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Development and Validation of Montelukast with Grape/Licorice Juices and its Application to Pharmacokinetic Studies by LC/MS

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ABSTRACT

A simple, rapid and sensitive simultaneous method for validation and determination of Montelukast in rat plasma in the presence of grape and licorice juices has been done by using High Performance Liquid Chromatography–Mass Spectrometry (HPLC/MS). A mixture of (77.5 % methanol, 22.5 % of 0.2% FA in water) was used as a mobile phase, ACE 5 C8 column (50 X 2.1 mm, 5µ), and a flow rate of 0.1 mL/min were used, the auto-sampler injection volume was 5 microliters, and candesartan was used as an internal standard. According to the result obtained, the precision of predicted measurements for Montelukast was good (mean CV % 0.05) of grape on Montelukast Cmax (163.492 ng/mL ±113.27). Neither licorice gives significant decrease in the Montelukast Cmax (143.861ng/mL ±54.52), its only delay the average Montelukast Cmax to half an h. Even for the AUC, there were no significant difference between Montelukast alone and Montelukast with grape, and Montelukast with licorice (P>0.05). The kinetic data shows that Montelukast plasma level was the same with both combination of Montelukast and grape, and Montelukast and licorice.

Keywords: Montelukast, Validation, LC/MS, Grape, Licorice, Pharmacokinetic.

INTRODUCTION

Drug interactions represent an important and widely under recognized source of medication errors. An interaction is said to occur when the effects of one drug are changed by the presence of another drug(s), food, drink or an environmental chemical (Bista *et al*., 2006). The relationships and interactions between foods, the nutrients they contain and drugs are gaining recognition in the health care and medical fields. Certain foods and specific nutrients in foods, if ingested concurrently with some drugs, may affect the overall bioavailability, pharmacokinetics, pharmacodynamics and therapeutic efficacy of the medications. Furthermore, the therapeutic efficacy of many drugs depends on the nutritional status of the individual (Ismail *et al*., 2009). Cysteinyl leukotriene-1 inhibitor (Montelukast) is one of the oral drugs used for long-term management of asthma in adults and children ages of 12months and older. Montelukast sodium is an oral, potent and selective antagonist at CysLT1Rs, which mediate the Broncho constrictor and pro-inflammatory actions of the CysTLs in asthma and AR. (Diamant *et al*., 2009) It is described chemically as [R-(E)]-1-[[[1-[3-[2-(7-chloro-2-quinolinyl)ethenyl]phenyl]-3-[2-(1-hydroxy-1-methylethyl)phenyl]propyl] thio] methyl]cyclopropaneacetic acid, monosodium salt. The empirical formula is C35H35ClNNaO3S, and its molecular weight is 608.18 (Figure 1) (Merck and co 2009; Riccioni *et al*., 2004; AmLani and McIvor 2011). Grapes have many biological activities such as antioxidant (Yilmaz and Toledo 2004), anticarcinogenic (Hakimmudin *et al*., 2004), anti-inflammatory, antiarthritic (Donnelly *et al*., 2004), antidiabetic (Zhang *et al*., 2004), cardio protective (Miura *et al*., 2003), neuroprotective, antipyretic (Mendes *et al*., 2003), antiviral (Docherty *et al*., 2004) and anti-encephalitozoon.

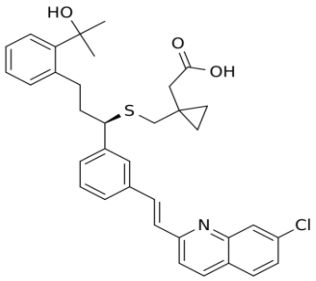

Figure 1. Montelukast chemical structure

Licorice Glycyrrhizaglabra, also known as licorice and sweet wood, is derived from the ancient Greek term glykos, meaning sweet, and rhiza, meaning root. A number of components have been isolated from licorice; it is composed of triterpenesaponins, flavonoids, polysaccharides, pectins, simple sugars, amino acids, mineral salts, and various other substances (Obolentseva *et al* 1999). Glycyrrhizin, a triterpenoid compound, accounts for the sweet taste of licorice root. This compound represents a mixture of potassium-calcium-magnesium salts of glycyrrhizic acid (Tamir *et al*., 2001). Glycyrrhizin and its metabolites inhibit hepatic metabolism of aldosterone and suppress 5-ß-reductase properties responsible for the well documented pseudoaldosterone syndrome.

There are many studies focused on Montelukast –drug interaction, Montelukast –food (beverages interactions, or grape drug interaction and licorice drug interaction, by using different techniques (Cingi *et al*., 2013; Hegazy *et al*., 2012; Karonen *et al*., 2010; Singh *et al*., 2012; Rashmitha *et al*., 2010; Madhavi *et al*., 2010; Zhou *et al*., 2011.

