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Determination of Carvedilol in Rabbit Plasma by Gas Chromatography/ Mass Spectrometry and Its Application to Pharmacokinetic Study

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This paper describes a gas chromatography/mass spectrometry (GC/MS) method with selected-ion monitoring (SIM) for the measurement of plasma carvedilol levels. Carvedilol and atenolol (internal standard, IS) were extracted from rabbit plasma with a mixture of diethyl ether and ethyl acetate at basic pH with liquid-liquid extraction. The extracts were derivatized with N-methyl-N-(trimethylsilyl)trifluoro acetamide (MSTFA) and analyzed by GC/MS. Calibration curves were linear over the concentration range 15-500 ng/mL. Intra- and inter-day precision values for carvedilol in rabbit plasma were less than 8 and accuracy (relative error) was better than 11 %. The analytical recovery of carvedilol from rabbit plasma averaged out to 88.70 %. The limit of detection (LOD) and limit of quantification (LOQ) of carvedilol were 5 and 15 ng/mL, respectively. The developed and validated GC/MS method was also successfully applied to a pharmacokinetic study of carvedilol in New Zealand white rabbits.

Key Words: Carvedilol, Gas chromatography/mass spectrometry, Validation, Pharmacokinetics.

INTRODUCTION

Carvedilol is an antihypertensive agent with non-selective β- and α_1-adrenergic receptor blocking activities[1-3]. Carvedilol has been used in treatment of congestive heart failure[4-6]. Chemically, it is 1-(9*H*-carbazol-4-yloxy)-3-[[2-(2-methoxy-phenoxy)ethyl]amino]-2-propanol[7].

Several methods have been reported for determination of carvedilol including high-performance liquid chromatography (HPLC)[8-19], capillary electrophoresis[20], LC/MS/MS[21,22] and GC/MS[23].

On extensive survey of literature, no method is reported till date for determination of carvedilol by GC/MS in plasma. Therefore, we report a GC/MS method for determination of carvedilol after a derivatization procedure in rabbit plasma using internal standard methodology. The developed method was validated by using linearity, stability, precision, accuracy and sensitivity parameters according to center for drug evaluation and research (CDER) guidance for bio-analytical method validation[24].

The advantages of present method include simple and single step extraction procedure using inexpensive chemicals and short run time. This method was also used to assay the carvedilol in plasma sample obtained from six rabbits which had been given an oral tablet of Dilatrend (25 mg carvedilol).

EXPERIMENTAL

Carvedilol was obtained from Department of Cardiology, Faculty of Medicine, Ataturk University (Erzurum, Turkey). Atenolol as internal standard (IS) was kindly donated from Abdi Ibrahim Pharmaceutical Industry (Istanbul, Turkey). N-Methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA), ethyl acetate, diethyl ether and acetonitrile were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dilatrend tablet (25 mg carvedilol) was obtained from Roche Pharmaceutical Industry (Istanbul, Turkey).

Chromatographic analysis was carried out on an Agilent 6890N gas chromatography system equipped with 5973 series mass selective detector, 7673 series autosampler and chemstation (Agilent Technologies, Palo Alto, CA). HP-5 MS column with 0.25 μm film thickness (30 m × 0.25 mm i.d., USA) was used for separation. Splitless injection was used and the carrier gas was helium at a flow rate of 1.5 mL/min. The MS detector parameters were transfer line temperature 280 ºC, solvent delay 3 min and electron energy 70 eV.

Preparation of stock and working solutions: 10 mg of each carvedilol and atenolol were weighed, transferred in 500 mL volumetric flask. 250 mL acetonitrile was added and the flask was sonicated. The flask was filled to volume with acetonitrile (20 μg/mL). After, 5000 ng/mL standard solution

was prepared by diluting with acetonitrile with appropriate volumes of 20 µg/mL stock solution and stored at -20 ºC under refrigeration. Internal standard *i.e.*, atenolol working solution was prepared at final concentration of 2500 ng/mL. The working solutions were prepared by diluting the standard stock solution from 15-500 ng/mL. The quality control (QC) solutions were also prepared from stock solution at concentrations of 75, 250 and 450 ng/mL together with 500 ng/mL IS.

