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Validation of the Analytical Method for *In-Vivo* Determination of Meloxicam and Bioequivalence Study from Meloxicam Containing Microparticle Formulations in Rabbits

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Introduction

Inflammation reaction is a rapid process induced by prostaglandin E_2 (PGE_2) which is one of the yield products of cyclooxygenase (COX) enzyme. Mechanical trauma, corrosive chemicals and antigen-antibody reactions are the most common stimulants of inflammation reaction in the body. PGE_2 affects the neurons resulting in systemic inflammation symptoms such as edema, pain and fever [1-2]. Three different types of COX enzymes exist in the human body, which are named as COX-1, COX-2 and COX-3. The COX-3 enzyme is a splice variant of COX-1 enzyme that retains intron one and has a frame shift mutation. Depending on this similarity it is sometimes named as COX-1b or COX-1 variant. COX-1 and COX-2 types are expressed at different levels in human tissues. Although they are very much similar in fashion, selective inhibition is the discriminating factor in terms of side effects. COX-1 enzyme is generally

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expressed in the tissues which are mainly responsible for maintaining the uniformity of the body such as blood cell aggregation, gastrointestinal channel and kidney homeostasis. On the other hand, COX-2 enzyme is expressed as a response to cytokines, growth factors, tumor inducing agents and bacterial endotoxins [2-4].

Meloxicam (MLX), 4-hydroxy-2-methyl-N-(5-methyl-2-thiazolyl)-2H-1, 2-benzothiazine-3-carboxamide-1,1-dioxide, is a member of non-steroidal anti-inflammatory drugs having analgesic and antipyretic effects. MLX is a yellow, odorless powder that is practically insoluble in water (12µg/mL), but on the other hand it is relatively more soluble in acidic and basic solutions [5]. According to the Biopharmaceutics Classification System (BCS), the drugs are classified as Class I to Class IV according to their solubility and permeability properties [6]. MLX is a good representative of BCS Class II drugs with its low solubility and high permeability. In this study we have used chitosan coated sodium alginate microparticles containing MLX which were previously prepared by orifice-ionic gelation method [7-8]. The major focus of this study was to validate the analytical method for determination of MLX from rabbit plasma and compare the bioavailabilities of the microparticle formulations containing MLX after oral administration to New Zealand rabbits.

Materials and Methods

Materials

The model drug MLX was from Dr. Reddy's Laboratories (India, Batch No: MX20141T06). Tenoxicam was obtained from Madex Pharmaceuticals (Switzerland; Batch No:330018202) and all other chemicals used were analytical grade and used without further purification.

Chromatography

For the *in-vivo* determination of MLX from plasma, a high performance liquid chromatography system (HPLC) (HP 1100, Hewlett Packard GmBH, Germany) which has been equipped with a quaternary pump, an auto sampler, an injector with a 100µL loop, a column oven, a UV detector and a HP Chem Station software was used [9]. As the internal standard, tenoxicam (TNX) was used. 75 µL of TNX solution in methanol (200 µg/mL) was added

into 200 µL of plasma sample and vortexed for 15 seconds. After addition of 200 µL 1 M HCl, for the precipitation of proteins, the solution was again vortexed for 30 seconds. No degradation has been observed for MLX and TNX as seen in Figure 1a and b. The solution was then extracted with 2 mL of chloroform for 3 minutes and the samples were centrifuged at 4500 rpm for 15 minutes. 1 mL of sample from the organic phase was transferred into a clear tube and evaporated under nitrogen gas. The residue was reconstructed with the mobile phase (100 µL) and 15 µL of this final solution was analyzed for quantification of MLX and TNX. The chromatographic conditions were set as follows: UV detection at wavelength (λ) of 363.4 nm; Nucleosil C18 reverse phase column (length x internal diameter); mobile phase: 50 mM diammoniumhydrogenphosph ate:methanol:acetonitril (5:4:1-v:v:v); flow rate was 1 mL/min at ambient temperature.

