Asian Journal of Chemistry; Vol. 23, No. 8 (2011), 3329-3333

ASIAN JOURNAL OF CHEMISTRY

www.asianjournalofchemistry.co.in

Determination of a Novel COX-2 Inhibitor in Mouse Plasma: Application to Preclinical Pharmacokinetic Studies

A. HAERI[1], F.M. SHAHAB[1], S. ARFAEE[2], A. ZARGHI[2] and S. DADASHZADEH[1,3*]

[1]Biopharmacy and Pharmacokinetic Division, Department of Pharmaceutics, Faculty of Pharmacy, Shaheed Beheshti Medical University, P.O. Box 14155-6153, Tehran, Iran
[2]Department of Medicinal Chemistry, Faculty of Pharmacy, Shaheed Beheshti Medical University, P.O. Box 14155-6153, Tehran, Iran
[3]Pharmaceutical Sciences Research Centre, Shaheed Beheshti Medical University, P.O. Box 14155-6153, Tehran, Iran

*Corresponding author: Fax: +98 21 88209620; Tel: +98 21 8820070; E-mail: sdadashzadeh@sbmu.ac.ir

(*Received*: 21 April 2010; *Accepted*: 11 April 2011) AJC-9795

For preclinical study an HPLC method was developed and validated for the determination of a novel and selective cyclooxygenase-2 (COX-2) inhibitor in mouse plasma. The sample preparation procedure involved simple protein precipitation following extraction of analyte and internal standard (IS) from plasma. The separation was achieved on a Lichrospher C8 column using an isocratic mobile phase consisting of water, acetonitrile and triethylamine (48:52:0.1, v/v) as eluent with UV detection at 330 nm. A linear response was observed over a concentration range of 30-2000 ng/mL. The limits of detection and quantification were 10 and 30 ng/mL, respectively. The coefficients of variation for inter-day and intra-day assay were found to be less than 5.0 %. The assay was successfully applied to determine the pharmacokinetic parameters in mice after IV administration of the analyte. The method showed good characteristics of linearity, sensitivity, accuracy, selectivity and precision that made it suitable for preclinical pharmacokinetic studies.

Key Words: Cyclooxygenase-2 inhibitor, HPLC, Plasma, UV detection, Pharmacokinetic studies.

INTRODUCTION

Non-steroidal antiinflammatory drugs (NSAIDs) are among the most widely prescribed therapeutic agents worldwide because of their proven analgesic and antiinflammatory properties. The non-steroidal antiinflammatory drugs exert most of their activities through the inhibition of cyclooxygenase (COX), a key enzyme for prostanoid synthesis. Non-steroidal antiinflammatory drugs as a class comprise both non-selective NSAIDs that non-specifically inhibit both COX-1 and COX-2 and selective COX-2 inhibitors[1-3]. In spite of the beneficial effects of non-selective NSAIDs, they inhibit the synthesis of the prostaglandins that are required to protect the gastrointestinal mucosa as well as protect platelet function[4-7]. This paved the way for the discovery and development of newer agents called COX-2-specific inhibitors[8,9]. Selective inhibition of COX-2, the main isoenzyme expressed during inflammation is the main specification which reduces the serious side effects associated with the inhibition of COX-1 seen with non-selective COX inhibitors[10]. Selective COX-2 inhibitors are used for the treatment of acute pain states (*e.g.*, primary dysmenorrhea, dental surgery and orthopedic surgery) and the treatment of signs and symptoms of osteoarthritis and rheumatoid arthritis[11,12]. Because COX-2 is overexpressed in a broad range of pre-malignant, malignant and metastatic human epithelial cancers[13], clinical studies are ongoing to evaluate the use of selective COX-2 inhibitors in cancer prevention and chemotherapy. In addition, they offer potential for the prophylactic prevention of inflammatory neurodegenerative disorders such as Alzheimer's disease[14].

In order to achieve more potent COX-2 inhibitors a novel class of (E)-1,3-diarylprop-2-en-1-one derivatives possessing a COX-2 SO_2Me pharmacophore at the *para* position of the C-1 or C-3 phenyl ring, in conjunction with a C-3 or C-1 phenyl (4-H) or substituted phenyl ring, has been designed for evaluation as selective COX-2 inhibitors[15]. *In vitro* COX-1 and COX-2 isozyme inhibition studies identified (E)-1-(4-methanesulfonylphenyl)-3-(4-methylphenyl)prop-2-en-1-one (Zar-Me) (Fig. 1) as a potent COX-2 inhibitor (IC_{50} = 0.3 μM) with a high COX-2 selectivity index (SI = 106) comparable to that of the reference drug rofecoxib (COX-2 IC_{50} = 0.5 μM; COX-2 SI > 200)[15].