Derivatization procedure and sample preparation: MSTFA is an effective trimethylsilyl donor. N-Methyl-N-trimethylsilyl-trifluoroacetamide reacts to replace labile hydrogens on a wide range of polar compounds with a-Si$(CH_3)_3$ (trimethyl silane) group and is used to prepare volatile and thermally stable derivatives for GC/MS[25]. In this study, the purpose of the derivatization reaction is the raise of sensitivity thus the possibility of working in low concentrations has been occurred. Therefore, carvedilol and internal standard were derivatized using MSTFA. The secondary amine (-NH) and hydroxy (-OH) groups, which render the compounds non-volatile and polar, were converted to the corrosponding silyl (-N-TMS) and (-O-TMS) groups, thereby rendering them volatile and non-polar.

A 1 mL blank plasma of New Zealand white rabbit was transferred to a 12 mL centrifuge tube. 0.1 mL of standard carvedilol solutions together with 0.2 mL internal standard solution (500 ng/mL) and 0.5 mL of 1 M sodium hydroxide solution were added. After vortex mixing for 5 s, 4 mL of diethyl ether and ethylacetate was added (3:1, v/v), the mixture was vortexed for 30 s and then centrifuged at 3000 × g for 3 min. The organic layer was transferred into another tube and evaporated to dryness at room temperature under nitrogen gas. The dry residue was dissolved in 100 µL of a mixture of acetonitrile and MSTFA (30:70, v/v). The mixture was vigorously shaken and then left at room temperature for 10 min. Then 1 µL of aliquot was injected into the GC/MS system. The mass spectra of the carvedilol and internal standard are shown in Fig. 1.

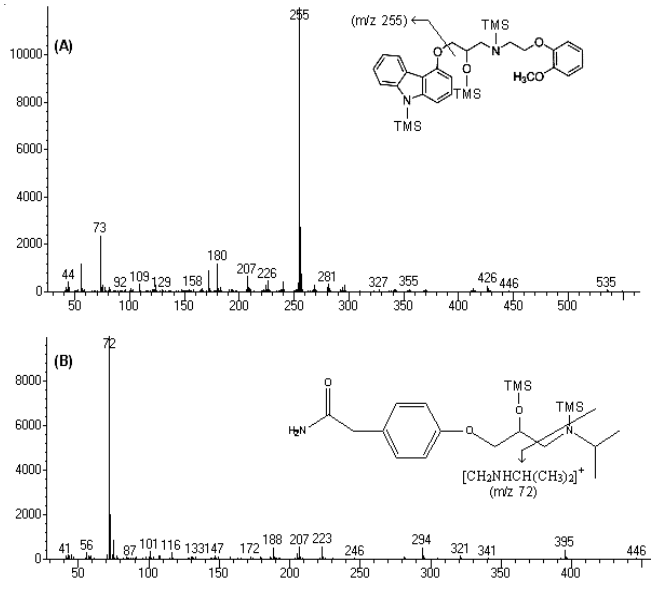

Fig. 1. Chemical structures and full scan mass spectra of carvedilol (A) and atenolol (IS) (B) after the derivatization of with MSTFA

RESULTS AND DISCUSSION

Method development: The developed method for the assay of carvedilol was based on its chemical properties. Carvedilol is a polar molecule. Therefore, the capillary column coated with 5 % phenyl-, 95 % dimethylpolysiloxane is a good choice for separation of this analyte since they elute as symmetrical peaks at a wide range of concentrations. Different temperature programs were investigated for GC oven. The temperature programs of the GC oven was as follows: initial temperature 230 ºC for 1 min, increased to 250 ºC at a rate of 25 ºC/min for 1 min and then increased to 315 ºC at a rate of 20 ºC/min for 5 min. The splitless injection mode was chosen. To confirm the complete derivatization of carvedilol and internal standard, since only one peak appears on the chromatogram, each compound was derivatized and analyzed separately. After establishing the optimum reaction conditions, the compounds were mixed together and then derivatized in order to perform a simultaneous analysis. Additionally, preliminary precision and linearity studies performed during the development of the method showed that the 1 mL injection volume was reproducible and the peak response was significant at the analytical concentration chosen.

