New High Performance Liquid Chromatographic Method for Simultaneous Determination of Diclofenac and Meloxicam in Oral Formulation of Liposomes and Human Plasma

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Summary: A new, easy and consistent reversed-phase high-performance liquid chromatographic method with diode array detection has been developed and validated for the simultaneous determination of diclofenac potassium (DP) and meloxicam (MLX) in liposome, marketed brands and human plasma. Microextraction method based on liquid-liquid extraction principal was used to extract drug from plasma matrix. The optimized mobile phase was used in the molar ratio of 20:20:60 (v/v/v) mixture of acetonitrile, methanol and 20 x 10^{-3} M potassium dihydrogen phosphate buffer (pH 3.7), pumped at an optimized flow rate of 1.0 mL.min^{-1}. The linearity was performed in the concentration range of 15 ng.mL^{-1} to 10μg.mL^{-1} with r^2 of 0.9989 ± 0.13 and 0.9979 ± 0.11 (n = 6) for DP and MLX, respectively. The assay was repeatable at concentration levels of 10 ng.mL^{-1}, 1 μg.mL^{-1} and 10 μg.mL^{-1} with coefficient of variation of 0.168 - 0.603% for 10 ng.mL^{-1} (DP), 15 ng.mL^{-1} (MLX) and 1 μg.mL^{-1} &10 μg.mL^{-1} for DP and MLX. The LOD values were 3 and 5 ng.mL^{-1}, while values of LOQ were 10 and 15 ng.mL^{-1}, for DP and MLX. The present method is applicable in routine quality control operations, advanced drug delivery formulations (Liposomes) and in human Plasma samples for pharmacokinetics and bioequivalence studies.

Introduction

The NSAIDs remain first-line drug therapy for a wide range of rheumatic and other pain conditions. The combination of drugs for the complete eradication of disease is requirement of era [1]. Diclofenac potassium (DP) is potassium-[(2, 6-dichlorophenyl) amino]-phenyl acetate (Fig. 1 a) [2]. It possesses analgesic, anti-inflammatory, and antipyretic activity. It inhibits prostaglandin synthesis by interfering with the action of prostaglandin synthetase (cyclooxygenase) [3]. Another novel drug Meloxicam (4-hydroxy-2-methyl-*N*-(5-methyl-l-2-thiazolyl)-2*H*-1,2-benzothiazine-3-carboxamide-1,1-dioxide) of acidic enolcarbox-amide class (Fig. 1 b) yield peak plasma concentrations in 5–11 hours and peak time is much longer under pain condition; therefore, a combination is prescribed to fill this gap and numerous studies have been taken account for using combination of analgesics [2-4]. The late onset of Meloxicam can be advantageously gapped with use of diclofenac potassium whose blood level appears with in 30 minutes after administration.

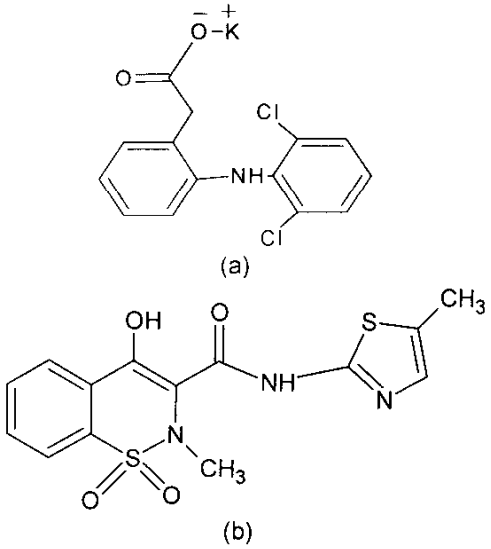

Fig. 1: Structural formula for DP (a) and MLX (b.

