3,5-dimethylphenylcarbamate) column. The (+)-isomers, which may be assigned to the *R*-configuration, were eluted first in all cases. In addition, the methodology has also been applied by others for the resolution of the enantiomers of propranolol [22].

EXPERIMENTAL

Reagents and chemicals

The individual metoprolol enantiomers were a gift from Ciba-Geigy (Basle, Switzerland). The internal standard (+)-propranolol, was a gift from Wyeth-Ayerst (Princeton, NJ, U.S.A.). (+)-Propranolol was a 0.2 μg/ml solution in methanol (Mallinckrodt, Paris, KY, U.S.A.). HPLC-grade hexane, isopropyl alcohol (American Burdick and Jackson, Muskegon, MI, U.S.A.) and diethylamine (Aldrich, Milwaukee, WI, U.S.A.) were used for the mobile phase. Anhydrous diethyl ether was reagent grade (Fisher Scientific, Fair Lawn, NJ, U.S.A.). 4.0 *M* Sodium hydroxide (Mallinckrodt) was prepared from deionized water.

Instrumentation

The solvent delivery system was a constant-flow reciprocating pump (ConstaMetric III, LDC Milton Roy, Riviera Beach, FL, U.S.A.) and sample injection was performed using a Rheodyne Model 7126 syringe loading valve fitted with a 100.0-μl sample loop. The analytical stainless-steel precolumn (50 mm × 4.6 mm I.D.) and column (250 mm × 4.6 mm I.D.) was a CSP (J.T. Baker, Phillipsburg, NJ, U.S.A.) containing 10 μm of a cellulose tris(3,5-dimethylphenylcarbamate) polymer absorbed on macroporous silica.

The column effluent was monitored using a Schoeffel Model FS980 fluorescence detector (Kratos Analytical, Ramsey, NJ, U.S.A.) equipped with a 25.0-μl flow cell and utilizing an excitation wavelength of 274 nm; a 320-nm custom bandpass emission filter was employed (Andover, Lawrence, MA, U.S.A.). A Fisher Recordall Series 5000 strip chart recorder was used to record the detector response (Fisher Scientific, Lexington, MA, U.S.A.)

Chromatographic conditions

The mobile phase was hexane–2-propanol–diethylamine (91:8:1, v/v). The flow-rate was 1 ml/min, maintained by a pressure of 33.3 bar. The analysis was performed at room temperature. The chromatography on this system is illustrated in Fig. 1.

Emission spectra

Characterization of the absorbance spectra of the mobile phase with and without racemic metoprolol was carried out on a Beckman DU-8B spectropho-

tometer. Selection of the appropriate excitation wavelength was based on the maximum absorbance in the presence of the mobile phase.

Validation study

Stock solutions of metoprolol were prepared by dissolving 10 mg of base enantiomer in 100 ml of methanol. Serum standards were prepared by adding aliquots of the stock solution to drug-free human serum. Standard serum solutions containing 25.0, 50.0, 100.0, 200.0 and 400.0 ng/ml racemic metoprolol, i.e., 12.5–200 ng/ml for each enantiomer, were prepared for calibration curves with 35.0 and 300.0 ng/ml prepared to test accuracy and precision of the assay ($n=10$), i.e., 17.5 and 150 ng/ml for each isomer. Lower limits of detection were determined using serum standards with the limit of sensitivity defined as a signal-to-noise ratio of 4:1. Percentage extraction recovery ($n=3$) was determined by comparing peak-height ratios of chromatograms obtained from extracted plasma samples to those of the standard stock methanol solutions at a concentration of 100.0 ng/ml. The stability of a 100.0 ng/ml standard was determined after storage at −20°C for thirty days. Potential interfering substances were added to serum and extracted.

Extraction procedure

Metoprolol was extracted from serum by a single-step basic extraction procedure. To 1.0 ml of serum were added 100 µl of a methanolic solution of internal standard, 0.2 ng/µl (+)-propranolol. A 4 *M* sodium hydroxide solution (200 µl) was added along with 5 ml of diethyl ether into a borosilicate glass round-bottom test tube. The contents of the test tube were vortex-mixed for 1 min and centrifuged at 2000 *g* for 10 min. The ether layer was removed from the original tube by transfer pipette and collected into a glass conical tip test tube, then evaporated to dryness under a nitrogen stream in a water bath (37°C). For HPLC analysis, the extracted residue was redissolved in 100 µl of mobile phase, and a portion of the reconstituted residue (approximately 75 µl) was injected into the sample loop of the injection valve.