Several HPLC methods have been developed for the determination of COX-2 inhibitors in plasma and serum. Some of the published methods involve the use of solid-phase extraction procedure[16-20] and therefore, sample preparation is

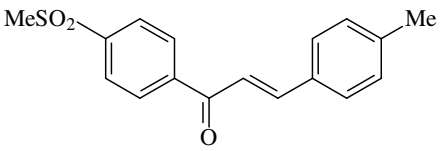

Fig. 1. Chemical structure of Zar-Me

time-consuming and expensive. Some of the reported liquid-liquid extraction methods suffer from either large volume of organic solvent or multiple steps sample preparation, so these published methods are tedious as well as time-consuming[21-27]. Some of previous analytical methods for COX-2 inhibitors required large plasma sample volumes which make them inappropriate for preclinical study[23,25,26,28]. The column-switching method proposed by Rose *et al.*[16], has the problem of the availability of the required instruments. Chromatographic methods based on mass-spectrometry[23,29,30,31] or post-column fluorescence derivatization[17,32], in spite of having higher sensitivity, are much more complex than the conventional UV detection.

For conducting preclinical evaluation of Zar-Me and understanding the efficacy and toxicity of the drug, a simple bioanalytical assay employing small volume of plasma is required. Therefore in the present study, an HPLC-UV reversed phase method using single-step extraction procedure, which involves protein precipitation was developed and validated for the determination of Zar-Me in mouse plasma and successfully applied for investigation the pharmacokinetics of Zar-Me in mouse.

EXPERIMENTAL

Zar-Me was synthesized by the Medicinal Chemistry Group, School of Pharmacy, Shaheed Beheshti University of Medical Sciences. Tolmetin sodium was a generous donation from Modava Pharmaceuticals (Tehran, Iran). HPLC-grade acetonitrile, sodium chloride, triethylamine and phosphoric acid (analytical grade) were purchased from Merck (Darmstadt, Germany).

Chromatographic system and conditions: The HPLC system consisted of a model K-1001 solvent delivery pump, a Wellchrom online degasser, a rheodyne auto injector equipped with a 100 µL loop, a model K-2600 UV detector (all from Knauer, Germany). Chromgate software (Version 317) was used to acquire and process all chromatographic data. The analytical column was Lichrospher-C8-250, 4.6 mm × 250 mm, 5 µm particle size (Merck, Germany). The isocratic mobile phase consisted of a mixture of water, acetonitrile and triethylamine (48:52:0.1, v/v) (adjusted to pH = 3 by adding 10 % phosphoric acid). The mobile phase was run at a flow rate of 1.0 mL/min at ambient temperature. The column eluent was monitored at 330 nm and the total analytical run time was 12 min.

Stock solution and standards: Ten mg of Zar-Me were accurately weighed and dissolved in HPLC-grade acetonitrile to achieve a final concentration of 1.0 mg/mL. This standard was used to prepare a 100 µg/mL solution in HPLC-grade acetonitrile. Working solutions were prepared daily in the concentration range of 0.2-15.0 µg/mL in acetonitrile by serial dilutions of intermediate solution and were stored at -20 ºC prior to use. The internal standard solution was prepared by dissolving 10 mg of tolmetin sodium in 100 mL methanol to give a final concentration of 100 µg/mL. This solution was diluted further by methanol yielding a final concentration of 4 µg/mL and stored at 2-8 ºC prior to apply.

Preparation of calibration standards and quality control samples: Spiked plasma samples used as calibration standards were prepared daily by addition of appropriate amounts of Zar-Me working solutions to drug-free human plasma, resulting in calibration standards of 30, 50, 100, 200, 300, 400. 500, 600, 800, 1000 and 2000 ng/mL. Spiked quality control (QC) samples were prepared by spiking blank plasma at low, medium and high concentration levels of 50, 500 and 1000 ng/mL, respectively. The plasma used as the matrix was obtained from whole blood anticoagulated with heparin.