Many analytical methods were established for measuring Montelukast such as spectrophotometry (Saeed Arayne *et al*., 2009), liquid chromatography (LC) (Bharathi *et al*., 2009; Sripalakit *et al*., 2008; Papp *et al*., 2007), capillary electrophoresis (Shakalisava and Regan, 2008) , and spectroflurometry (Alsarra *et al*., 2005). Some of these procedures include many steps that do not fulfill the determination of the samples. Also, LC-MS/MS and HPLC Coupled with ESI-MS/MS used for determination of montelukast human plasma (Bharathi *et al*., 2009; Challah *et al*., 2010).

The Aim of this project is to develop a sensitive and simple chromatographic method for quantifying Montelukast in rat plasma and examine the possible interaction of Montelukast with different fruit juices (grape and licorice) on experimental animals, and the effect of these juices on the pharmacokinetics of Montelukast.

MATERIALS AND METHODS

Chemicals Reagents and Instrumentation

Reagents

Deionized Water, Nano pure (Fisher Scientific, B# 1207702). Rats Plasma, (Harvested from animals from animal house in UOP). Methanol advanced gradient grade (Fisher scientific, B# 1155904). Acetonitrile advanced gradient grade (Fisher scientific, B# 1156250). Formic acid advanced gradient grade (Across). Montelukast sodium (99.5% potency) (Dar Al Daw Pharma, Jordan) and Candesartan (99.5% potency) (JPM, Jordan).

Instrumentation

API Mass spectrometer with On-line vacuum Degasser (Agilent 1200), Solvent delivery systems pump (Agilent 1200), Auto-sampler (Agilent 1200), Thermostat column compartment (Agilent 1200), API 3000 Mass Spectrometer. ACE 5, C8 (50 x 2.1mm, 5µm). Computer System, Windows XP, SP3, Data Management Software 1.5.1. Bath Sonicator Crest model-175T (Ultra Sonics CORP.). Sartorius balance BP 2215. Sartorius pH meter (Professional meter PP-25). Centrifuge (Eppendorf 5417C)

Preclinical study

The study protocol was approved by the Research Committee at the Faculty of Pharmacy and Medical Sciences, University of Petra, Amman, Jordan. All animal experiments were performed in compliance with FELASA guidelines (Federation of European Laboratory Animal Science Association).

Animals

Adult male Sprague Dawley laboratory rats were supplied by the animal house of Petra University. The rat's average weight was about 270.0g, and they were in healthy condition. They were placed in air-conditioned environment (20-25°C) and exposed to a photoperiod cycle (12h light/ 12h dark) daily. The rats were divided into 12 groups, every group contain an average of six rats, four groups received Montelukast only and another four groups received Montelukast with grape, while the last four groups received Montelukast with licorice. After preparation of the drug solution, the rats received a certain amount of a drug solution (according to their weights).

Multiple doses of fruit juices were given to the rats before the administration of the drug. Blood samples were taken in Eppendorf tube by cutting the tip of the tail. Then drug solution was given to the rats orally, after that a blood sampling was done in Eppendorf tubes after 15, 30min and 1, 2, 3, 4 and 6h of administration. Eppendorf tubes were centrifuged for 10min and plasma samples were kept at – 20°C until analysis.

Stock and working solutions Preparation
Montelukast drug solution

A concentration of 0.4mg/mL of Montelukast was prepared by dissolving 0.4g of Montelukast sodium in 10mL methanol, and then 1mL of this solution was diluted in100mL distilled water.

Montelukast stock solution

An equivalent weight of 10.00mg of Montelukast dissolved it in 10mL of methanol to get 1.0mg/mL as stock solution

Stock solution of candesartan (Internal Standard, IS)

An equivalent weight of 10.00mg of candesartan dissolved it in 10mL of methanol to get 1.0mg/mL stock solution.

Working solution of Candesartan, I.S

A 300µL of candesartan stock solution (1.0mg/mL) was diluted in 100mL of methanol to get 3.0 ug/mL concentration of candesartan.

Preparation of Montelukast serial spiking samples in plasma:

Samples of standard curve in plasma were prepared by spiking 20.0 µL from serial solution into 1.0mL of plasma, using seven concentrations other than zero to obtain STD concentrations of: 10, 20, 50, 100, 200, 400 and 600ng/mL for Montelukast in plasma. Then each concentration of the plasma sample was divided to 25µL in 1.5mL Eppendorf tube and kept at (-30°C), standard samples were given daily together with the quality control samples.

Preparation of Montelukast Quality Control Samples in plasma

Samples of QC in plasma were prepared by spiking 20.0µL from serial solution into 1.0mL of plasma to obtain QC concentrations of 30, 225 and 500ng/mL for Montelukast in plasma (Table IV). Each concentration of the plasma sample was divided to 25µL in 1.5mL Eppendorf tube and kept at (-30°C), standard samples were given daily together with the quality control samples.

Preparation of grape and licorice juices

Grape had been bought from local market in Jordan. Cleaned, divided and freshly squeezed at the day of experimentation without any further treatment or additives. Licorice juice was prepared from licorice root as in (Hamad *et al*., 2017).