Literature search revealed that no HPLC method has yet been available for the analysis of DP and MX in plasma, advance drug delivery systems as well as in quality control samples. Limited

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simultaneous methods exist in literature for DP and MX separately in different combinations using spectrophotometric technique simultaneous determination of diclofenac potassium and tizanidine in tablet dosage form [5], Simultaneous narrow bore HPLC using column-switching analysis for aceclofenac and diclofenac in human plasma [6], reverse phase HPLC method for simultaneous determination of meloxicam and pridinol mesylate in drug formulations [7], LC-MS method for the simultaneous determination of piroxicam, meloxicam and tenoxiam in human plasma. Different assays are available for determination of pharmacokinetics of individual drugs, either DP or MX, using HPLC technique [8-19]. A method employing CZE (capillary zone electrophoresis) is also available for the determination of meloxicam in tablets, although benefits using less solvent and simplicity but the method are not available for simultaneous determination of meloxicam with any other drug or combinations or in plasma matrix [20]. A method demonstrated by Silvana *et al.*, (2008) determining the contents of meloxicam and pridinol mesylate in commercial pharmaceutical formulations, although the method is simple but is not applicable in plasma analysis. LC–MS/MS technique for the determination of piroxicam, meloxicam and tenoxicam in plasma was developed by Young *et al.*, (2005) seems worthless as the combination of three drugs is not prescribed for a patient suffering any kind of pain condition. A study reported by Malliou *et al.*, (2005) describing simultaneous determination of Clobutinol with some anti-inflammatory drugs in urine samples while the present method quantitated both analgesics in plasma matrix. Further, difference exists in condition of experiments and mobile phase compositions as well as results of validation parameters [21]. Microextraction methods are becoming important in analysis owing to reduced extraction time, increased efficiency, minimum solvent expenditure, minimum exposure of toxic solvents and cost effective [22-24]. No Microextraction method is available for the determination of diclofenac potassium and meloxicam. One study is available using microextraction method for analysis of diclofenac only in dosage forms [25]. Therefore, the HPLC method developed and validated in the present study can advantageously applied for the determination of DP and MLX in pharmaceuticals, advanced drug delivery systems, and therapeutic drug monitoring studies for better patient care.

Results and Discussion

Screening and Optimization

Selection of the Detection Wavelength

The HPLC-Diode array Chromatogram (Fig. 2) for solution containing both drugs showed maximum wavelength of 280 and 340 for DP and MLX, respectively. HPLC attached with DAD was used for this purpose. This suggested optimum detection wavelengths were determined in order to favor the quantification of both drugs in samples.

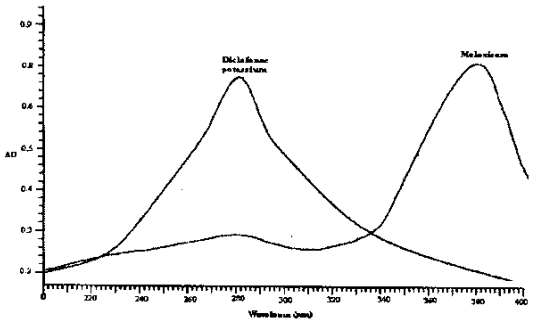

Fig. 2: UV scan of DP and MLX drug solutions in Diode array detector of HPLC.

Choice of the Mobile Phase Composition

After a series of viewing trials, the phosphate buffers provided improved and better peak resolution and separation than their acetate and citrate counterparts. It was also found rational to use methanol-acetonitrile in molar ratio of (20:20) and phosphate buffer with 60% of aqueous phase produced satisfactory peak symmetry and selectivity. The phosphate buffer of strength of 20 x 10^{-3} was selected and found better to avoid peak tailing and broadening. The effect of increased pH and buffer concentration reduce the retention time while a smooth increase of the t_r was evidenced with increments in the ionic strength of the aqueous phase. The pH of 3.7 was selected because it produces the capacity factor (k'), Separation factor (α) and Resolution (R) values of 2.115 ± 0.18, 1.16 ± 0.22 and 1.68 ± 0.32 (mean ± S.D), respectively. In reverse-phase chromatography, separation is governed by the interactions in mobile phase because the stationary phase surface is saturated by the

molecules of organic modifier of the mobile phase. A monolayer of methanol is adsorbed on to the surface of stationary phase if the concentration is greater than 10% of methanol. The addition of acetonitrile further facilitated the saturation of stationary phase layer. The changes in controlled variables like pH, buffer concentration and flow rate affect elution time, peak separation and resolution. Therefore, these conditions were optimized to adjust best retention time (t_r) of DP and MLX suitable for analysis of drugs (Fig. 3).