Subject

After fasting for 10 h, one healthy male volunteer (32 years old) took a single commercial metoprolol tablet containing 50 mg of racemic metoprolol every 12 h for five days. On the morning of the sixth day following an overnight fast, a single 50-mg tablet was again ingested. Fasting continued for 3 h. At timed intervals venous blood was drawn by venipuncture. Serum was collected and frozen (−20°C) until analyzed within 48 h. The area under the (+)- and (−)-metoprolol serum concentration versus time curves was measured by the linear trapezoidal method for the ascending portion of the curve and by the logarithmic trapezoidal method for the descending portion. The terminal log-linear phase of the serum concentration versus time profile for the enantiomers

was identified visually and the slope (terminal elimination rate constant, k_{el}) determined by log-linear regression analysis. The isomer area under the concentration versus time curve at steady state was calculated over the 12-h dosing interval. Metoprolol apparent oral clearance at steady state was calculated as dose divided by area under the curve from 0 to 12 h. The sum of the isomer concentrations were compared to total metoprolol concentrations using a previously published assay for metoprolol and its α-hydroxy metabolite [23].

RESULTS AND DISCUSSION

HPLC conditions

After characterization of the absorbance spectra, a plot of relative absorbance versus wavelength was made using a sample of racemic metoprolol in the mobile phase and comparing it with a sample of mobile phase alone. Peak absorbance was recorded at approximately 276 nm and therefore 275 nm was used for the excitation wavelength. This is relevant information because the usual excitation wavelength for most fluorescence assays for metoprolol is approximately 225 nm [10]. Mobile phases for the determination of racemic metoprolol and its α-hydroxy metabolite usually consist of a mixture of two solutions. The first solution is 1 l of a 1% acetic acid solution with 25 ml of 0.005 *M* 1-heptanesulfonic acid, i.e., solution A. Solution B is simply 1 l of acetonitrile with 25 ml of 0.005 *M* 1-heptanesulfonic acid. The mobile phase, therefore, consists of a mixture of the two solutions in a ratio of 70:30 (v/v) for

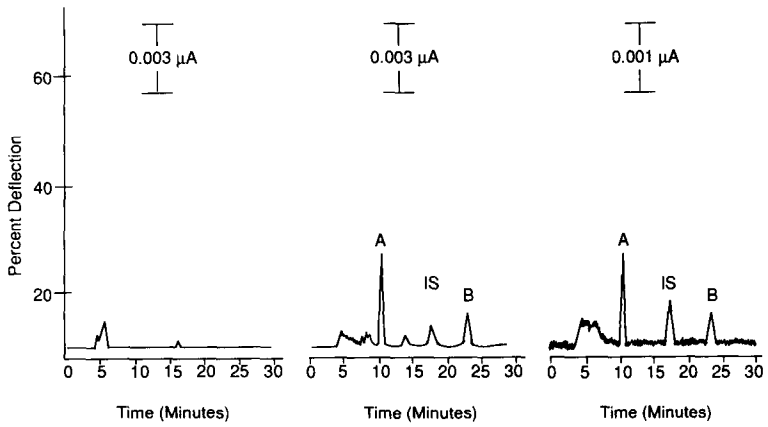

Fig. 1. Chromatograms obtained on analysis of a drug-free serum sample (left), a 3-h sample from the volunteer [101.2 ng/ml (+)-metoprolol and 97.7 ng/ml (−)-metoprolol] (middle) and a 50 ng/ml extracted serum standard (right). The column was a 25 cm×4.6 mm I.D. stainless-steel tube packed with cellulose tris(3,5-dimethylphenylcarbamate) polymer absorbed on macroporous silica. The mobile phase was hexane–isopropyl alcohol–diethylamine (91:8:1, v/v). Peaks: A = (+)-metoprolol; B = (−)-metoprolol; IS = internal standard.

solutions A and B, respectively. However, under the present conditions the wavelength of 225 nm is not opaque and maximum absorbance will not occur. The fact that there is a difference in the excitation wavelength when different mobile phases are used is not unexpected. Chromatographers should check the effect of mobile phase on the ultraviolet, fluorescence, optical, etc. properties of the solute.