Method validation: The validation of the method was carried out by establishing specifity, linearity, intra- and inter-day precision, accuracy, recovery, limit of detection (LOD) and limit of quantification (LOQ) according to CDER guidance for bio-analytical method validation[24].

Specificity: The specificity criterion tries to demonstrate that the result of the method is not affected by the presence of interferences[25]. The specificity of method was determined by checking the chromatograms obtained from blank plasma samples and no endogenous interferences were encountered (Fig. 2). The fragment ions (m/z 255 and 72) were used for quantitation of carvedilol and internal standard. The retention time of carvedilol and internal standard in rabbit plasma was approximately 9.5 and 6.3 min.

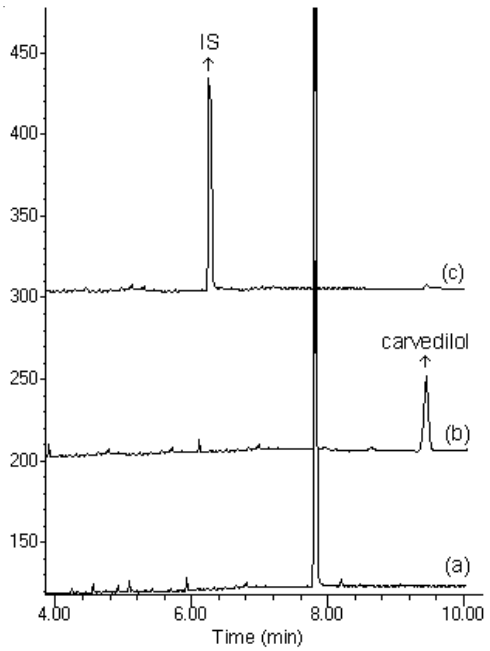

Fig. 2. Typical SIM chromatogram of blank plasma (a), plasma spiked with 500 ng/mL carvedilol (b) and 500 ng/mL atenolol (IS) (c)

Linearity: The linearity of an analytical method is its ability within a definite range to obtain results directly proportional to the concentrations (quantities) of an analyte in the sample. The concentrations of the spiked carvedilol in rabbit plasma were 15, 50, 100, 200, 300, 400 and 500 ng/mL with constant concentration of internal standard (500 ng/mL). The calibration curves were established by plotting the ratio of the peak areas of carvedilol and internal standard obtained after extraction of the spiked plasma sample. The linear regression equation was calculated by the least squares method using Microsoft Excel® program and summarized in Table-1.

TABLE-1
LINEARITY OF CARVEDILOL IN RABBIT PLASMA

Linearity (ng/mL)	15-500
Regression equation*	$y = 0.0033x + 0.0973$
Standard deviation of slope	1.01×10^{-4}
Standard deviation of intercept	4.03×10^{-3}
Correlation coefficient	0.9955
Standard deviation of correlation coefficient	4.28×10^{-3}
Limit of detection (LOD) (ng/mL)	5.0
Limit of quantification (LOQ) (ng/mL)	15

*Based on six calibration curves, y: peak-area ratio, x: carvedilol concentration (ng/mL).

Precision and accuracy: Assay precision was determined by repeatability (intra-day) and intermediate precision (inter-day). Repeatability during the same day and intermediate precision on different days (3 days) were evaluated with six replicates of quality control samples. The accuracy of this analytic method was assessed as the percentage relative error (RE %). The accuracy and precision of the method were evaluated with quality control samples at concentrations of 75, 250 and 450 ng/mL. The intra- and inter-day accuracy and precision results are shown in Table-2. The intra- and inter-day precisions of the quality control samples were satisfactory with RSD less than ± 8 % and accuracy with relative error (RE) within ± 11 % (should be less than 15 according to CDER guidance for bio-analytical method validation)[24].