Analytical method validation

Calibration and control samples

The working solutions for calibration were prepared from the stock solution of MLX (400 µg/mL) in methanol. The samples were spiked with TNX as the internal standard at concentration of 16µg/mL. Calibration curve was constructed from blank sample and 7 non-zero samples covering the total range of 66.66 ng/mL up to 2133.33 ng/mL of MLX. Calibration curves were generated on 6 different batches and linearity was assessed by weighed ($1/x^2$) least squares regression analysis. The acceptance criterion was set as the 2% coefficient of variation at same concentration of the 6 different batches.

Specificity

The chromatogram of 1.2 µg/mL MLX sample and TNX (15 µg/mL) was compared with blank plasma HPLC chromatogram for the investigation of possible peak interactions.

Accuracy

Three concentrations as low, medium and high (123.07, 1230.76 and 2133.33 ng/mL) were set as the control points of the analytical method.

Six different solutions at these three concentrations were prepared and analyzed with the HPLC method. The recovery of the MLX was then calculated with the calibration curve and the results were evaluated over the coefficient of variation at these concentrations.

2.3.4. Precision

Within batch accuracy and precision evaluations were performed by repeated analysis of MLX solution having concentrations of 66.66, 666 and 2133.33 ng/mL as low, medium and high concentrations. The reproducibility of the determined concentrations was evaluated over six different batches. The repeatability of the stock solutions was assessed by six replicates over the same batch. The results are expressed in terms of means, standard deviations and coefficient of variation.

2.3.5. Stability

The short term stability of MLX in plasma was evaluated by keeping the sample containing 666 ng/mL of MLX at -20°C for 10 days. On day 0, 1, 3, 6 and 10, the samples were analyzed and the concentration of MLX was determined. For each day, six replicates were analyzed in one analytical batch. The concentration of MLX after each storage period was related to its initial concentration as determined for the samples that were freshly prepared and possessed immediately.

2.3.6. Sensitivity

The sensitivity of the analytical method has been evaluated by the determination of limit of detection (LOD) and limit of quantification (LOQ) parameters [10-11]. The LOD value was recorded as the concentration of MLX with the signal to noise ratio of the HPLC chromatogram was 3:1. Similarly the LOQ value was recorded as the signal to noise ratio was 10:1.

2.4. *In-Vivo* Experiments

The *in-vivo* studies are conducted by using six healthy New Zealand (NZ) rabbits weighing 2.5-3.5 kg. The experimental protocols were approved by the Hacettepe University Local Ethics Committee for Animal Experiments with the protocol number 2006/40-5. The study was designed as a single dose, two-way cross over study with a washout period of 10 days using formulations that contain 15 mg MLX as *formulation A and formulation B (-fast and -slow release).* Formulation A and B denotes

for microparticle formulations prepared by using 1% (w/v) and 2% (w/v) sodium alginate, respectively [7].

Animals were randomly assigned in equal numbers to two sequences of formulations so that they would receive all two formulations upon the completion of the study. The formulations were administered to NZ rabbits by oral gavage and 10 mL of water was administered by the same way after each dose. Blood samples of 0.5 mL were collected into heparinized tubes prior to dosing (0-hour) and at 1, 2, 3, 4, 5, 6, 8, 12, 16, 24, 36, 48, 60 and 72 hours after dosing in each period. The samples were kept at -20°C until analysis.

Pharmacokinetic parameters of area under curve (AUC), maximum plasma concentration (C_{max}), time for maximum plasma concentration (t_{max}), plasma elimination half life ($t_{1/2}$) were determined from the plasma samples, by exponential stripping computer programme (ESTRIP) software.

2.5. Statistical Evaluations of the In-vivo Results

According to the guidelines on the design of bioequivalence studies, the design should be in the way that minimizes the variances in the experimental design. Therefore, crossover studies are needed to be used and the order of administration for test and reference products should be specifically defined before starting the experiments [12-13]. In the evaluation phase of the study, log transformed $AUC_{0-\infty}$ and C_{max} values were compared using analysis of variance (ANOVA). The values of the test and the reference products were evaluated within the 90% confidence interval between 80%-125% range. After the determination of plasma profiles for two microparticle formulations (Formulation-A and –B), the possible bioequivalency of the two formulations were investigated with ANOVA after log transformation of the raw data of $AUC_{0-\infty}$.