Fig. 1 shows typical chromatograms of, from left to right, an extracted drug-free blank serum sample, the 3-h sample from the volunteer (101.2 and 97.7 ng/ml for the (+)- and (−)-isomer, respectively) and a 50.0 ng/ml extracted serum standard. The elution order is (+)-metoprolol (A) (+)-propranolol, internal standard (IS), and (−)-metoprolol (B). The elution order is consistent with previous reports of the separation of the enantiomers of metoprolol and propranolol [9–11].

Standard curve

Standard curves were prepared by adding known amounts of drug to blank serum and determining the appropriate peak-height ratios, e.g., (+)-metoprolol/internal standard. The peak-height ratios were then plotted as a function of the concentration of the drug added. Metoprolol isomer standard curves were consistently linear over a range of 12.5–200.0 ng/ml with correlation coefficients ranging from 0.991 to 0.999. The slope and y-intercept values for (+)-metoprolol were 0.01 and −0.03, respectively. The slope and y-intercept values for (−)-metoprolol were 0.006 and −0.01, respectively.

Accuracy and precision

The mean extraction efficiencies of metoprolol at 100.0 ng/ml were $75.4 \pm 4.6\%$ and $78.1 \pm 5.7\%$ for the (+)- and (−)-enantiomer, respectively. The coefficient of variation (C.V.) of extraction was less than 8.0% for each determination.

The reproducibility of the procedure was evaluated by analyzing ten replicate samples containing metoprolol on the same day and by analyzing one sample on ten consecutive days. The results are shown in Table I. The C.V. values on replicate metoprolol determinations on the same day and over ten consecutive days were less than 10.0%.

Limit of detection

The limit of detection of the assay determined from extracted serum standards was 8.0 ng/ml for the racemic drug, i.e., 4.0 ng/ml for each enantiomer. The limit of detection was defined at a signal-to-noise ratio of 4:1.

Stability and interferences

Comparisons of peak-height ratios for metoprolol enantiomers from a freshly prepared serum standard (100.0 ng/ml) to ratios from a sample frozen at

TABLE I

INTRA-ASSAY PRECISION AND REPRODUCIBILITY

Column: 25 cm × 4.6 mm I.D. packed with cellulose tris(3,5-dimethylphenylcarbamate) polymer absorbed on macroporous silica; mobile phase: hexane–isopropyl alcohol–diethylamine (91:8:1, v/v); flow-rate: 1 ml/min. Values given are mean ± S.D. of ten determinations.

Compound	Concentration added (ng/ml)	Within-day		Between-day	
		Concentration (ng/ml)	C.V. (%)	Concentration (ng/ml)	C.V. (%)
(+)-Metoprolol	17.5	16.8 ± 1.3	7.7	16.5 ± 1.2	7.6
(−)-Metoprolol	150	153.4 ± 11.6	7.6	155.4 ± 11.0	7.0
(+)-Metoprolol	17.5	19.0 ± 1.8	9.7	18.1 ± 1.4	8.0
(−)-Metoprolol	150	151.8 ± 12.7	8.4	156.2 ± 12.5	8.0

−20°C for a period of thirty days showed no differences. These data suggest samples drawn for pharmacokinetic analysis can be kept for a minimum of thirty days at −20°C with no degradation.

No endogenous sources of interference have been observed (Fig. 1). To assess the potential for chromatographic interference from drugs that are commonly coadministered with metoprolol in the clinical setting, we added and extracted from serum various cardiovascular drugs. Propranolol (100.0 ng/ml), gallopamil (50.0 ng/ml) and procainamide (6.0 μg/ml) were detected if present at therapeutic concentrations. The capacity factors for (+)- and (−)-metoprolol were 2.4 and 5.3, respectively. Stereoselectivity (α) and stereochemical resolution (R_s) were 2.2 and 5.5, respectively. The capacity factors for the (+)- and (−)-isomers of propranolol were 4.0 and 7.5, respectively. Stereoselectivity and R_s were 1.875 and 4.4, respectively. There were two peaks following the gallopamil injection having capacity factors of 4.6 and 5.1. Procainamide eluted having a capacity factor of 33.3 Imipramine (100.0 ng/ml), flecanide (500.0 ng/ml), α-hydroxymetoprolol (50 ng/ml), diltiazem (500.0 ng/ml), quinidine (6.0 μg/ml) and disopyramide (6.0 μg/ml) were either not extracted and/or not detected.