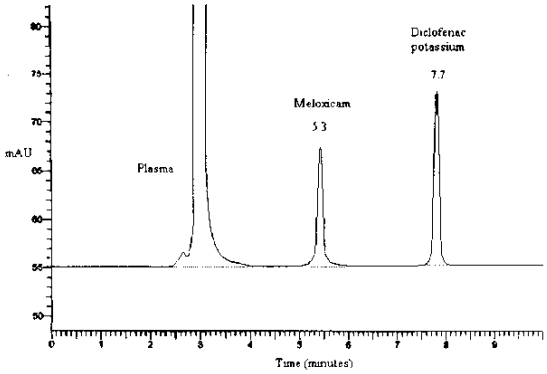

Fig. 3: Separation of DP and MLX plasma samples on C_{18} Column.

Method Validation

Linearity

Linearity is evaluated by assessing coefficient of correlation (r), slope and intercept. The present method was linear as the values were with in acceptable ranges of ICH guidelines [26]. The method was linear in concentrations range of 0.01-10 μg. mL^{-1} for DP and 0.015-10 $\mu g.mL^{-1}$ for MLX spiked in plasma. The chromatograms were gathered and peak area was recorded. The graph between concentration and peak area were constructed and presented in Fig. 4. The values of standard curve parameters were computed by Kinetica® software and shown in Table-1. Six (n=6) replicates were tested at each level. The mean value of (r^2 was 0.9989 and 0.9979 for DP and MLX, respectively) showed that method produced good linearity over entire range of concentrations and found better when compared to the previous studies available in literature [6-8, 19, 20].

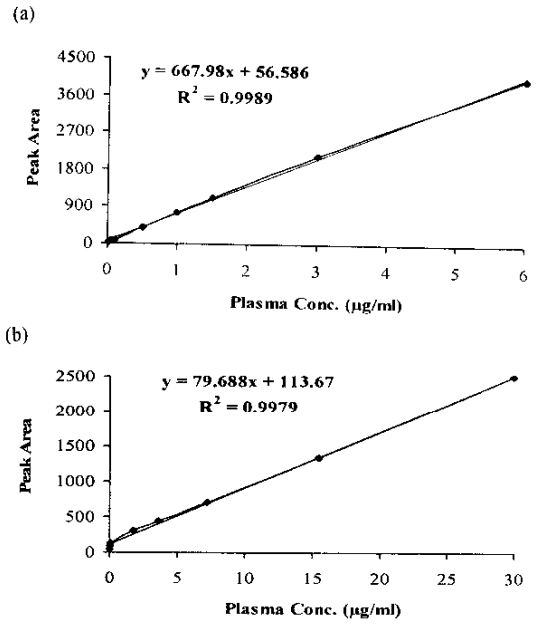

Fig. 4: Standard Curve of DP (a) and MLX (b) in spiked human plasma.

Table-1: Standard curve parameters of DP (a) and MLX (b) in plasma samples.