3. Results and Discussion

3.1. Chromatography

MLX and TNX were well separated from the materials existing in the plasma at retention times of 3.97 min. and 6.11 min. for TNX and MLX respectively (Figure 1). The peaks were of good shape, completely resolved from another peaks originating from plasma without any interference.

Analytical Method Validation

Linearity and Specificity of the Assay

Linear least-square regression analysis of the calibration graph on six different batches demonstrated linearity between the response and nominal concentration of MLX/TNX over the range of 66.66-2133.33 ng/mL. After the evaluation of the standard curves of MLX/TNX in 6 different batches, linear regression analysis revealed determination coefficient (r^2) value of 0.9932 with the equation of y=0.0004x-0.0138. The statistical parameters for the average calibration curve are presented in Table 1 with LOD and LOQ values.

Also the specificity of the assay was well established after spiking the blank plasma with MLX and TNX solutions. There were no interfering peaks and the retention times for TNX and MLX were recorded as 3.97 and 6.11 minutes.

TABLE I

The statistical parameters for the average calibration curve for HPLC assay of MLX/TNX

	Slope	**Intercept**	**r^2**	**LOD (ng/mL)**	**LOQ (ng/mL)**
Mean	0.0004	0.0138	0.9932	41.37	66.66
S.D.	0.0002	0.0006	0.0011		
S.E.	$4.083x10^{-5}$	$0.024x10^{-2}$	$0.045x10^{-2}$		

S.D.= Standard Deviation; **C.V.=**Coefficient of Variation; **LOD:** Limit of Determination; **LOQ:** Limit of Quantification; **r^2:** Determination Coefficient

Precision

The precision of the analytical method was investigated over the subparameters of repeatability and reproducibility. The results of the precision parameters are given in Table 2 and Table 3 with the mean±standard deviations. The data showed excellent reproducibility of the sample analysis and perfect recovery with the coefficients of variations smaller than 10%.