Sample preparation: To 150 µL of plasma in a polypropylene eppendorf tube were added 30 µL of tolmetin solution as internal standard (4 µg/mL), 1000 µL of acetonitrile and 50 mg sodium chloride. After mixing (2 min), the mixture centrifuged for 5 min at 12000 rpm. The upper layer was separated and evaporated to dryness under a gentle nitrogen stream at 40 ºC and the residue was dissolved in 150 µL mobile phase. Reconstituted solutions were carefully vortexed for 30 s and a 100 µL aliquot was injected into the HPLC system.

Validation of the assay: Assay performance was validated regarding selectivity, linearity, accuracy, precision, recovery and sensitivity based on the International Conference on Harmonization (ICH) guidelines[33].

Linearity, accuracy, precision and selectivity: The linearity of the method was tested by constructing an 8 points standard curve in the concentration range of 30-2000 ng/mL of Zar-Me (three replicates) by plotting the peak area ratios of the analyte to IS *versus* the nominal analyte concentration. Linear least squares regression analysis without weighing was applied to calculate the slope, intercept and linear correlation coefficient (r^2).

The precision of the analytical procedure was evaluated by determining the intra- and inter-day coefficients of variation. The intra-day precision and accuracy of the method was estimated by analysis of six replicates of the spiked quality control samples prepared at three concentrations using mouse plasma. Inter-day precision and accuracy of the method was evaluated through duplicate determination of the same three low, medium and high concentrations as used in the study of intra-day precision, with samples at each concentration being analyzed on each daily experiment over five consecutive days. The accuracy at each concentration was expressed as the relative differences of measured and nominal concentration. The accuracy at each concentration, expressed as relative error (RE), was calculated by the following equation:

$$\text{Relative error (\%)} = \frac{\text{(Determined vlaue} - \text{Theoretical value)}}{\text{Theoretical value}} \times 100$$

In order to study the selectivity of the assay, six independent blank plasma samples were subjected to the same sample processing and analyzed.

Recovery: The recovery of the extraction procedure for Zar-Me was evaluated by comparing the peak response of three

replicate analyses of quality control samples at three different concentrations to the peak response of equivalent plain standards prepared in the mobile phase.

Sensitivity: Limit of detection (LOD) and limit of quantitation (LOQ) represent the sensitivity of the method. The lower limit of quantitation (LOQ) was defined as the lowest concentration at which the precision expressed by relative standard deviation (RSD) is lower than 20 % and accuracy expressed by relative error is also lower than 20 %. Limit of detection (LOD) was determined at the lowest concentration to be detected, taking into consideration of a signal-to-baseline noise ratio of 3.

Stability studies: Stability of the analyte in mouse plasma was assessed using quality control samples stored at room temperature for a period that exceeded the routine preparation time of samples (around 2 h). Quality control samples were also stored frozen at -20 ºC and analyzed at 0, 3 and 7 days. Zar-Me stock solution was stored at -20 ºC and measured weekly through 1 month to determine the change in drug concentration. The stability of the compound in dried samples on storage at 4 ºC was studied at low, medium and high concentrations. To evaluate the stability of the analyte during sample preparation (evaporation of acetonitrile at 40 ºC), the results of evaporation at ambient temperature were taken as standard (100 %) and the subsequent results were compared with the standard. Samples were considered to be stable if assay values were within the acceptable levels of accuracy (*i.e.*, ± 15 % relative error) and precision (*i.e.*, ± 15 % RSD).

Application of the method to a pharmacokinetic study

Animal treatment: The study was approved by the local ethics committee for animal experiment of Shaheed Beheshti University (Tehran, Iran). Male NMRI mice weighing 25-32 g obtained from Pasture Institute (Tehran, Iran) were used for these studies. Animals were maintained in a controlled environment of about 25 ºC, 50 % relative humidity and a 12 h light/12 h dark cycle and allowed free access to food and water. The animals were allowed to acclimatize to animal facility condition for at least 1 week before starting the experiment. All animals were fasted overnight before dosing.

A single dose of Zar-Me solution was administered to mice intravenously *via* tail vein at dosage of 40 mg/kg. 500 µL of blood samples were taken under ether anesthesia from five mice per time point at pre-determined times after drug administration. Plasma was separated by centrifugation and stored at -20 ºC until analysis. Dosing solutions of Zar-Me was prepared in a vehicle containing PEG400/DMSO/Water (50:5:45, v/v).