(a)

Curve Code	Slope	Intercept	r^2	Conc. Range
DP-01	667.94	56.670	0.9993	
DP-02	668.48	56.542	0.9990	0.01-10 ($\mu g.mL^{-1}$)
DP-03	667.53	56.546	0.9985	
Mean	667.98	6.586	0.9989	
S.D.	0.5	0.0728	0.0004	
%CV	0.072	0.13	0.040	
%error	0.61	0.58	0.52	

(b)

Curve Code	Slope	Intercept	r^2	Conc. Range
MLX-01	79.539	113.781	0.9982	
MLX-02	80.143	113.658	0.9981	0.015-10($\mu g.mL^{-1}$)
MLX-03	79.382	113.571	0.9975	
Mean	79.688	113.670	0.9979	
S.D.	0.4	0.11	0.0004	
%CV	0.504	0.093	0.038	
%error	0.72	0.75	0.67	

Limit of Detection and Quantification

Limit of detection (LOD) is the concentration of analyte that can be reliably detected under the stated experimental conditions and give signal-to-noise ratio of 2 or 3. The signal is the analyte response (peak) and noise is amplitude of short term noise appeared in the baseline. The limit of detection (LOD) was established from the relative

standard deviation (R.S.D) of the response and the slope of calibration curves prepared with known concentrations of DP and MLX. The values were 3 and 5 ng.mL^{-1} for DP and MLX, respectively. The limit of quantification (LOQ) is the amount of analyte that can be quantified under the same experimental conditions. This was calculated at 3 multiple of LOD for both drugs. The values were first values of standard curve as 10 and 15 ng.mL^{-1} for DP and MLX, respectively (Table-1). The LOD and LOQ values were better than previous studies [5-7,12,19,20] and comparable with studies employing microextraction methods [21-25].

Precision and Accuracy

Precision is the degree of closeness of results and is reported as % C.V. Repeatability (intra-day) and reproducibility (inter-day) are divisions of precision. Accuracy is the measure of systematic error or biasness. An assay is accurate if the mean value of the result is same as the true value. Nine replicate injections of the spiked standard solutions in plasma at lowest, mid and highest standard curve concentration levels of DP and MLX. The concentrations studied were 10 ng.mL^{-1}, 1 µg.mL^{-1} and 10 µg.mL^{-1} for DP and 15 ng.mL^{-1}, 1µg.mL^{-1} and 10 µg.mL^{-1} for MLX. The values of intra-day precision and accuracy (Table-2) were 99.22%-99.37% and 99.17%-99.50% while values of %bias were -0.63 to -0.78 and -0.50 to -0.83 for DP and MLX, respectively. The values of Inter-day precision and accuracy were calculated at same concentrations of DP and MLX as Intra-day studies (Table-3). 99.26-99.44% and 98.67%-99.67% precision and accuracy were recorded while values of %bias were -0.62 to -1.55 and -0.33 to -1.33 for DP and MLX, respectively. The values were found better than available in literature [5,8, 12]

Recovery

Microextraction procedure was adopted for its added benefits of higher recovery and sensitivity besides its low solvent consumption and fast. The method was validated by studying recovery of both drugs from the spiked plasma samples and it was found that extraction efficiency was better than conducted in previous studies [8,10,11,19-24]. The recovery was calculated at three concentration levels of standard dilutions of 0.01, 1 1and 10 µg.mL^{-1} for DP and 0.015, 1 1and 10 µg.mL^{-1} for MLX (Table-4).

Table-2: Inter-day precision and accuracy of DP and MLX in human plasma.

	DP			MLX		
Curve Code	LQC	MQC	HQC	LQC	MQC	HQC
Units	ng/mL	µg/mL	µg/mL	ng/mL	µg/mL	µg/mL
Nominal	10.00	1.00	10.00	15.00	1.00	10.00
Batch-01	9.92	0.99	9.98	14.990	0.98	9.99
	9.9	0.99	9.96	14.950	0.99	9.97
	9.94	0.98	9.99	14.970	0.98	9.96
Batch-02	9.96	0.99	9.90	14.940	0.99	9.94
	9.94	0.98	9.86	14.960	0.99	9.92
	9.96	0.97	9.88	14.940	0.99	9.96
Batch-03	9.92	0.99	9.94	14.900	0.98	9.9
	9.96	0.98	9.92	14.980	0.99	9.88
	9.94	0.99	9.90	14.920	0.99	9.93
Mean	9.938	0.984	9.926	14.950	0.987	9.939
S.D.	0.0211	0.007	0.045	0.029	0.005	0.035
%CV	0.2121	0.738	0.454	0.192	0.507	0.354
%Bias	-0.622	-1.556	-0.744	-0.33	-1.333	-0.611
%Accuracy	99.38	98.44	99.26	99.67	98.67	99.39