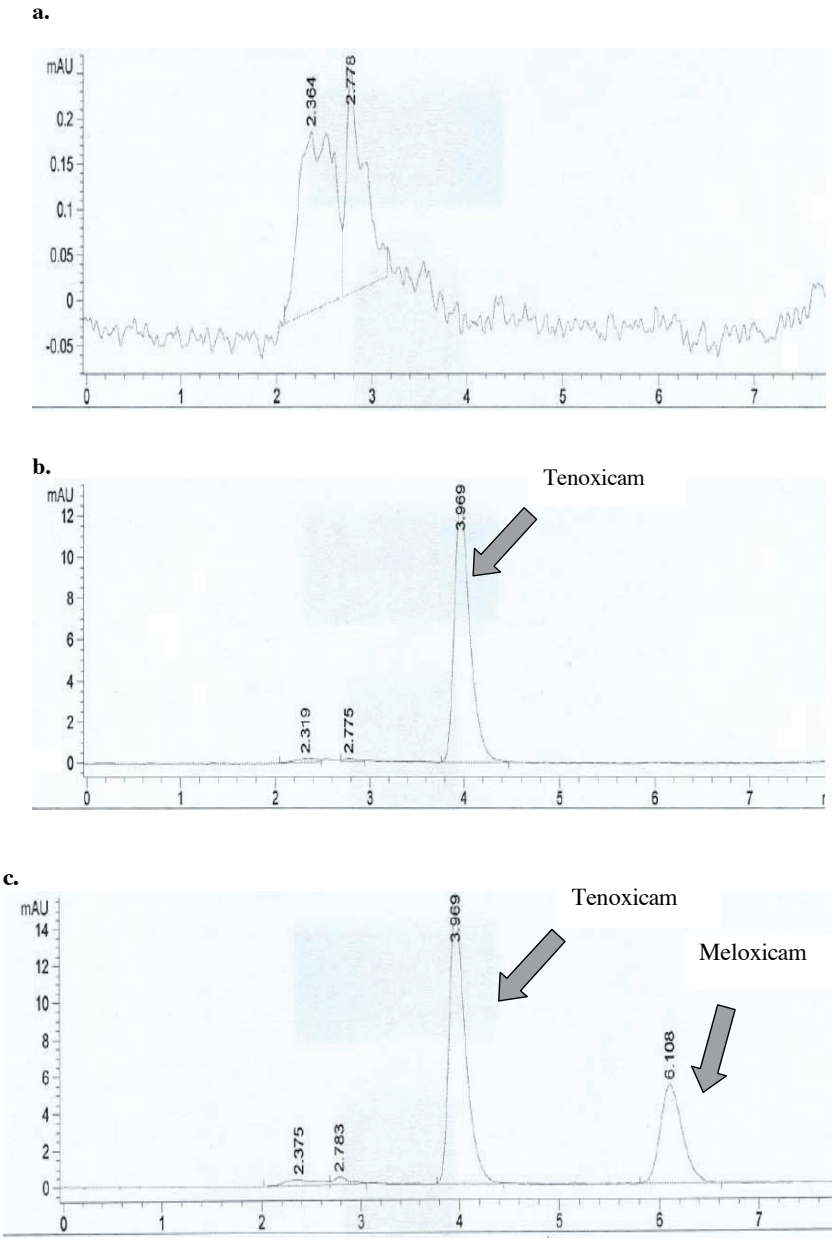

Figure 1
HPLC chromatogram of **a.** blank plasma, **b.** plasma spiked with TNX **c.** plasma spiked with TNX and MLX.

TABLE II

Reproducibility Results

	Concentration (ng/mL)		
N	66.66	666	2133.33
1	63.69	660.40	2117.36
2	77.19	657.04	2170.55
3	62.24	664.12	2062.80
4	69.25	653.98	2196.64
5	69.14	658.29	2036.38
6	64.16	695.39	2118.93
Mean	67.614	664.869	2117.111
S.D.	5.534	15.330	61.077
C.V.	8.184	2.305	2.884

S.D.= Standard Deviation; **C.V.=**Coefficient of Variation

TABLE III

Repeatability parameters

	Concentration (ng/mL)		
N	66.66	666	2133.33
1	64.07	660.41	2117.37
2	67.25	657.04	2170.55
3	63.32	664.12	2062.80
4	69.68	653.98	2196.64
5	63.42	658.29	2036.38
6	66.13	695.39	2118.92
Mean	65.642	664.786	2117.111
S.D.	2.527	15.330	61.077
C.V.	3.849	2.306	2.884

S.D.= Standard Deviation; **C.V.=**Coefficient of Variation

Accuracy

The accuracy of the method was evaluated with three concentrations of MLX as 123.07, 1230.76 and 2133.33 ng/mL) over six replicates. The recovery of the MLX was then calculated with the calibration curve and the results were evaluated over the coefficient of variation at these concentrations (Table 4).

TABLE IV

The recovery results of the three concentrations of MLX (n=6)

	Spiked Concentration (ng/mL)			Recovery %		
	123.07	1230.76	2133.33			
Determined Concentration (ng/mL)	132.95	1217.77	2312.09	108.04	98.94	108.38
	132.21	1214.82	2223.58	107.43	98.70	104.23
	125.69	1293.44	2131.23	102.13	105.09	99.90
	127.66	1237.76	2087.94	103.73	100.57	97.87
	123.22	1202.72	2133.52	100.12	97.72	100.01
	118.58	1182.78	2158.21	96.35	96.10	101.17
Mean	126.72	1224.88	2174.43	102.97	99.52	101.97
S.D.	5.467	38.163	80.806	4.443	3.101	3.787
C.V.	4.314	3.115	3.716	4.315	3.116	3.716

S.D.= Standard Deviation; **C.V.=**Coefficient of Variation

Stability

The stability results for MLX in plasma are summarized in Table 5. From the spiked plasma with MLX (666 ng/mL), the concentration was determined on certain time points with six replicates.