Ruggedness of the method

Under the present conditions the system worked properly for more than seven months. Approximately twenty samples per day can be analyzed. The useful column life for this analysis is at least seven months. Column equilibration occurs within roughly 30 min. It is important to recognize that, although not presented in this paper, column-to-column variations do exist and therefore reproducibility is sometimes poor. Should interfering substances that have not been evaluated in this study elute and interfere with peaks of interest,

modification of the mobile phase would be one appropriate response, i.e., to increase retention decrease isopropyl alcohol.

Clinical application

The serum samples from the subject taking racemic metoprolol were analyzed using this procedure. The 3-h sample is shown in Fig. 1. The total analysis time is less than 24 min. The pharmacokinetic disposition of metoprolol isomers over the 12-h dosing interval is shown in Fig. 2. The area under the concentration versus time curve and the apparent oral clearance were 984.02 ng·h/ml and 0.25 l/h/kg for the (+)-isomer and 969.71 ng·h/ml and 0.25 l/h/kg for the (−)-isomer, respectively. The k_{el} for the (+)- and (−)-isomer was 0.08 h^{-1} and 0.06 h^{-1}, respectively.

Comparison with a non-stereoselective assay

The results were compared to an analysis of these samples for metoprolol and the α-hydroxy metabolite using a non-stereoselective assay [23]. There was a statistically significant correlation ($r=0.93$, $P<0.001$) between total metoprolol concentrations from the racemic metoprolol assay and the sum of the isomer concentrations from the present assay (Fig. 3). Area under the concentration versus time curve for racemic metoprolol and the sum of the areas for the two isomer curves were 1954.17 and 1953.73 ng·h/ml, respectively. The area under the α-hydroxymetoprolol concentration versus time curve was 78.6 ng·h/ml with concentrations ranging from 4.1 to 16.5 ng/ml.

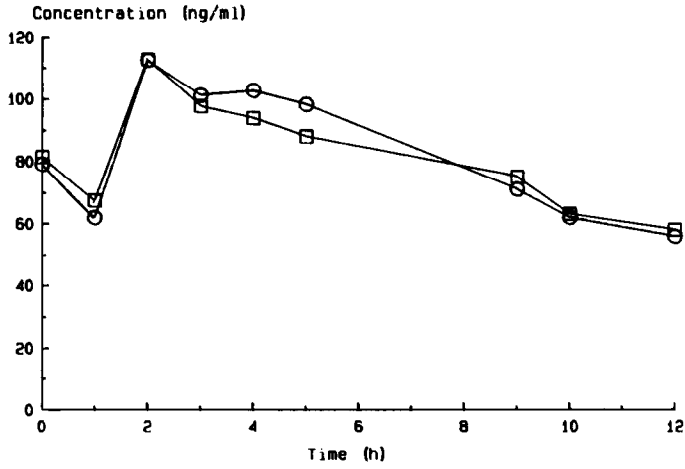

Fig. 2. Serum concentrations of (+)-metoprolol (o) and (−)-metoprolol (□) from one subject receiving a 50-mg commercial tablet after taking 50 mg every 12 h for five days.

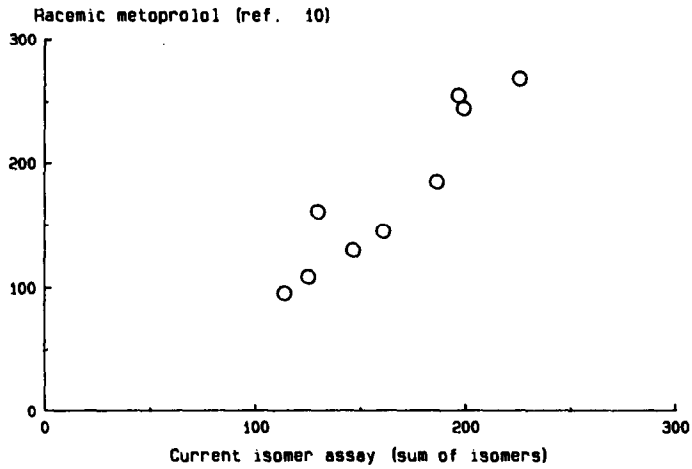

Fig. 3. Correlation of total concentrations from a racemic metoprolol assay [10] compared to the present 'sum' of the isomer assay. There was a significant correlation ($r=0.93, P<0.001$) between the two methods.