Pharmacokinetic analysis: Pharmacokinetic analysis was performed by two compartmental open model using WinNonlin software (version 3.2.) based on the following exponential equation[34]:

$$C_t = A \times e^{-\alpha t} + B \times e^{-\beta t}$$

where C_t is the drug concentration (Y-axis) at time t (X-axis). A and B are the Y-intercepts and α and β are the apparent first order distribution and elimination rate constants. Elimination rate constant (β) was estimated by least square regression of plasma concentration-time data points lying in the terminal log-linear region of the curve. Rate constant for distribution phase (α) was obtained by the method of residuals. The area under the plasma concentration-*versus*-time curve (AUC) was calculated using the trapezoidal rule with extrapolation to infinity. Clearance (Cl) was calculated by dividing dose over AUC. Volume of distribution at steady state (V_{ss}) and mean residence time (MRT) were calculated using following non-compartmental equations:

$$V_{ss} = \text{Dose} \times \frac{\text{AUMC}}{(\text{AUC})^2}$$

$$\text{MRT} = \frac{\text{AUMC}}{\text{AUC}}$$

where AUMC (area under the first moment curve) is the area under the C × t plotted against t from time 0 to infinity[34].

RESULTS AND DISCUSSION

Chromatographic conditions: Representative chromatograms of control mouse plasma (free of analyte and internal standard), mouse plasma spiked with Zar-Me at 500 ng/mL and an *in vivo* plasma sample obtained at 0.5 h after IV administration of Zar-Me at 40 mg/kg are shown in Fig. 2. As shown the chromatographic method provided sharp, well-resolved and symmetrical peaks without interference from endogenous plasma components.

Various chromatography systems were tested and finally the use of a RP-C8 column in combination with a mobile phase composition of acetonitril-water-triethylamine (48:52:0.1 v/v) with adjusted pH of 3 made it possible to determine the analyte under optimum condition. An endogenous interference was eluting just prior to analyte and the level of acetonitrile was critical for providing an acceptable resolution between Zar-Me and endogenous peaks in a relatively short run time. Although RP-C_{18} column demonstrated acceptable separation of the component from endogenous peaks, but higher percentage of acetonitrile was required for the elution of the analyte within 12 min total run time. The effect of pH of mobile phase on the resolution and retention times was also investigated by varying pH within the range of 3-7. It was observed that pH changes had no marked effect on Zar-Me analysis while led to a significant decrease in the sharpness of tolmetin peak, thereby a pH of 3 was chosen. Under the given HPLC conditions, internal standard and the analyte eluted at retention time of 4.9 and 9.7 min, respectively and the total time of the chromatogram was 12 min.

Selection of internal standard: A number of substances were screened to select a suitable internal standard. Considering the good extractability, proper retention time and no interference of drug-free plasma, tolmetin sodium was selected as the most suitable internal standard.

Assay validation

Linearity and selectivity: Eight points' calibration curves were constructed and there was a good linear relationship between peak area ratio (y) of Zar-Me to internal standard and C(x) over the range of 30-2000 ng/mL. The mean correlation coefficients of calibration curves were more than 0.997 and the relevant slope was statistically different from 0 ($p < 0.00$). A typical calibration curve had the regression equation of y = 0.0031 (6.26×10^{-5})x - 0.036(0.03).