(LQC= Lowest Quality Concentration; MQC= Middle Quality Concentration; HQC= Highest Quality Concentration)

Table-3: Intra-day precision and accuracy of DP and MLX in human plasma.

Curve Code	LQC (ng.mL^{-1})		MQC (µg.mL^{-1})		HQC (µg.mL^{-1})	
	DP	MLX	DP	MLX	DP	MLX
Nominal	10	15	1.0	1.0	10.0	10.0
	9.96	14.94	0.994	0.990	9.97	9.96
	9.94	14.92	0.989	0.996	9.92	9.98
	9.96	14.88	0.994	0.989	9.94	9.86
	9.88	14.95	0.992	0.992	9.95	9.94
	9.96	14.92	0.994	0.990	9.88	9.84
	9.92	14.94	0.998	0.993	9.87	9.97
	9.86	14.98	0.998	0.992	9.95	9.94
	9.88	14.96	0.996	0.990	9.88	9.84
	9.94	14.94	0.994	0.993	9.87	9.97
Mean	9.94	14.93	0.99	0.99	9.92	9.93
S.D.	0.03	0.03	0.00	0.00	0.04	0.06
%CV	0.32	0.168	0.297	0.260	0.400	0.603
%Bias	-0.63	-0.500	-0.650	-0.833	-0.783	-0.750
%Accuracy	99.37	99.50	99.35	99.17	99.22	99.25

(LQC= Lowest Quality Concentration; MQC= Middle Quality Concentration; HQC= Highest Quality Concentration)

Table-4: Recovery (Percent Extraction Yield) of DP (a) and MLX (b) in plasma samples (a).

Curve Code	LQC		HQC	
	Extracted	Non-extracted	Extracted	Non-extracted
DP-01	9.88	10.00	9.86	9.92
	9.90	10.01	9.88	9.94
	9.94	9.99	9.90	9.98
Mean	9.91	10.00	9.88	9.95
S.D.	0.03	0.01	0.02	0.03
%CV	0.308	0.100	0.101	0.153
%PEY	99.07		99.67	

Percent Extraction Yield (PEY) is calculated as 99.07 and 99.67 for Lowest Quality Concentration and Highest Quality Concentration, respectively.

(b)

Curve Code	LQC		HQC	
	Extracted	Non-extracted	Extracted	Non-extracted
MLX-01	14.86	14.94	19.84	19.96
	14.88	14.95	19.86	19.98
	14.90	14.96	19.88	19.95
Mean	14.88	14.95	19.86	19.96
S.D.	0.02	0.01	0.02	0.02
%CV	0.134	0.067	0.101	0.077
%PEY	99.53		99.48	

Percent Extraction Yield (PEY) is calculated as 99.53 and 99.48 for Lowest Quality Concentration and Highest Quality Concentration, respectively.

Peak response of each analyte in spiked plasma was compared with standard dilution dissolved in the mobile phase. The extraction efficiency was 99.987% and 99.988% for DP and MLX, respectively. The results were higher than those of 73.1–95.1% in other published method [11, 20-25].

