Comparative in vitro Dissolution and in vivo Bioequivalence of 2 Pentoxifylline Sustained Release Formulations

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Key words
- pentoxifylline
- dissolution
- bioavailability
- bioequivalence

Abstract

Pentoxifylline is a xanthine derivative that is indicated for the treatment of patients with intermittent claudication on the basis of chronic occlusive arterial disease of the limbs. In the present study, prior to the in vivo study, an in vitro comparative dissolution test was performed by the paddle method for 2 oral sustained release pentoxifylline tablets (400 mg) following the bioequivalence guidance of FDA. Metrics of peak exposure (C_{max}) and total exposure to 24 h (AUC_{24}) were compared using a randomized, single oral, open-label, 2-period, 2-sequence, 2 treatments crossover study in 24 healthy male volunteers under fasted conditions. After an overnight fast, the volunteers received 400 mg pentoxifylline and the blood samples were collected over a 24-h period following drug administration. Plasma drug concentrations were measured by a reverse-phase HPLC method with ultraviolet detection. In vitro dissolution tests requirements were met by both formulations. Observed exposure metrics for test and reference products were 140.6 ± 51.5 and 132.6 ± 48.5 ng/ml for C_{max} and 986.4 ± 350.7and 1 035.8 ± 350.3 ng.h/ml for AUC_{0-24} respectively. The confidence intervals (90 %) around ratios (test/reference) of least squares means derived from logarithmic transformed exposure metrics were 0.9912–1.1564 % for C_{max} and 0.8886–1.0535 % for AUC_{0-24}. Therefore it can be concluded that both products are bioequivalent in terms of peak and total exposure and therefore interchangeable.

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Introduction

Pentoxifylline [1-(5-oxohcxyl)-3,7-dimethylxanthine] (CAS-6493–05–6) is a xanthine derivative useful in intermittent claudication on the basis of chronic occlusive arterial disease of the limbs [1]. Intermittent claudication (IC) is defined by leg muscle pain, cramping and fatigue brought on by ambulation/exercise; relieved on rest; and caused by inadequate blood supply and is the primary symptom of peripheral arterial disease (PAD) [2]. It can improve function and symptoms but is not intended to replace more definitive therapy, such as surgical bypass, or removal of arterial obstructions when treating peripheral vascular disease [3]. The mechanism by which pentoxifylline (PTX) works is not well known, but appears to be related to erythrocyte adenosine triphosphate (ATP) concentrations and the phosphorylation of erythrocyte membrane proteins, both mechanisms resulting in an improvement in erythrocyte flexibility [2]. PTX is a highly water-soluble drug (~77 mg/mL) and is well absorbed from the gastrointestinal tract (>95 %), however the amount of drug bioavailable to the body is only about 20–30 % because of extensive hepatic first-pass metabolism. After oral and intravenous dosing, pentoxifylline is extensively metabolized. One of the major metabolites in plasma is l-(S'-hydroxyhexyl) 3,7- dimethylxamhine, while the 2 major urinary metabolites are 1-carboxypropyl-7-dimethylxanthine and 1-carboxybutyl-3,7-dimethylxanthine. Pentoxifylline has a short half-life of approximately 1–2 h [1,4–7]. PTX is not known to induce its own metabolism after multiple oral doses of the drug. Other recognized metabolites have lower plasma concentrations and less or no activity [1]. Pentoxiphylline excretion is almost totally urinary and no parent drug is essentially found in urine. Small amount (4 %) of the administered dose is recovered in feces. Food intake shortly before dosing delays absorption of an immediate-release dosage form but does not affect total absorption. However in the case of PTX sustained release tablets the total extent of absorption is also increased by co-administration of drug with meals.

Pentoxiphylline is made and marketed by more than one pharmaceutical manufacturer. There is evidence that the bioavailability of different formulations of the same drug, in the same dosage form and the same strength may be different which could be a special challenge to physicians and pharmacists regarding the therapeutic effect of the products. For this purpose the bioavailability of drug products are determined and compared according to FDA guidelines. The objective of this study was to assess the in vitro and in vivo bioequivalence of generic pentoxifylline preparations at 400 mg after a single oral dose administration in healthy Iranian male volunteers.