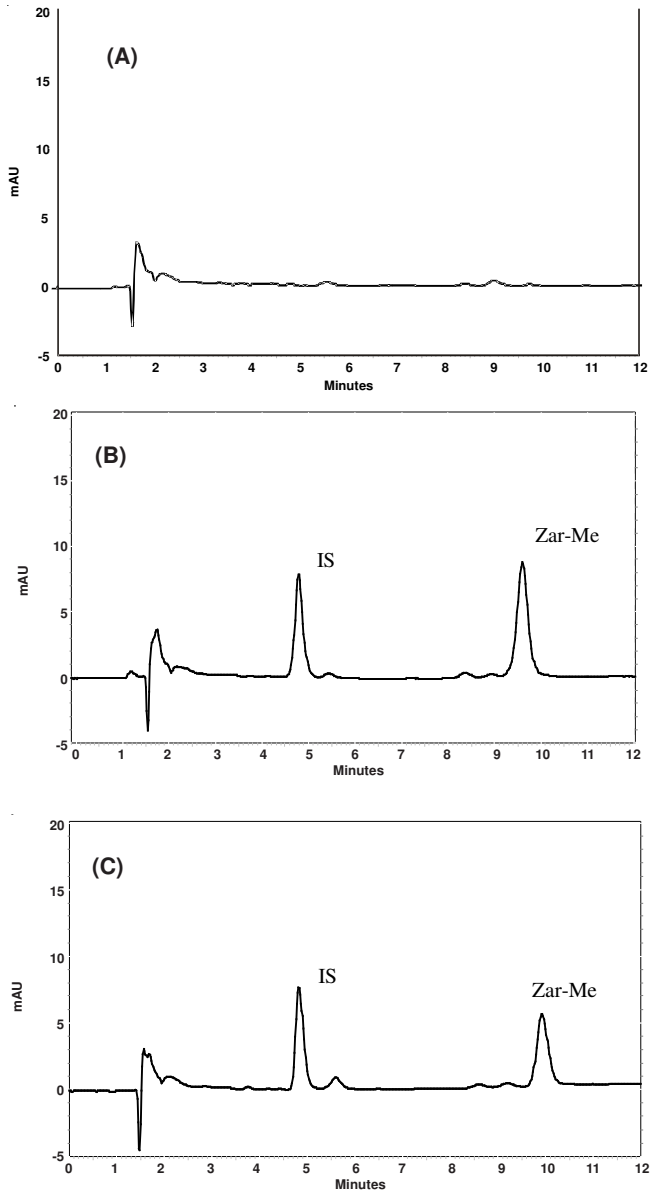

Fig. 2. Chromatograms obtained: (A) blank mouse plasma; (B) blank plasma spiked with Zar-Me at 500 ng/mL and 677 ng/mL of IS; (C) mouse plasma 0.5 h after an IV dose of 40 mg/kg of the compound

Precision and accuracy: The precision of the assay method was validated by the determination of the intra-day and inter-day coefficient of variation (% RSD). Table-1 shows the inter-day and intra-day precision data for the proposed method using mouse plasma spiked with Zar-Me over the concentration range of 30-2000 ng/mL. As shown all percentage RSDs were less than 5 % ranging from 3.9-4.9. As the averages inter- and intraday precision of the analyte in plasma were within 5 %, the assay is highly reproducible and robust.

The intra-day and inter-day accuracy data obtained by calculating the percentage of difference between amount found and amount added (relative error) in mouse plasma at the three concentrations is included in Table-1. The intra-day accuracies were from -1.29 to 3.45 % and the inter-day accuracies were from -1.6 to 1.2 %. These results were considered satisfactory.

Selectivity: No detectable interfering peak was found with retention times close to those of internal standard and Zar-Me due to the matrix in the extracts from drug-free plasma samples.

Per cent recovery: The recovery of Zar-Me in plasma samples after sample preparation employed here was tested at three concentration levels (50, 500, 1000 ng/mL) using quality control samples compared to the same concentrations of analyte in mobile phase (Table-2). The overall mean recovery was calculated at 84 %.

TABLE-2
RECOVERY OF Zar-Me FROM PLASMA AT VARIOUS CONCENTRATIONS, (MEAN ± SD, n = 3)

Concentration (ng/mL)	Recovery (%)
50	81.56 ± 2.78
500	88.74 ± 4.43
1000	82.92 ± 1.46

Quantitation limits: Using a 150 µL plasma sample LOQ and LOD (defined as a minimum signal-to-noise of three) of the assay were 30 and 10 ng/mL, respectively.

Stability studies: Stability studies were carried out at three concentration levels (50, 500 and 1000 ng/mL). The results of ambient temperature stability were found to be within the assay variability limits. The dried samples stored refrigerated were found to be stable for at least 24 h. No significant variation of concentrations was observed at -20 ºC temperatures after 1 week conservation. After 1 month stock solutions of Zar-Me and tolmetin did not show any degradation. This was confirmed by replicate injections of standards. Consequently, stock solutions were found to be stable for at least 30 days at -20 ºC. The analyte and internal standard were adequately stable under sample preparation conditions over a sufficient period of time to cover the sample preparation process.