TABLE-2
PRECISION AND ACCURACY OF CARVEDILOL IN RABBIT PLASMA

Added (ng/mL)	Found (mean ± SD[a])	Precision (%) RSD[b]	Accuracy[c]
Intra-day			
Plasma pools[d]			
75	78.6 ± 3.467	4.41	4.80
250	241.2 ± 16.484	6.83	-3.52
450	459.9 ± 9.229	2.01	2.20
Inter-day			
75	83.1 ± 4.745	5.71	10.80
250	255.6 ± 20.258	7.93	2.24
450	458.5 ± 16.832	3.67	1.89

a: Standard deviation of six replicate determinations, RSD: Relative standard deviation, b: Average of six replicate determinations, c: (relative error %) (found-added)/added × 100, d: Plasma volume (1 mL).

Limit of detection (LOD) and limit of quantification (LOQ): The limit of quantification (LOQ) is defined as the lowest concentration of analyte that can be determined with acceptable precision and accuracy. The limit of detection (LOD) is a parameter that provides the lowest concentration in a sample that can be detected from background noise but not quantitated. The LOD and LOQ were studied to test the sensitivity of the method. The LOD defined as signal/noise = 3 in method (RSD > 20 % for LOD) was found to be 5 ng/mL. The LOQ defined as signal/noise = 10 in method (RSD > 10 % for LOQ) was found to be 15 ng/mL (Table-1). Both accuracy and precision of these values were well within the proposed criteria (RSD % < 20 %).

Recovery: The recovery of an analyte is the extraction efficiency of an analytical process, reported as a percentage of the known amount of an analyte carried through the sample extraction and processing steps of the method[26]. Several solvents (ethyl acetate, diethyl ether, dichloromethane, acetonitrile, butanol and chloroform) were tested for the extraction. Finally, diethyl ether and ethyl acetate mixture (3:1, v/v) proved to be the most efficient in extracting carvedilol from rabbit plasma. Spiked plasma samples were prepared six time at all levels of the calibration graph of carvedilol. The recovery of carvedilol was determined by comparing the peak areas measured after analysis of spiked plasma samples with those found after direct injection of standard solutions at the same concentration levels. The recovery of carvedilol from rabbit plasma was satisfactorily ranged from 85.9-91.1 % (RSD was less than 9.13) at all the concentration levels (Table-3).

TABLE-3
RECOVERY OF CARVEDILOL IN RABBIT PLASMA

Added (ng/mL)	Found (mean ± SD[a])	Recovery (%)	RSD[b] (%)
15	12.93 ± 1.181	86.2	9.13
50	44.65 ± 3.456	89.3	7.74
100	90.10 ± 5.775	90.1	6.41
200	177.4 ± 10.18	88.7	5.74
300	268.8 ± 22.01	89.6	8.19
400	364.4 ± 31.81	91.1	8.73
500	429.5 ± 32.13	85.9	7.48

a: Standard deviation of six replicate determinations, RSD: Relative standard deviation, b: Average of six replicate determinations.

Matrix effect: The matrix effect was defined as the direct or indirect alteration or interference in response due to the presence of unintended analytes or other interfering substances in the sample[24]. The plasmas used in this study were from six different batches of rabbit blank plasma. If the ratio < 85 % or >115 %, a matrix effect was implied.

The matrix effect data at three concentration levels of carvedilol in rabbit plasma were presented in Table-4. The results showed that there was no matrix effect of the analytes observed from the matrix of plasma in this study.

Stability: The stability of carvedilol in rabbit plasma was studied under a variety of storage and handling conditions at low (200 ng/mL) and high (500 ng/mL) concentration levels. The short-term temperature stability was assessed by analyzing three aliquots of each of the low and high concentration samples that were thawed at room temperature and kept at this temperature for 8 h. Freze-thaw stability (-20 ºC in rabbit plasma) was checked through three cycles. Three aliquots at

TABLE-4
MATRIX EFFECT EVALUATION OF CARVEDILOL AND IS IN RABBIT PLASMA (n = 3)

Samples	Concentration level (ng/mL)	A (mean ± SD)	B (mean ± SD)	Matrix effect (%)
Carvedilol	150	138±8.74	146±6.22	94.5
	350	318±15.75	327±12.42	97.2
	500	432±14.41	472±11.34	91.5
IS	500	419±13.57	447±8.62	93.7

(A) The amount of carvedilol and IS derivatized in blank plasma sample's reconstituted solution (the final solution of blank plasma after extraction and reconstitution), (B) The amount of carvedilol and IS derivatized with MSTFA.

each of the low and high concentrations were stored at -20 ºC for 24 h and thawed unassisted at room temperature. When completely thawed, the samples were refrozen for 24 h under the same conditions. The freze-thaw cycles were repeated three times and then analyzed on the third cycle. The long-term stability was determined by analyzing three aliquots of each of the low and high concentrations stored at -20 ºC for 1 week. The results of the stability studies was given in Table-5 and no significant degradation of carvedilol was observed under the tested conditions.