Methods

Dissolution testing

Dissolution studies on test and reference sustained release tablets of PTX were conducted in USP apparatus 2 (paddle method) with 12 replicates. The dissolution medium was 900 mL water. The paddle rotation speed was kept at 75 rpm. Samples were withdrawn and filtered at 0.5, 1, 2, 4, 6, 8, 10, 12, and 24 h, and assayed by UV spectrophotometry at 274 nm (Shimadzu UV-160A, Japan). Cumulative percentages of the drug dissolved from the tablets were calculated. In vitro bioequivalence was demonstrated by comparison of the dissolution profile after fitting into the mathematical model, similarity factor, f_2. The similarity factor (f_2) was calculated to compare the dissolution profiles of a pair of sustained release PTX tablet formulations using the following formula.

$$f_2 = 50 + \log \{[1+ (1/n) \sum_{t=1} * n (R_t-T_t)^2]^{-0.5} *100\}$$

Where, f_2 is the similarity factor; n is the number of time points; R_t is the mean percent drug dissolved e.g. a reference product; T_t is the mean percent drug dissolved of e.g. a test product. An f_2 value between 50 and 100 suggests that the 2 dissolution profiles are similar. The uniformity of dosage units was demonstrated by 2 methods; weight variation and content uniformity, according to the drug monograph in USP30. Finally the assay of drug content which is a global requirement was analyzed for both products using the method described in USP30.

Subjects

24 Iranian healthy male volunteers were enrolled in this study. All of them aged between 20 and 29 years (23.6 ± 2.1 years) and weight from 62 to 100 kg (75.1 ± 10.7 kg). Volunteers were studied for hypersensitivity to pentoxifylline, alcohol dependency or drug addiction, smoking, treatment with other drugs during the last 2 weeks and undergoing a medical examination at another hospital during the study. Before the studies subjects underwent physical examination such as status of the heart, lungs and blood circulation, as well as medical history was recorded. The volunteers were informed about aims, methods, objectives and potential hazards of the study, and written consent was obtained. The study was reviewed and approved by the local ethical review board of Tabriz University of Medical Sciences in Iran and conducted in conformity with the Declaration of Helsinki (World Medical Assembly 1964) as revised in Edinburgh (2000).

Study design

400 mg PTX as a single sustained release dosage form of the test (Exir Pharmaceutical Co, Boroujerd, Iran) and reference (batch no. 025560) products were randomized and administered to the volunteers after 10 h of overnight fasting in an open label, randomized, 2 sequence, 2 period, crossover study with a 2 week washout period between 2 treatments. A crossover study is a longitudinal study in which subjects receive a sequence of exposures. In crossover studies the influence of confounding covariates is reduced because each patient serves as his or her own control. Subjects were given standard breakfast 2 h, lunch 6 h and dinner 12 h after the morning drug administration and were only allowed drinking mineral water during the day getting sample. Serial blood samples from subject's forearm veins were drawn prior to the dosing at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12 and 24 h after each administration. All samples were centrifuged in heparinized tube. The plasma samples were separated and kept frozen at −20 °C for subsequent analysis. Each tube was properly labeled indicating patient identity and the sampling time after drug administration.