Pharmacokinetic study: Plasma samples of mice receiving single 40 mg kg^{-1} IV dose were analyzed for Zar-Me by the above mentioned HPLC method. The log plasma concentration-time profile of the analyte was shown in Fig. 3. Pharmacokinetic parameters were obtained by both two compartmental and non-compartmental analysis, as described earlier. The mean pharmacokinetic parameters for Zar-Me after IV administration in mice were presented in Table-3. As shown in Fig. 3 after IV bolus administration Zar-Me followed a biphasic pattern.

TABLE-1
ACCURACY AND PRECISION IN SPIKED PLASMA

Concentration added (ng/mL)	Intra-day variation (n = 6)			Inter-day variation (n = 10)		
	Mean concentration found (ng/mL)	Precision (% RSD)	Accuracy (% RE)	Mean concentration found (ng/mL)	Precision (% RSD)	Accuracy (% RE)
50	50.72	4.91	2.97	49.90	4.68	-1.60
500	496.48	3.90	-1.29	502.35	4.37	1.225
1000	1034.55	4.09	3.45	1006.83	4.76	1.20

RE = Relative error, RSD = Relative standard deviation.

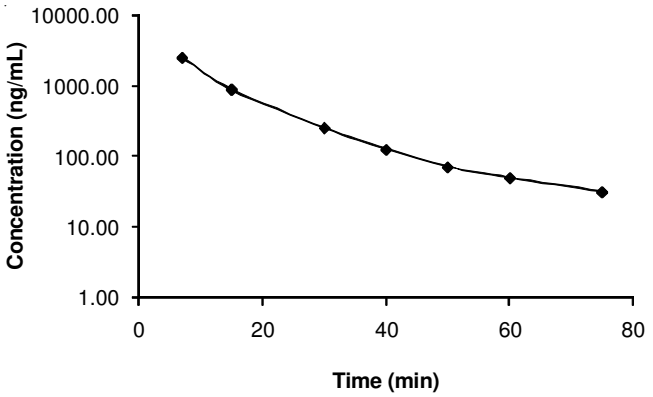

Fig. 3. Mean plasma concentration-time profile of Zar-Me after IV administration of 40 mg/kg to mice

TABLE-3
PHARMACOKINETIC PARAMETERS OF Zar-Me AFTER SINGLE IV ADMINISTRATION OF 40 mg/kg IN MICE

Parameter	Mean (SEM)
A (ng/mL)	8119.06 (243.22)
B (ng/mL)	982.47 (64.81)
α (min^{-1})	0.198 (0.01)
β (min^{-1})	0.047 (0.00)
$t_{1/2}(\alpha)$ (min)	3.50 (0.04)
$t_{1/2}$ (β) (min)	14.54 (0.15)
AUC (ng min/mL)	57842.62 (178.97)
MRT (min)	10.37 (0.39)
Cl (mL/min kg)	648.25 (8.18)
V_{ss} (mL/kg)	6728.59 (296.69)

Conclusion

Determination of drug candidate concentrations in biological fluids continues to be of great importance in preclinical and clinical phase of drug development. A rapid and sensitive isocratic reversed-phase HPLC method was developed in the present study for the determination of Zar-Me, a novel COX-2 inhibitor, in mouse plasma. The analysis technique meets ICH standards for selectivity, linearity, precision and accuracy. Sample preparation procedure proved to be an adequate way for the separation of the analytes from matrix interferences. The method was linear in the concentration range of 30-2000 ng/mL. The intra-day and inter-day coefficients of variation for the analyte averaged ranging from 3.9-4.9. Simple work up procedure, the good sensitivity and resolution, small sample volume (150 µL) as well as the short analysis time (*ca.* 12 min) could provide the basis for further development the examined compound.

ACKNOWLEDGEMENTS

The authors acknowledged Ms. Zahra Abbasian and Ms. Zoreh Solemani for their valuable technical assistances.