TABLE-5
STABILITY DATA OF CARVEDILOL IN RABBIT PLASMA UNDER VARIOUS STORAGE CONDITIONS (n = 3)

Storage conditions	Concentration level (ng/mL)	Calculated concentration (ng/mL)	RSD (%)	Relative error (%)
Room temperature for 8 h	200	191	6.91	-4.50
	500	475	7.98	-5.00
Three freze-thaw cycles	200	187	7.83	-6.50
	500	483	8.91	-3.40
1 weeks at -20 ºC	200	189	6.73	-5.50
	500	481	9.14	-3.80

Application of the method: The method was applied to determine the plasma concentration of carvedilol after an oral administration of 25 mg dilatrend tablet to 6 rabbits. The study was conducted in accordance with the Animal Ethical Guidelines for Investigations in Laboratory Animals and was approved by the Ethical Committee for Medical Experimental Research and Application Centre of Ataturk University. The rabbits are male which is 3.5-3.8 kg weight. The rabbits were housed with free access to food and water, except for the final 2 h before experimentation. After a single oral administration of 25 mg of carvedilol (dilatrend tablet), 2.5 mL of blood samples were collected from the marginal ear vein at 0, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0 and 8.0 h time-points into EDTA collection tubes. The blood was immediately centrifuged 6000 × g for 10 min at ambient temperature. The supernatant plasma layer was separated and analyzed for carvedilol concentrations as described above.

The total area under the observed plasma concentration-time curve (AUC) was calculated by using the linear trapezoidal rule. The first order elimination rate constant (k_{el}) was estimated by the least square regression of the points describing the terminal log-linear decaying phase. $T_{1/2}$ was derived from k_{el} ($T_{1/2} = \ln 2/k_{el}$). The maximum observed carvedilol concentration (C_{max}) and the time at which C_{max} was observed (T_{max}) were directly reported from the profile.

The method was applied to quantify the plasma concentration of carvedilol in a single-dose pharmacokinetic study conducted on six New Zealand white rabbits. GC/MS chromatogram of rabbit plasma is shown in Fig. 3.

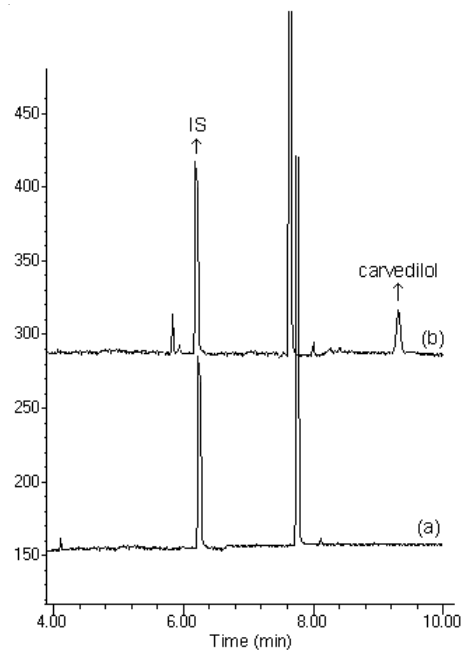

Fig. 3. Typical SIM chromatogram of plasma obtained from a rabbit before (a) and after 1.5 h (b) oral administration of dilatrend tablet (25 mg carvedilol)

Representative mean plasma concentrations *versus* time profiles following a single oral administration of carvedilol to 6 rabbits are presented in Fig. 4. Various pharmacokinetic parameters have been summarized in Table-6.