TABLE V

Stability results for MLX in plasma

	Determined Concentration (ng/mL)							
Time (Day)	0	1	3	6	10	Mean	S.D.	C.V.
Spiked Concentration 666 ng/mL	660.40	661.37	655.12	649.09	657.83	656.765	4.928	0.750
	657.03	655.97	679.23	665.55	663.67	664.294	9.316	1.402
	664.12	660.52	651.08	654.78	650.76	656.257	5.897	0.898
	653.98	660.83	681.83	675.73	665.59	667.596	11.224	1.681
	658.29	671.76	651.30	662.80	665.95	662.023	7.740	1.169
	695.38	688.01	649.26	688.47	671.10	678.449	18.605	2.742

S.D.= Standard Deviation; **C.V.=**Coefficient of Variation

Sensitivity

The sensitivity of the HPLC method for in-vivo determination of MLX has been investigated by determining the LOD and LOQ. The minimum detected concentration was 41.37 ng/mL with the signal:noise ratio (3:1) and the limit of quantification was 66.66 ng/mL with the signal:noise ratio of 10:1.

In-Vivo experiments

The plasma profiles after administration of microparticle formulations have been determined and the pharmacokinetic parameters were calculated (Table 6). As the concentration of sodium alginate used in the preparation of microparticle formulations was increased, longer t_{max} values were observed. Similar to this, the $t_{1/2}$ values also increased with respect to the sodium alginate concentrations. The total plasma profiles of the rabbits after administration of MLX containing formulations are given in Figure 2.

TABLE VI

Pharmacokinetic parameters for MLX in NZ Rabbits (n=6). Data are means ± Standard Deviation

Parameter and Unit	Formulation-A (1%-w/v Na Alginate)	Formulation-B (2%-w/v Na Alginate)
*$AUC_{0-\infty}$ (µg.h/mL)	19.84 ± 12.70	20.85 ± 35.38
Min-Max	8.39-44.74	17.25-27.56
*t_{max} (h)	9.66 ± 3.88	16.33 ± 12.02
Min-Max	6-16	4-36
*$t_{1/2}$ (h)	23.37 ± 10.47	34.75 ± 28.44
Min-Max	13.47-43.04	10.67-87.72
*C_{max} (µg/mL)	0.78 ± 0.44	0.68 ± 0.33
Min-Max	0.44-1.66	0.47-1.29

*$\mathbf{AUC_{0-\infty}}$: Area under the concentration-time plasma curve extrapolated to infinity; $\mathbf{t_{max}}$: Time for maximum plasma concentration; $\mathbf{t_{1/2}}$: Elimination half life; $\mathbf{C_{max}}$: Maximum plasma concentration

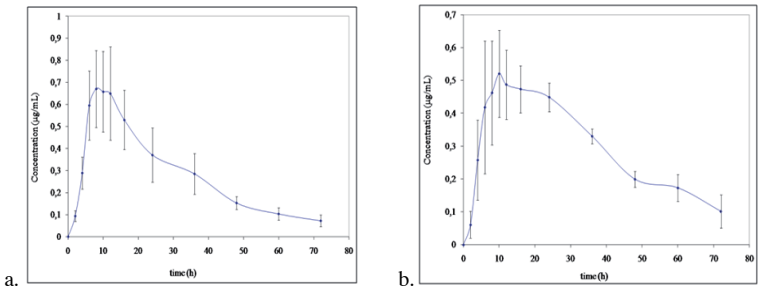

Figure 2

The plasma profiles of MLX after oral administration to rabbits (n=6) after oral administration of **a.** Formulation A **b.** Formualtion B to NZ Rabbits

After determination of the plasma profiles of the microparticle formulations, the necessary log transformations for C_{max} and $AUC_{0-\infty}$ values were calculated and summarized in Table 7-8 and the other statistical parameters degree of freedom, sum of squares, mean of squares which are used in the determination of bioequivalence limits and coefficients of inter and intra subject variations, are summarized in Table 9-10.

For microparticle formulations A and B, the $AUC_{0-\infty}$ values were found within the range of 68.38%-147.87% (90 % Confidence Interval-C.I.). Intra-subject variation coefficient and inter-subject variation coefficient were calculated as 31.35 and 24.31, respectively. The values for C_{max} were within the range of 78.85%-161.49% (90% C.I.). The intra-subject variation and inter-subject variation for C_{max} were 29.15 and 35.96, respectively.