Analysis of plasma samples

Several extraction procedures have been described for the assay of PTX and its metabolites in biological fluids [8–11]. PTX and its metabolites have been assayed in biological fluids by thin-layer chromatography (TLC) [4], high performance thin-layer chromatography [10, 11], gas chromatography (GC) [4, 12], rapid high-performance liquid chromatography-tandem mass spectrometry [9], and several high performance liquid chromatography (HPLC) [1, 4, 10, 13, 14]. In this study pentoxifylline plasma concentrations was determined using a modified extraction procedure followed by a high performance liquid chromatographic (HPLC) method which was validated for specificity, accuracy, precision and sensitivity. Runs contained quality control samples (QC) at 4 concentration levels. The recoveries of PTX analysis at calibration curve concentration range were evaluated by comparison of peak areas obtained after extraction of known amount of drug from plasma with those of obtained from the same amounts of unextracted PTX in water. For sample preparation, on 1 ml of plasma 50 µl of the internal standard (10 µg/ml of theophylline) (Sigma, St Louis, MO, USA) was added, then all samples were extracted with 5 ml diethyl ether (Merck, Darmstadt, Germany) by vortexing for 5 min. Extraction with diethyl ether gave better recovery and cleaner chromatograms with lesser number of peaks. After centrifugation for 5 min at 1 000 g, the upper organic phase was transferred to a 7 ml glass evaporation tube. The tubes were placed in a 45 °C water bath. After dryness the residue reconstituted with 200 µL of the mobile phase by vortex mixing for 20 s. 150 µL of the resulting solution was injected onto the HPLC column. The mobile phase consisted of methanol-tetrahydrofurane-0.02 M monobasic potassium phosphate (Merck, Darmstadt, Germany) pH 4.0 adjusted with o-phosphoric acid (45:1:55, v/v) (Merck, Darmstadt, Germany), Analytical column used for chromatographic separations was Shimpack CLC C18 (250×4.6 mm) 5 µm (Shimadzu, Columbia, MD, USA) with a Shimpack C18 (10×4 mm) 5 µm precolumn guard (Shimadzu, Columbia, MD, USA); the flow rate was 1.5 ml/min; the detector wavelength was set at 273 nm. Under these conditions the retention times for pentoxifylline and the internal standard (theophylline) were 6 and 4 min respectively. A liquid chromatographic system (Beckman, Fullerton, CA, USA) comprising of 126 gold solvent delivery module equipped with a Rheodyne (Rheodyne, Cotati, CA, USA) injector and a variable wavelength ultraviolet spectrophotometric detector (166 gold, Beckman, CA, USA) set at 273 nm. System Gold software was used for data acquisition and System Gold nouveau software was used for data reporting and analysis.

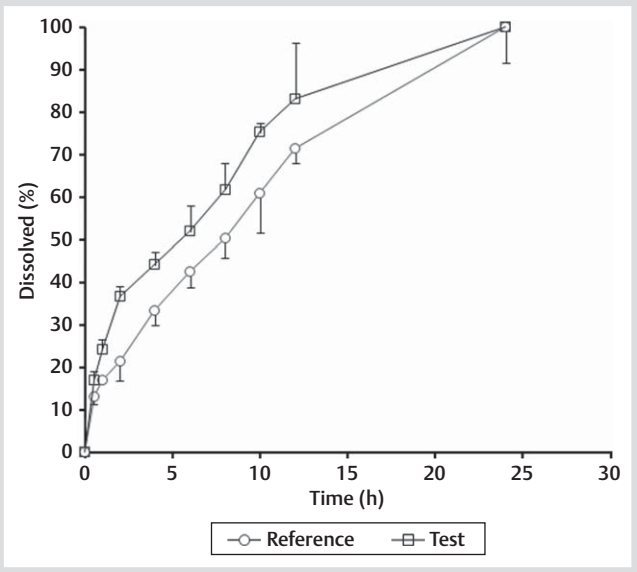

Fig. 1 Dissolution profiles of PTX. Circles and squares are the data for Reference and Test formulations, respectively.