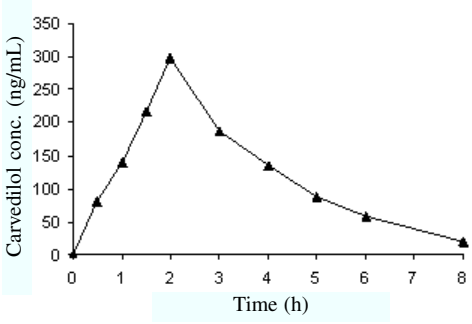

Fig. 4. Mean plasma concentration-time profile of carvedilol in rabbits (n = 6) after application of dilatrend tablet

β-Blockers have similar chemical structures with highly polar functional groups that yield them unsuitable for determination by GC methods. Recently the use of mass selective detectors with a capilllar GC coupled to MS as a mode of detection has considerably increased. Suitable derivatization should improve the gas chromatographic properties of the compounds and yield compounds with mass spectra containing high relative intensity and high-mass fragments suitable for SIM mode[27].

TABLE-6
PHARMACOKINETIC PARAMETERS OF CARVEDILOL FOR SIX RABBITS AFTER ORAL ADMINISTRATION OF DILATREND TABLET (25 mg)

Parameters	Mean ± SD	RSD (%)
Maximum plasma concentration C_{max} (ng/mL)	305.2 ± 43.55	14.27
Time required for maximum plasma concentration T_{max} (h)	2.0 ± 0.289	14.45
Area under curve $AUC_{(0\rightarrow 8h)}$ (ng/mL h)	987.5 ± 208.67	21.13
Area under curve at infinite time $AUC_{(0\rightarrow\infty)}$ (ng/mL h)	4192.6 ± 1010.54	24.10
Plasma half life, $T_{1/2}$ (h)	2.37 ± 0.389	16.41

Several methods have been reported for the determination of carvedilol by LC/MS/MS[21,22]. Detection using LC/MS/MS would be a more sensitive approach but is costly and not yet available for every laboratory.

Now-a-days, GC/MS is a powerful technique for highly specific and quantitative measurements of low levels of analytes in biological samples. As compared to HPLC, high-resolution capillary GC has been less frequently used. Because it requires pre-conversion of multifunctional β-blockers into thermally stable volatile derivatives. However, it has inherently high resolving power and high sensitivity with excellent precision and accuracy allowed simultaneous detection of expected and unexpected β-blockers, their metabolites and contaminants. Also, the detection limits were lowered to pg levels by GC combined with MS in SIM mode[28].

In this study, the specificity of developed GC/MS method has been demonstrated by the representative chromatograms for carvedilol in rabbit plasma (Figs. 2 and 3). The retention time of carvedilol in rabbit plasma is 9.5 min. The samples received from six different batches of blank plasma have also been tested and showed no significant interference at the retention time of compound of carvedilol. The recovery of carvedilol was achieved by developed liquid-liquid extraction procedure in rabbit plasma. Carvedilol was extracted from rabbit plasma with a mixture of diethyl ether and ethyl acetate at basic pH. This solvent mixture gave an excellent recovery. The recovery of carvedilol from rabbit plasma averaged out to 88.70 %. Intra- and inter-day precision values for carvedilol in rabbit plasma were less than 8.0 %. The LOD and LOQ were found to be 5 and 15 ng/mL, respectively.

When this method is applied to plasma samples, its sensitivity was found to be adequate for pharmacokinetic studies. The present method has the following advantage over the reported method[17]. The LOQ of the reported method was 50 ng/mL whereas the present method LOQ was 15 ng/mL. Additionally, this method was applied to six rabbits which had been given an oral tablet of 25 mg carvedilol. The amount of carvedilol was determined between 0 and 8 h in rabbit plasma.

Conclusion

In the present work, a simple and sensitive GC/MS method has been developed for the determination of carvedilol in rabbit plasma. The method was completely validated by using stability, specificity, linearity, sensitivity, accuracy and precision parameters for determination of carvedilol in rabbit plasma. Also, the extraction and derivatization procedures in this study were simple. No significant interferences and matrix effect caused by endogenous compounds were observed. To our best of knowledge, this is the first description of carvedilol pharmacokinetics in plasma by GC/MS method in the literature. It can be very useful and an alternate to performing pharmacokinetic studies in determination of carvedilol for clinical use.