TABLE VII

Comparison of C_{max} values for Formulation-A (Test) and Formulation-B (Reference) and logarithmic transformation

		Raw Data			**Log-Transformed Data**	
ID	**Sequence**	**Test *C_{max}**	**Ref. C_{max}**	**Relative C_{max} (%)**	**Test Ln (C_{max})**	**Ref Ln (C_{max})**
1	A-B	446.82	755.631	59.132	6.102	6.628
2	B-A	724.7232	661.384	109.577	6.586	6.494
3	A-B	1664.056	1294.244	128.574	7.417	7.166
4	A-B	633.494	352.1181	179.910	6.451	5.864
5	B-A	502.1441	478.31	104.983	6.219	6.170
6	B-A	725.2886	552.4317	131.290	6.587	6.314
Mean		782.75	682.35	118.9108	6.5603	6.4394
*S.D.		446.59	330.99	39.5527	0.4634	0.4439
*C.V.		57.05	48.51	33.2625	7.0636	6.8931

***S.D.=** Standard deviation; **C.V.=**Coefficient of variation;

C_{max}: Maximum plasma concentration (ng/mL)

TABLE VIII

Comparison of $AUC_{0-\infty}$ values for Formulation A (Test) and Formulation-B (Reference) and logarithmic transformation

		Raw Data			Log-Transformed Data	
ID	**Sequence**	**Test *AUC$_{0-\infty}$**	**Ref. AUC$_{0-\infty}$**	**Relative AUC (%)**	**Test Ln (AUC$_{0-\infty}$)**	**Ref. Ln (AUC$_{0-\infty}$)**
1	A-B	17223.17	20499.9	84.016	9.754	9.928
2	B-A	14807.26	20627.97	71.782	9.603	9.934
3	A-B	44747.65	27560.84	162.360	10.709	10.224
4	A-B	15096.87	18802.25	80.293	9.622	9.842
5	B-A	8390.334	20364.69	41.200	9.035	9.922
6	B-A	18787.73	17247.23	108.932	9.841	9.755
	Mean	19842.17	20850.48	91.4305	9.7606	9.9342
	*S.D.	12706.43	3538.81	41.0496	0.5434	0.1580
	*C.V.	64.04	16.97	44.8971	5.5671	1.5902

***S.D.**= Standard deviation; **C.V.**=Coefficient of variation; **$AUC_{0-\infty}$**: Area under plasma concentration versus time curve for MLX

TABLE IX

Statistical Data for *C_{max}

Sources of Variation	*D.F.	Sum of Squares	Mean of Squares	P
Subjects	5	1.71800	0.34360	
Period	1	0.00081	0.00081	0.00954
Formulation	1	0.04387	0.04387	0.51639
Error	4	0.33980	0.08495	
Total	11	0.33979	0.03089	

***D.F.**=Degree of Freedom; **C_{max}**: Maximum plasma concentration

TABLE X

Statistical data for *$AUC_{0-\infty}$

Sources of Variation	*D.F.	Sum of Squares	Mean of Squares	P
Subjects	5	1.08310	0.21662	
Period	1	0.12478	0.12478	1.26932
Formulation	1	0.09043	0.09043	0.91986
Error	4	0.39323	0.09831	
Total	11	1.69154	0.15378	

***D.F.**=Degree of Freedom; **AUC:** Area Under Curve

Conclusion

In this study, major aim was to partially validate a simple and rapid HPLC analysis method for the determination of MLX from microparticle formulations and determine the pharmacokinetic parameters after administration to NZ rabbits by oral route. For this purpose, TNX was used as the internal standard. In this method, liquid-liquid extraction was applied and the amounts of MLX and TNX were determined. This method was selected because the extraction procedure was simple and rapid; also the amount of plasma in this procedure was 100 µL [9]. In each sampling time point, maximum 300-350 µL of plasma was obtained from rabbits, so this model was the best choice for determination of MLX from plasma.

The validation results of the HPLC method were selected as linearity, accuracy, precision, specificity, sensitivity and stability. For the linearity parameter, the calibration curve was constructed between concentrations of 66.66-2133.33 ng/mL of MLX with six replicates. The slopes of these 6 different batches were compared and the variation coefficients were found as smaller than 15%. Similar to these, the variation coefficients of accuracy, precision and stability were also found as smaller than 15%. The specificity of the HPLC method indicated that, there were no other peaks interfering with the MLX and TNX peaks determined from plasma sample. The stability of MLX in plasma sample was investigated until 10 days and the statistical comparison of the results between day 0-1; day 0-3; day 0-6 and day 0-10 were evaluated by Mann-Whitney U test. As a result, no significant change was found in the concentration of MLX in plasma in 10 days time ($p>0.05$).