REFERENCES

1. J.R. Vane, *Nat. New Biol*., **231**, 232 (1971).
2. L. Laine, *Gastroenterology*, **120**, 594 (2001).
3. R.C. Harris and M.D. Breyer, *Clin. J. Am. Soc. Nephrol*., **1**, 236 (2006).
4. C. Richardson and P. Emery, *Drug Saf*., **15**, 249 (1996).
5. G. Singh, *Am. J. Med*., **105**, 31S (1998).
6. M.M. Wolfe, D.R. Lichtenstein and G. Singh, *N. Engl. J. Med*., **340**, 1888 (1999).
7. C.K. Ong, P. Lirk, C.H. Tan and R.A. Seymour, *Clin. Med. Res*., **5**, 19 (2007).
8. G.W. Cannon, *Drugs Today (Barc)*, **36**, 255 (2000).
9. P. Bertin, J.M. Behier, E. Noel and J.L. Leroux, *J. Int. Med. Res*., **31**, 102 (2003).
10. G.S. Geis, *Scand. J. Rheumatol. Suppl*., **109**, 31 (1999).
11. G.W. Cannon, *Drugs Today (Barc)*, **35**, 487 (1999).
12. D. Clemett and K.L. Goa, *Drugs*, **59**, 957 (2000).
13. A.T. Koki, K.M. Leahy and J.L. Masferrer, *Exp. Opin. Investig. Drugs*, **8**, 1623 (1999).
14. G.M. Pasinetti, *Arch. Gerontol. Geriatr*., **33**, 13 (2001).
15. A. Zarghi, S. Arfaee, P.N. Praveen Rao and E.E. Knaus, *Bioorg. Med. Chem*., **14**, 2600 (2006).
16. M.J. Rose, E.J. Woolf and B.K. Matuszewski, *J. Chromatogr. B: Biomed. Sci. Appl*., **738**, 377 (2000).
17. P.T. Vallano, R.S. Mazenko, E.J. Woolf and B.K. Matuszewski, *J. Chromatogr. B Anal. Technol. Biomed. Life Sci*., **779**, 249 (2002).
18. E. Stormer, S. Bauer, J. Kirchheiner, J. Brockmoller and I. Roots, *J. Chromatogr. B Anal. Technol. Biomed. Life Sci*., **783**, 207 (2003).
19. H.H. Chow, N. Anavy, D. Salazar, D.H. Frank and D.S. Alberts, *J. Pharm. Biomed. Anal*., **34**, 167 (2004).
20. L. Salgado, G. Encina, R. Farran, S. Puig and L. Martinez, *Chirality*, **16**, 302 (2004).
21. D.J. Jaworowicz Jr., M.T. Filipowski and K.M. Boje, *J. Chromatogr. B: Biomed. Sci. Appl*., **723**, 293 (1999).
22. F. Jamali and S. Sattari, *J. Pharm. Pharm. Sci*., **3**, 312 (2000).
23. U. Werner, D. Werner, A. Pahl, R. Mundkowski, M. Gillich and K. Brune, *Biomed. Chromatogr*., **16**, 56 (2002).
24. H. Jalalizadeh, M. Amini, V. Ziaee, A. Safa, H. Farsam and A. Shafiee, *J. Pharm. Biomed. Anal*., **35**, 665 (2004).
25. N.V. Ramakrishna, K.N. Vishwottam, S. Wishu and M. Koteshwara, *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci*., **802**, 271 (2004).
26. N.V. Ramakrishna, K.N. Vishwottam, S. Wishu and M. Koteshwara, *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci*., **816**, 215 (2005).
27. C.M. Rolim, V. Porta and S. Storpirtis, *Arzneimittelforschung*, **57**, 537 (2007).
28. G. Khaksa and N. Udupa, *J. Chromatogr. B Biomed. Sci. Appl*., **727**, 241 (1999).
29. L. Brautigam, G. Vetter, I. Tegeder, G. Heinkele and G. Geisslinger, *J. Chromatogr. B: Biomed. Sci. Appl*., **761**, 203 (2001).
30. U. Werner, D. Werner, B. Hinz, C. Lambrecht and K. Brune, *Biomed. Chromatogr*., **19**, 113 (2005).
31. H.G. Gika, A. Theodoridou, F. Michopoulos, G. Theodoridis, E. Diza, L. Settas, P. Nikolaidis, C. Smith and I.D. Wilson, *J. Pharm. Biomed. Anal*., **49**, 579 (2009).
32. C.Z. Matthews, E.J. Woolf and B.K. Matuszewski, *J. Chromatogr. A*, **949**, 83 (2002).
33. ICH, International Conference on Harmonization. Guidance for Industry, Q2B: Validation of Analytical Procedures: Methodology (1996).
34. L. Shargel, S. Wu-Pong and A.B.C. Yu, Applied Biopharmaceutics and Pharmacokinetics, Appleton & Lange, Connecticut, edn. 5, p. 67 (2005).