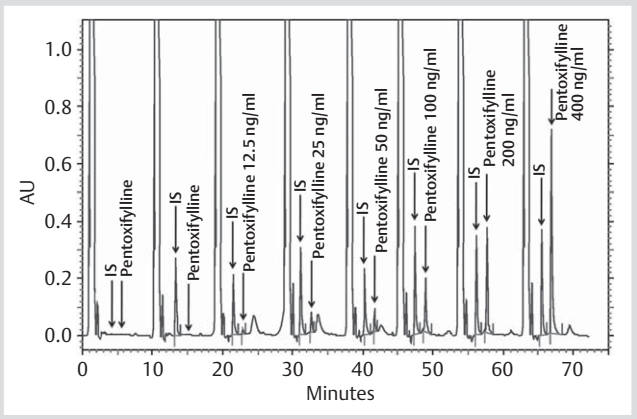

Fig. 2 Representative chromatogram of blank human plasma, blank human plasma spiked with internal standard, and standard concentrations of PTX.

Pharmacokinetic study

To calculate pharmacokinetic parameters for pentoxifylline the individual plasma concentration-time profile was constructed using standard non-compartmental methods for each treatment. Maximum plasma concentration (C_{max}) and the time at which this occurred (T_{max}) obtained from the individual subject plasma concentration-time profiles. The area under the plasma concentration-time curve from time zero to t (AUC_{0-t}) was calculated using the linear trapezoidal rule. The terminal first order constant (k_{el}) was determined by a least squares fit of the terminal plasma concentrations (using Excel® for Windows®). The elemination half life ($t_{1/2}$) was determined with the quotient of $0.693/k_{el}$. The constant k_{el} was used to extrapolate $AUC_{t-\infty}$. $AUC_{0-\infty}$ was obtained from AUC_{0-t} plus $AUC_{t-\infty}$. Bioequivalence between the formulations was determined by calculating 90% confidence intervals (90% C.I.) for the ratio of C_{max}, AUC_{0-t}, and $AUC_{0-\infty}$ values for the test and reference products and were compared to the reference intervals (0.8–1.25) as suggested by the FDA [15,16]. Treatments were compared by analysis of variance (ANOVA) with effects for sequence, subject nested within sequence, period and treatment. Moreover a computer program

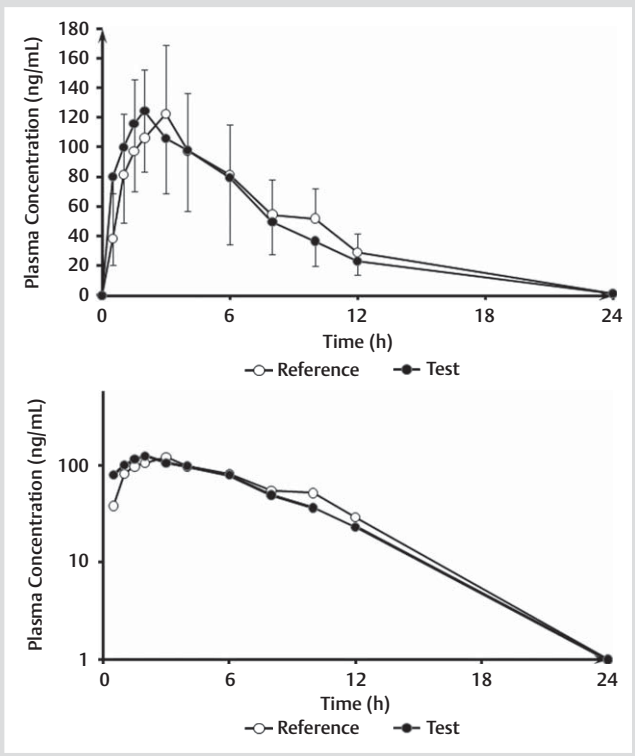

Fig. 3 Average (±SD) plasma concentration of PTX vs. time plots of 24 subjects after administration of 400 mg PTX tablets of test and reference formulations (Top: Linear scale, Bottom: Log-Linear scale).

(kinetica 5.0 PK/PD Analysis) was used for pharmacokinetic analyses. Peroral data from each volunteer were fitted to 1, 2 and 3 compartment models with first order absorption, with lag time and elimination from the central compartment.