According to the previous literature findings, the clinical effective dose of MLX in rabbits was determined as 0.3 mg/kg. In repeated toxicology studies, it was shown that 20 mg/kg MLX has no toxic effect in rabbits. As a result, the dose of MLX used in this study was selected as 15 mg (~5mg/kg) [14].

The *in-vivo* experiments were designed in a cross-over design. The washout period was 10 days which ensures the complete clearance of MLX from the plasma. According to the pharmacokinetic results summarized in Table 6, the increase in sodium alginate concentration in microparticle formulations elevated the $AUC_{0-\infty}$; t_{max} and $t_{1/2}$ values

in rabbits. In the relative bioavailability comparison between these two microparticle formulations, ANOVA test was used.

For microparticle formulations A and B, the $AUC_{0-\infty}$ values were found within the range of 68.38%-147.87% (90 % Confidence Interval-C.I.). Intra-subject variation coefficient and inter-subject variation coefficient were calculated as 31.35 and 24.31, respectively. The values for C_{max} were within the range of 78.85%-161.49% (90% C.I.). The intra-subject variation and inter-subject variation for C_{max} were 29.15 and 35.96, respectively. These confidence interval limits are out of the range for the establishment of a bioequivalence which is 80-125%; as a result these two microparticle formulations can not be considered as bioequivalent.

Summary

Meloxicam is a member of non-steroidal anti-inflammatory drugs having analgesic and anti-pyretic effects. It belongs to the Biopharmaceutics Classification System Class II with its low solubility and high permeability profile. In this study, we have validated a rapid and simple HPLC method for the in-vivo determination of meloxicam from rabbit plasma after oral administration of microparticle formulations containing meloxicam. The pharmacokinetic parameters of $AUC_{0-\infty}$, t_{max}, $t_{1/2}$, and C_{max} were determined for the microparticle formulations. After necessary logarithmic transformation of the $AUC_{0-\infty}$ and C_{max} values, the confidence interval limits for both $AUC_{0-\infty}$ and C_{max} values were calculated as 68.38-147.87% and 78.85-161.49%, respectively. These results indicated that these two microparticle formulations were not bioequivalent, since they were both out of the range for the establishment of bioequivalence which is 80-125%.

Key Words: Meloxicam, Bioequivalence, Microparticle

Özet

***In-vivo* Meloksikam Miktar Tayininin Validasyonu ve Tavşanlarda Meloksikam İçeren Mikropartiküller ile Biyoeşdeğerlik Çalışması**

Meloksikam, analjezik ve antipiretik etkileri olan ve steroidal olmayan anti-inflamatuvar ilaçlar grubuna dahil bir etkin maddedir. Düşük çözünürlük ve yüksek permeabilite özellikleri ile Biyofarmasötik Sınıflandırma Sitemine gore Sınıf II grubuna dahildir. Bu çalışmada

meloksikam içeren mikropartikül formülasyonlarının oral olarak tavşanlara uygulanmasından sonra miktar tayininin yapılabilmesine yönelik olarak hızlı ve basit bir HPLC yöntemi valide edilmiştir. $AUC_{0-\infty}$, t_{maks}, $t_{1/2}$, and C_{maks} değerleri üzerinden farmakokinetik parametreler değerlendirilmiştir. $AUC_{0-\infty}$ and C_{maks} değerlerinin gerekli logaritmik dönüşümleri sonrasında güven aralığı değerleri sırasıyla %68.38-147.87 ve %78.85-161.49 olarak bulunmuştur. Bu sonuçlar doğrultusunda, biyoeşdeğer olarak kabul edilme sınırları olan %80-125 aralığının dışında kalmalarından dolayı bu iki mikropartikül formülasyonlarının biyoeşdeğer olmadıkları gösterilmiştir.

Anahtar Kelimeler: Meloksikam, Biyoeşdeğerlik, Mikropartikül

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