Stability

Lowest and highest concentrations (extremes) of each drug were selected for freezing and thawing. Three cycles freeze-thaw study showed values of difference were -1.77 (LQC) and -0.543 (HQC) for DP and -4.154 (LQC) and -2.272 (HQC) for MLX, indicating that samples were stable after freezing and thawing (Table-5). Bench top stability of spiked plasma stored at room temperature was evaluated for 12 hours (Table-6). The values of % C.V. at 12 hour were 0.215 (LQC) and 0.078 (HQC) for DP and 0.815 (LQC) and 0.0213 (HQC) for MLX. The values (mean ± SD) of long-term stability studies were at LQC (9.893 ± 0.019 for DP and 14.963 ± 0.01 for MLX). The values of MQC and HQC for both drugs were presented in Table-7. It was observed that plasma samples were stable in three types of studies and comparable to the previous studies [5-8, 20, 22-25].

Table-5: Freeze-thaw stability DP (a) and MLX (b) in human Plasma.

(a)

	Cycle							
	Cycle 0		Cycle 1		Cycle 2		Cycle 3	
Curve Code	LQC	HQC	LQC	HQC	LQC	HQC	LQC	HQC
	9.68	9.56	9.52	9.81	9.54	9.75	9.46	9.97
DP	9.81	9.98	9.91	9.71	9.42	9.68	9.81	9.64
	9.89	9.92	9.42	9.84	9.83	9.79	9.59	9.69
Mean	9.79	9.82	9.62	9.79	9.60	9.74	9.62	9.77
S.D.	0.106	0.227	0.259	0.068	0.211	0.056	0.177	0.178
%CV	1.082	2.313	2.692	0.696	2.197	0.572	1.839	1.821
%Difference	-	-	-1.804	-0.340	-2.008	-0.815	-1.77	-0.543

(b)

	Cycle							
	Cycle 0		Cycle 1		Cycle 2		Cycle 3	
Curve Code	LQC	HQC	LQC	HQC	LQC	HQC	LQC	HQC
	14.96	9.86	14.87	9.78	14.45	9.68	13.85	9.75
MLX	14.12	9.79	14.78	9.85	14.52	9.81	14.75	9.62
	14.97	9.84	14.51	9.71	14.7	9.10	13.62	9.45
Mean	14.683	9.830	14.720	9.780	14.557	9.530	14.073	9.607
S.D.	0.488	0.036	0.187	0.070	0.129	0.378	0.597	0.150
%CV	3.323	0.367	1.273	0.716	0.886	3.967	4.243	1.566
%Difference	-	-	0.250	-0.509	-0.863	-3.052	-4.154	-2.272

Application of Present Method

The present method was validated successfully and applied for the simultaneous determination of DP and MLX in pharmaceutical drug products, advanced drug delivery system and human plasma drug analysis (Table-8).

Table-6: Bench top stability DP (a) and MLX (b) in human Plasma.

(a)

Curve Code	0 h		3 h		6h		12 h	
	LQC	HQC	LQC	HQC	LQC	HQC	LQC	HQC
DP-01	10.013	9.990	9.968	9.989	9.968	9.989	10.013	9.990
	10.000	9.980	9.997	9.970	9.997	9.970	10.000	9.980
	10.042	9.970	9.992	9.960	9.992	9.960	10.042	9.970
Mean	10.0183	9.980	9.9857	9.973	9.9857	9.973	10.018	9.985
S.D.	0.022	0.010	0.016	0.015	0.016	0.015	0.022	0.007
%CV	0.2146	0.1002	0.1552	0.1477	0.155	0.148	0.215	0.071
%Difference	-	-	-0.33	-0.07	-0.33	-0.07	0.00	0.05

(b)

Curve Code	0 h		3 h		6 h		12 h	
	LQC	HQC	LQC	HQC	LQC	HQC	LQC	HQC
MLX-01	15.013	9.989	14.773	9.982	14.656	9.971	14.613	9.967
	15.000	9.973	14.879	9.965	14.864	9.974	14.844	9.964
	14.990	9.977	14.982	9.961	14.974	9.984	14.786	9.956
Mean	15.001	9.980	14.878	9.969	14.831	9.976	14.748	9.966
S.D.	0.012	0.0083	0.105	0.0112	0.162	0.007	0.120	0.002
%CV	0.077	0.0834	0.702	0.1118	1.089	1900.0	0.815	0.021
%Difference	-	-	-0.82	-0.10	-1.13	-0.04	-1.69	-0.15

Table-7: Long term stability DP and MLX in human Plasma.