Results and Discussion

An oral dosage form is normally composed of a drug substance and excipients. The type of excipients, proportion between ingredients, and the manufacturing method of the final product gives each product certain dissolution characteristics, which varies from one brand to the other. According the obtained results both PTX sustained release formulations met the general pharmaceutical specifications for weight variation, content assay and content uniformity test. Both products released not more than 30% in first hour and not less than 80% within 20 h and passed the USP standard for dissolution test of PTX sustained release tablets. Therefore the obtained results preclude any possibility of problems in bioavailability resulting from drug dissolution and consider the test product interchangeable with the innovator brand. The release profile of PTX from 2 products is shown in **Fig. 1**. Moreover for 2 dissolution profiles to be considered similar and bioequivalent, f_2 value should be between 50 and 100, therefore having similarity factor of 51, 2 tested brands are similar and interchangeable. Typical chromatogram of blank human plasma, blank human plasma spiked with theophylline as internal standard, and 5 standard concentrations of pentoxifylline are shown in **Fig. 2.** All chromatograms are free from any interfering peak at the retention times of PTX and internal standard, theophylline. The LOQ for pentoxifylline was 10 ng/ml and the assay was linear over the concentration ranges

Table 1 Pharmacokinetic parameters of PTX in healthy volunteers (n = 24) after a 400-mg oral dose of test and reference products.

parameters	Test product		Reference product	
	Mean	90%CI	Mean	90%CI
C_{max} (ng/ml)	140.6 ± 51.5	123.3–157.9	132.6 ± 48.5	117.4–149.9
T_{max}(h)	2.21 ± 1.22	1.8–2.62	2.77 ± 0.69	2.54–3.00
AUC_0^1 (ng.h/ml)	986.4 ± 350.7	868.6–1104.1	1035.8 ± 350.3	918.2–1153.4
AUC_0^∞ (ng.h/ml)	986.4 ± 350.7	868.6–1104.1	1035.8 ± 350.3	918.2–1153.4

Table 2 Analysis of variance (ANOVA) results.

Pharmacokinetic parameter	ANOVA (P-value)			C.I. 90% for the ratios
	Variation source			
	Product	Group	Period	
C_{max}	0.3136	0.1076	0.0962	99.12–115.64
AUC_0^1	0.3462	0.1763	0.790	88.86–105.35
AUC_0^∞	0.3462	0.1763	0.790	88.86–105.35

12.5–400 ng/ml, with a coefficient of correlation (r^2) of 0.999 [17–19], within-day reproducibility of ±4.46% for 100 ng/ml and a day-to-day reproducibility of ±5.55% for the same concentration. The recovery of pentoxifylline was 82.08, 84.53, and 80.50% at concentrations of 20, 200, and 400 ng/ml respectively. Recovery of the internal standard was 87%. The drug was well tolerated by all subjects and no adverse effects were reported which could have influenced the outcome of the study. The mean serum concentration-time profiles after single oral dose administration of reference and test products are illustrated in the following **Fig. 3**.