CONCLUSION

The stereoisomeric composition of drugs is becoming a significant issue in drug development, approval and clinical use. The lack of availability of simple analyses for enantiomers has significantly slowed the development of our understanding of these important aspects. The development of an analysis that does not require an additional procedure, i.e., a derivatization step, is essential to promote further investigations in this important area. The present analysis overcomes this specific limitation by utilizing a special, commercially prepared chiral HPLC pre-column and column. It is recognized that in todays market this column is relatively expensive.

The analyst must choose the right chiral bonded phase for a particular application. Today, there is no chiral column capable of separating all classes of compounds. The application of the methodology reported here appears suitable for separating the enantiomers of metoprolol.

ACKNOWLEDGEMENTS

This work was supported in part by a Research Stimulation Fund Award, Wayne State University (Detroit, MI, U.S.A.). The author wishes to thank the technical support of Ban Karmo and Ikhlas Karmo.

REFERENCES

1 B. Testa and P. Jenner, in P. Jenner and B. Testa (Editors), Concepts in Drug Metabolism, Marcel Dekker, New York, 1980, pp. 55–176.

2 M.S. Lennard, G.T. Tucker, J.H. Silas, S. Freestone, L.E. Ramsay and H.F. Woods, Clin. Pharmacol. Ther., 37 (1983) 732-737.
3 C. Harvengt and J.P. Dasager, Int. J. Clin. Pharmacol. Ther. Toxicol., 20 (1982) 57-71.
4 J. Moss, V.C. Mangeniello, B.E. Hom, S. Nakaya and M. Vaughan, Biochem. Pharmacol., 30 (1981) 1263-1269.
5 E.U. Keates and M.D. Stone, Am. J. Ophthamol., 98 (1984) 73-78.
6 S. Mason, New Sci., 101 (1984), 10-14.
7 K. Williams and E. Lee, Drugs, 30 (1985) 333-354.
8 M. Eichelbaum, Biochem. Pharmacol., 37 (1988) 93-96.
9 T. Walle, J.G. Webb, E.E. Bagwell, U.K. Walle, H.B. Daniell and T.E. Gaffney, Biochem. Pharmacol., 73 (1988) 115-124.
10 C.Y. Quon, K. Mai, G. Patil and H.F. Stampfli, Drug Metab. Dispos., 16(1988) 425-428.
11 H.M. Hoffmesiter and L. Seipel, Klin. Wochenschr., 66 (1988) 451-454.
12 F. Brunner and W.E. Muller, J. Pharm. Pharmacol., 39 (1987) 986-990.
13 S. Toon, E.M. Davidson, F.M. Garstang, H. Batra, R.J. Bowes and M. Rowland, Clin. Pharmacol. Ther., 43 (1988) 282-289.
14 P. Rauch, M. Puttmann, F. Oesch, Y. Okamoto and L.W. Robertson, Biochem. Pharmacol., 36 (1987) 4355-4359.
15 M.H. Chandler, S.R. Scott and R.A. Blouin, Clin. Pharmacol. Ther., 43 (1988) 436-441.
16 J. Hermansson and C. Von Bahr, J. Chromatogr., 227 (1982) 113-127.
17 A.J. Sedman and J. Gal, J. Chromatogr., 28 (1983) 199-203.
18 D. Schuster, M.W. Modi, D. Lalka and F.M. Gengo, J. Chromatogr., 433 (1988) 318-325.
19 Chiralpak Chiralcel, HPLC Column for Optical Resolution, New Chiral HPLC Column, Daicel Chemical Industries, Fort Lee, NJ.
20 Y. Okamoto, M. Kawashima, R. Aburatani, K. Hatada, T. Nishiyama and M. Masuda, Chem. Lett., (1986) 1237-1240.
21 Y. Okamoto, M. Kawashima and K. Hatada, J. Chromatogr., 363 (1986) 173-186.
22 R.J. Straka, R.L. Lalonde and I.W. Wainer, Pharm. Res., 5 (1988) 187-189.
23 D.R. Rutledge and C. Garrick, J. Chromatogr. Sci., 27 (1989) 561-565.