Conc.	Units	Week 1	Week 2	Week 3	Week 4	Mean	S.D.	%CV
DP LQC	$ng.mL^{-1}$	9.92	9.88	9.89	9.88	9.89	0.019	0.191
DP MQC	$\mu g.mL^{-1}$	0.99	0.97	0.96	0.97	0.97	0.013	1.294
DP LQC	$\mu g.mL^{-1}$	4.98	4.98	4.96	4.95	4.97	0.015	0.302
MLX LQC	$ng.mL^{-1}$	14.98	14.97	14.96	14.94	14.96	0.017	0.114
MLX MQC	$\mu g.mL^{-1}$	0.96	0.95	0.95	0.94	0.950	0.008	0.082
MLX HQC	$\mu g.mL^{-1}$	9.98	9.96	9.94	9.94	9.955	0.019	0.192

Table-8: Application of method in analysis of liposomes, marketed brands and human plasma.

Samples		Drug/Brands	Stated	Found	Percentage ± SEM
Liposome		DP	50 (mg)	49.94 (mg)	99.92 ± 0.014
Marketed Brands	DP	Caflam® Novartis	50	49.94	99.88 ± 0.011
		Diclorep® Sami	50	49.96	99.92 ± 0.010
		Maxit® Hilton	50	99.98	99.96 ± 0.009
	MLX	Xobix® Hilton	7.5	7.46	99.47 ± 0.011
		Melox® Kobec	7.5	7.43	99.10 ± 0.012
		Artex® Pharmadic	7.5	7.44	99.20 ± 0.010
Plasma		DP	10 (ng)	9.97	99.70 ± 0.011
			1 (µg)	0.998	99.80 ± 0.012
			10(µg)	9.996	99.96 ± 0.089
			15(ng)	14.89	99.26 ± 0.009
		MLX	1 (µg)	0.997	99.70 ± 0.010
			10 (µg)	9.965	99.65 ± 0.009

SEM= standard error of mean (n = 3)

Experimental

Materials and Solvents

Diclofenac potassium was received as research material donation from Novartis Pharmaceutical (Pakistan) Ltd. Meloxicam was

donated by Getz Pharma. (Pakistan) Ltd. Soya Lecithin, chloroform, acetonitrile, methanol and disodium hydrogen phosphate buffer, ortho phosphoric acid was purchased from Merck (Germany). HPLC-grade solvents were employed for analyses.

Chromatographic System and Optimized Conditions of Separation

The chromatographic system consisted of series 1200 Agilent Technologies. Series 1200 pumps, Series 1200 variable wavelength Uv and Vis (Programmable) diode array detector (DAD) which was automatically set at wavelengths of 280 nm and 340 nm. The drugs were separated ODS Hypersil (Thermo Electron Corporation) with dimensions of 250 mm Length, 4.6 mm ID, and 5 μm particle size of internal packing. The mobile phase composition was 20:20:60 (v/v/v) for acetonitrile, methanol and 20 x 10^{-3} M sodium dihydrogen phosphate buffer, respectively. The pH was maintained at 3.7 with a flow rate of 1.0 mL min^{-1}. An aliquot of 20 μL were injected into HPLC system and spectra was recorded.

Preparation of Stock and Working Standard Solutions

The stock solutions of DP in acetonitrile and MLX in acidic methanol were prepared in a concentration of 100 $\mu g.mL^{-1}$ by dissolving an accurately weighed (Shimadzu AUX 220) amount (10 mg). The solutions were preserved at 4 °C, in light-resistant containers and were left to attain room temperature before use. Working dilutions were prepared in concentration ranges of 10 $ng.mL^{-1}$ to 10 $\mu g.mL^{-1}$ for DP and 15 $ng.mL^{-1}$ to 10 $\mu g.mL^{-1}$ for MLX in anticipated range of plasma concentration. All the solutions were stored in refrigerator throughout the experiments, when not used.