A one-compartment model best described the decline in PTX plasma concentrations following oral administration in each subject. The estimated pharmacokinetic parameters by the model for k_{el} (elimination rate constant from the central compartment), V_z (Apparent volume of distribution during the terminal phase), T_{abs} (duration of absorption), and Cl (total clearance) were 0.28 (1/h), 2210 (L), 4.18 (h), 404 (L/h) for test and 0.16 (1/h), 2390 (L), 4.12 (h), 379 (L/h) for reference product respectively. The resulting pharmacokinetic parameters are shown in **Table 1**. Mean maximum serum concentrations of 140.6 ± 51.5 ng/ml (90% CI: 123.3–157.9) and 132.6 ± 48.5 ng/ml (90% CI: 117.4–149.9) were obtained for the test and reference products, respectively. T_{max}, the time required to reach the maximum serum concentration, was 2.21 ± 1.22 h (90% CI: 1.8–2.62) and 2.77 ± 0.69 h (90% CI: 2.54–3.00), respectively. Bioavailability parameters are in good agreement with the previous study by Yuen et al. where a generic preparation of PTX sustained release tablet was evaluated in comparison with a proprietary product, Trental 400 [20]. In that study, the PTX mean ± SD values for C_{max}, T_{max} and $AUC_{0-\infty}$ were 166.91 ± 69.43 ng/ml, 2.4 ± 0.8 h and 1078.19 ± 502.14 ng.h/ml for the reference and 181.69 ± 69.92 ng/ml, 2.3 ± 0.7 h and 973.12 ± 423.87 ng.h/ml for the test preparations. In addition to C_{max} and T_{max}, the ratio of $C_{max}/AUC_{0-\infty}$ also can be used as a parameter for evaluating the absorption rates in bioequivalence studies. These calculated ratios were 14.2% and 12.9% for the test and reference products. The parameters used as measures of the extent of absorption are AUC_{0-t}, $AUC_{0-\infty}$. The AUC_{0-24} and $AUC_{0-\infty}$ for the test formulation was 986.4 ± 350.7 ng.h/ml (90% CI: 868.6–1104.1). The calculated values for the reference product were 1035.8 ± 350.3 ng.h/ml (90% CI: 918.2–1153.4). The confidence limits shown in **Table 2** reveal that these values are entirely within the bioequivalence acceptable bounds of 80–125% for the primary pharmacokinetic parameters proposed by the FDA and EMEA [21,22]. All statistical tests used a 5% level of significance. The multivariate analysis accomplished through analysis of variance (ANOVA) indicated that there were no statistical differences between the 2 formulations with any of the pharmacokinetic parameters (**Table 2**). Furthermore, periods and sequence effects did not influence the outcome of the statistical analysis.

Conclusion

The results of in vitro tests confirm the presence of bioequivalence between the analyzed drugs, which could be a proof of the scientific basis for excipient and manufacturing process selection in the development of PTX test product. On the other hand, the currently accepted criterion for bioequivalence for most dosage forms requires that the mean pharmacokinetic parameters of the test dosage form should be within 80–120% of the reference dosage form using the 90% confidence interval. The multivariate analysis, accomplished through analysis of variance (ANOVA) for assessment of period, group and product effects, revealed the absence of any of these effects in the present study. The 90% confidence intervals for the ratio of C_{max} (99.12–115.64%), (88.86–105.35%) and (88.86–105.35%) values for the test and reference products were within the 80–120% interval proposed by FDA and EMA. Thus, from the results obtained it can be concluded that the pentoxifylline tablets of test and reference products are bioequivalent in terms of rate and extent of absorption.

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Conflict of Interest

The authors report no conflicts of interest.

References

1 *Best B, Burns J, DeVincenzo J et al.* Pharmacokinetic and tolerability assessment of a pediatric oral formulation of pentoxifylline in Kawasaki disease. Current Therapeutic Research 2003; 64 (2): 96–115
2 *Meru AV, Mittra S, Thyagarajan B et al.* Intermittent claudication: an overview. Atherosclerosis 2006; 187 (2): 221–237