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REFERENCES

1. R.R. Ruffolo Jr., M. Gellai, J.P. Hieble, R.N. Willette and A.J. Nichols, *Eur. J. Clin. Pharmacol*., **38**, 82 (1990).
2. A.J. Nichols, M. Gellai and R.R. Ruffolo Jr., *Fundam. Clin. Pharmacol*., **5**, 25 (1991).
3. C. de Mey, K. Breithaupt, J. Schloos, G. Neugebauer, D. Palm and G.G. Belz, *Clin. Pharmacol. Ther*., **55**, 329 (1994).
4. M.A. Pfeffer and L.W. Stevenson, *N. Engl. J. Med*., **334**, 1396 (1996).
5. M. Packer, M.R. Bristow, J.N. Cohn, W.S. Colucci, M.B. Fowler, E.M. Gilbert and N.H. Shusterman, *N. Engl. J. Med*., **334**, 1349 (1996).
6. W.H. Frishman, *N. Engl. J. Med*., **339**, 1759 (1998).
7. K. Parfitt, Martindale: The Complete Drug Reference, Pharmaceutical Press, UK, p. 838 (1999).
8. C. de Mey, K. Breithaupt, J. Schloos, G. Neugebauer, D. Palm and G.G. Belz, *Clin. Pharmacol. Ther*., **55**, 329 (1994).
9. F. Varin, L.X. Cubeddu and J.R. Powell, *J. Pharm. Sci*., **75**, 1195 (1986).
10. K. Reiff, *J. Chromatogr*., **413**, 355 (1987).
11. E.J. Eisenberg, W.R. Patterson and G.C. Kahn, *J. Chromatogr*., **493**, 105 (1989).
12. H. Spahn, W. Henke, P. Langguth, J. Schloos and E. Mutschler, *Arch. Pharm*., **323**, 465 (1990).
13. G. Neugebauer, W. Akpan, B. Kaufmann and K. Reiff, *Eur. J. Clin. Pharmacol*., **38**, 108 (1990).
14. N. Hokama, N. Hobara, H. Kameya, S. Ohshiro and M. Sakanashi, *J. Chromatogr. B*, **732**, 233 (1999).
15. D. Tenero, S. Boike, D. Boyle, B. Ilson, H.F. Fesniak, S. Brozena and D. Jorkasky, *J. Clin. Pharmacol*., **40**, 844 (2000).
16. P. Ptacek, J. Macek and J. Klima, *J. Chromatogr. B*, **789**, 405 (2003).
17. L. Clohs and K.M. McErlane, *J. Pharm. Biomed. Anal*., **31**, 407 (2003).
18. J. Oravcova, D. Sojkova and W. Lindner, *J. Chromatogr. B*, **682**, 349 (1996).
19. F. Behn, S. Laer and H. Scholz, *J. Chromatogr. Sci*., **39**, 121 (2001).
20. M. Machida, M. Watanabe, S. Takechi, S. Kakinoki and A. Nomura, *J. Chromatogr. B*, **789**, 187 (2003).
21. W.H. Schaefer, J. Politowski, B. Hwang, F. Dixon Jr., A. Goalwin, L. Gutzait, K. Anderson, C. DeBrosse, M. Bean and G.R. Rhodes, *Drug Metab. Dispos*., **26**, 958 (1998).
22. M. Gergov, J.N. Robson, E. Duchoslav and I. Ojanpera, *J. Mass Spectrom*., **35**, 912 (2000).
23. S.W. Myung and C.H. Jo, *J. Chromatogr. B*, **822**, 70 (2005).
24. Guidance for Industry, Bioanalytical Method Validation, US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Rockville, MD, (2001).
25. M. Donike, *J. Chromatogr*., **42**, 103 (1969).
26. M.J. Saiz-Abajo, J.M. Gonzalez-Saiz and C. Pizarro, *Anal. Chim. Acta*, **528**, 63 (2005).
27. J. Lee, D.S. Lho, M.S. Kim, K. Kim and Y.J. Kim, *Rapid Commun. Mass Spectrom*., **12**, 1150 (1998).
28. M.J. Paik, J. Lee and K.R. Kim, *Anal. Chim. Acta*, **601**, 230 (2007).