Preparation of Liposomes

Modified microencapsulation vesicle (MCV) method of Tomoko Nii and Fumiyoshi Ishi., (2005) was adopted for preparation of diclofenac potassium liposomes [27]. Soya lecithin (10 g) was dissolved in 25 mL chloroform and 2.5 gm DP dissolved in 25 mL water separately; both were mixed to form water in oil emulsion. The formed emulsion was homogenized at 10,000 rpm for half an hour. This primary emulsion was instantly poured to 100 mL of water in a round bottom flask at 45 °C at agitation speed of 700 rpm to form a water-in-oil-in-water multiple emulsion. The chloroform was recovered by rotary vacuum evaporator and DP loaded liposome suspension was freeze dried and dry powder was filled in capsules of weighing 50 mg for DP oral delivery.

Sample Preparation of Liposomes, Marketed Brands and Human Plasma

Diclofenac in liposomes equivalent to one dose were weighed dissolved in methanol and volume was adjusted to 100 mL. Similarly 20 tablets and capsules of DP and MLX were ground, LLME method was adopted for preparation of samples. 150 μL of the sample was transferred to poly propylene tube and equivalent amount of extraction solvent was added, vortexed and centrifuged at 3000 rpm for 2 minutes. The constituents were dried under gentle stream of nitrogen and reconstituted with 60 μL of mobile phased and 20 μL injected into HPLC system. Aliquot of the human plasma (200 μL) spiked with diclofenac sodium was taken in poly propylene tube and 200 μL of acetonitrile was added, vortexed for 30 seconds and centrifuged at 3000 rpm for 2 minutes. The supernatant layer was separated and filtered through cartridge filter (0.45 μm pore size and 13 mm diameter) and collected in poly propylene, dried under gentle stream of nitrogen and reconstituted with 60 μL of mobile phased and 20 μL injected into HPLC system.

Method Validation

The linearity, limit of detection, limit of quantification and precision and accuracy were established. The intra and inter-day precision and accuracy of the assay were determined by percent coefficient of variation (C.V) and percent relative error (R.E) values, respectively. Samples of lowest, middle and highest concentrations were spiked for the determination of precision and accuracy. Triplicates at each concentration were routed at day 1, 3, 5, and 7 for inter-day determination of precision and accuracy. Signal-to-noise ratio (s/n) of 3:1 was selected for the limit of detection (LOD) while three fold of LOD was finalized for determination of LOQ by comparing test results from spiked plasma samples with known concentrations of drugs with blank samples.

Recovery

The efficiency of Liquid-Liquid Micro Extraction (LLME) method was validated and confirmed by determination of extraction recovery. The peak response of spiked plasma was determined and compared of with standard QC samples at Lowest, Mid and Highest concentration. Triplicates of each concentration level was determined and compared.

Stability Studies

Three stability studies were assessed in this study. (1) Three cycles freeze and thaw stability was determined by thawing at room temperature for 2 h and then refreezing at −50 °C for 24 h. (2) bench top stability of spiked plasma stored at room temperature was evaluated for 12 h. (3) The long-term stability was assessed by carrying out the experiment for four weeks at storage temperature -20 °C.

Conclusions

The present research revealed simple and fast RP-HPLC method for the simultaneous determination of DP and MLX with DAD detection. The validation parameters were in acceptable limits of guidelines of FDA and ICH. The Microextraction procedure was simple and fast making method less time consuming and new composition of mobile phase was enable method to detect DP and MLX in shorter period of time. The method will be suitable for routine analysis of DP and MLX in routine quality control analysis in pharmaceutical industry, bioequivalence and pharmacokinetics studies as well as therapeutic drug monitoring of these drugs in optimizing dosage regimen.

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