3 *Raz I*, *Ben-David J*, *Hussein Z et al.* Comparative pharmacokinetic analysis of novel sustained-release dosage forms of pentoxifylline in healthy subjects. International Journal of Clinical Pharmacology Therapy and Toxicology 1988; 26 (4): 206–208
4 *Mancinelli A*, *Pace S*, *Marzo A et al.* Determination of pentoxifylline and its metabolites in human plasma by high-performance liquid chromatography with solid-phase extraction. J Chromatogr 1992; 575 (1): 101–107
5 *Shivhare UD*, *Ambulkar DU*, *Mathur VB et al.* Formulation and evaluation of pentoxifylline liposome formulation. Digest Journal of Nanomaterials and Biostructures 2009; 4 (4): 631–637
6 *Varshosaz J*, *Minayian M*, *Moazen E.* Enhancement of oral bioavailability of pentoxifylline by solid lipid nanoparticles. J Liposome Res 2010; 20 (2): 115–123
7 *Renke M*, *Rutkowski P*, *Tylicki L et al.* Pentoxifylline old drug or new hope for nephrology? Przeglad Lekarski 2008; 65 (7–8): 358–361
8 *Szymura-Oleksiak J*, *Sarczyk A*, *Cios A et al.* The kinetics of pentoxifylline release in vivo from drug-loaded hydroxyapatite implants. Ceramics International 2001; 27 (7): 767–772
9 *Vlase L*, *Kiss B*, *Muntean D et al.* Rapid high-performance liquid chromatography-tandem mass spectrometry method for determination of pentoxifylline and its active metabolites M1 and M5 in human plasma and its application in bioavailability study. Talanta 2010; 82 (3): 945–951
10 *Jia Woei W*, *Kah Hay Y*, *Kok Khiang P.* Simple high-performance liquid chromatographic method for determination of pentoxifylline in human plasma. J Chromatogr B Biomed Sci Appl 1998; 716 (1–2): 387–391
11 *Bauerova K*, *Soltes L*, *Kallay Z et al.* Determination of pentoxifylline in serum by high-performance thin-layer chromatography. Journal of Pharmaceutical and Biomedical Analysis 1991; 9 (3): 247–250
12 *Cleary JD*, *Evans PC*, *Hikal AH et al.* Administration of crushed extended-release pentoxifylline tablets: Bioavailability and adverse effects. American Journal of Health-System Pharmacy 1999; 56 (15): 1529–1534
13 *Marsella R*, *Nicklin CF*, *Munson JW et al.* Pharmacokinetics of pentoxifylline in dogs after oral and intravenous administration. American Journal of Veterinary Research 2000; 61 (6): 631–637
14 *Beermann B*, *Ings R*, *Mansby J.* Kinetics of intravenous and oral pentoxifylline in healthy subjects. Clinical Pharmacology and Therapeutics 1985; 37 (1): 25–28
15 *Zakeri-Milani P*, *Valizadeh H*, *Islambulchilar Z.* Comparative bioavailability study of two cefixime formulations administered orally in healthy male volunteers. Arzneimittel-Forschung/Drug Research 2008; 58 (2): 97–100
16 *Zakeri-Milani P*, *Valizadeh H*, *Ghanbarzadeh S et al.* Pharmacokinetics and comparative bioavailability study of two clarithromycin suspensions following administration of a single oral dose to healthy volunteers. Arzneimittel-Forschung/Drug Research 2009; 59 (8): 429–432
17 *Zakeri-Milani P*, *Barzegar-Jalali M*, *Tajerzadeh H et al.* Simultaneous determination of naproxen, ketoprofen and phenol red in samples from rat intestinal permeability studies: HPLC method development and validation. Journal of Pharmaceutical and Biomedical Analysis 2005; 39 (3–4): 624–630
18 *Valizadeh H*, *Zakeri-Milani P*, *Islambulchilar Z et al.* A simple and rapid high-performance liquid chromatography method for determining furosemide, hydrochlorothiazide, and phenol red: Applicability to intestinal permeability studies. Journal of AOAC International 2006; 89 (1): 88–93
19 *Nemati M*, *Valizadeh H*, *Ansarin M et al.* Development of a simple and sensitive high-performance liquid chromatography method for determination of glucosamine in pharmaceutical formulations. Journal of AOAC International 2007; 90 (2): 354–357
20 *Yuen KH*, *Wong JW*, *Peh KK et al.* Comparative bioavailability study of two controlled-release pentoxifylline tablet preparations. Drug Development and Industrial Pharmacy 2000; 26 (7): 803–807
21 *FDA.* Guidance for industry, bioavailability and bioequivalence studies for orally administered drug products; General considerations. U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), USA: http://www.fda.gov/cder/guidance/5356fnl.pdf 2003
22 *EMEA.* Guideline on the Investigation of Bioequivalence. European Medicines Agency, Pre-Authorisation Evaluation of Medicines for Human Use, Committee for Medicinal Products for Human Use (CHMP), London, UK: http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003011.pdf 2008