Extraction procedure

Sample preparation was done by pipetting 50.0µL aliquots of each test sample (blank, zero, standards, QCL, QCM, QCH and rat's plasma) into the appropriate tubes then 150µL of internal standard (3000ng/mL candesartan) solution and vigorously vortex for 1min, then centrifuge at 14000rpm for 15min. The supernatant was injected into HPLC.

Validation

Within-batch accuracy and precision evaluations were determined by analyzing 6 replicates of quality control samples. Also, the between-batch precision and accuracy were determined by analyzing three sets of within-batch quality control sequence in three separate batches. The quality control samples were randomized daily, processes and analyzed in position either immediately following the standard curve, or in the middle of batch or at the end of the batch. The specificity of the method was evaluated by screening six different lots of blank plasma according to (EMEA 2011). The chromatographic response of LLOQ must be ≥ 5 times that of blank response with accuracy 80-120% and precision ≤ 20%. Six replicates of LLOQ plasma samples were prepared along with the calibration curve. Three calibration curves consisting of a blank, zero and seven non-zero standards prepared in rat plasma for each analyte were prepared. The concentrations of calibration standards cover the range from LLOQ to the highest expected concentration. The linearity was evaluated by the linear regression (correlation coefficient, R^2. Stability of Montelukast in rat plasma is evaluated using low and high QC samples (blank plasma spiked with Montelukast at a concentration of a maximum of 3 times the LLOQ and to the ULOQ) which are analyzed immediately after preparation and after the applied storage conditions that are to be evaluated.

Table I. Chromatographic conditions and Mass Spectrometric conditions

HPLC Conditions	**Pump Flow Rate**		**Autosampler Injection Volume**			**Autosampler Temp**		**Column Oven Temp**	
	1.0mL/min		**5µL**			**4˚C**		**40˚C**	
Chromatography	Mobile phase		mixture of (77.5 % Methanol, 22.5 % (0.2% formic acid)),						
	column type		ACE 5 C8 Column (50 X 2.1 mm), 5µ						
	Expected Retention times (min)		Montelukast 0.74			Candesartn (I.S) 0.54			
MRM Detection Conditions	Analytes	Q1 Mass	Q3 Mass	Dwell	FP	DP	EP	CE	CXP
	Montelukast	586.400	568.400	150	70	81	10	19	22
	Candesartan (IS)	441.200	263.200	150	70	81	10	19	22
MS Conditions	CUR	CAD	IS		TEM		NEB		
	10	6	5500		400		5		

Mass spectrometer (MS), Curtain gas (CUR), Collision associated dissociation gas (CAD), Ion Spray voltage (IS), Temperature (TEM), Nebulizer Gas (NEB)

The QC samples are analyzed against a calibration curve, LXX obtained from freshly spiked calibration standards, and the obtained concentrations are compared to the nominal concentrations (Table I).

Pharmacokinetic analysis

No compartmental analysis using kinetica version 5 was done to calculate the area under the curve from zero to 6h (AUC), Cmax, Tmax, the K elimination was not calculated because the study time interval was up to 6h only.

Precision and accuracy

The precision and accuracy of this method were assessed using four concentrations of rat's plasma spiked with Montelukast; lower limit of quantification, low, middle and high concentrations. Intra-day precision and accuracy were assessed by analyzing the four concentrations. This was done five times daily for three different days for the inter-day precision and accuracy assessment. Also, precision was assessed by comparing each concentration with the CV% (coefficient of variation) while accuracy was assessed by comparing the mean calculated concentration with ± 20% of spiked concentration for the lower limit of quantification while it was compared with ± 15% for the other quality control samples.

Linearity

Montelukast calibration curve was drawn using the ratio of drug peak area (PAR) and area of internal standard against drug concentration (C). The unknown concentrations of montelukast calculated using the equation: (PAR = Slope × C + Intercept). The nominal values of the drug and the calculated PAR used to determine the slope and intercept. Correlation coefficient (R2) values used to determine the linearity of the plotted curve.

Stability

Montelukast stability in rats' plasma was assessed using low and high QC samples (blank plasma spiked with montelukast at a level of a maximum of 3 times the LLOQ and to the ULOQ) that analyzed at room temperature directly after preparation and after 24h. The QC samples were assessed by the calibration curve, which prepared from newly spiked calibration samples, and the resulted concentrations are compared to the nominal concentrations. The accepted ranges of mean concentrations are ±15% of the nominal concentration.

Statistical analysis

Statistical significance of difference in the mean of the variable, such as Cmax, Tmax and AUC between groups was evaluated by ANOVA and Fisher test used to detect significance in pharmacokinetic parameters with 95% confidence interval. The SYSTAT version 5 applied and $P<0.05$ was considered significant.

RESULTS AND DISCUSSION

HPLC/MS is a sensitive, selective and reproducible analytical procedure. This study established a simple, rapid and sensitive simultaneous method for validation and determination of Montelukast in rat plasma in the presence of grape and licorice juices.

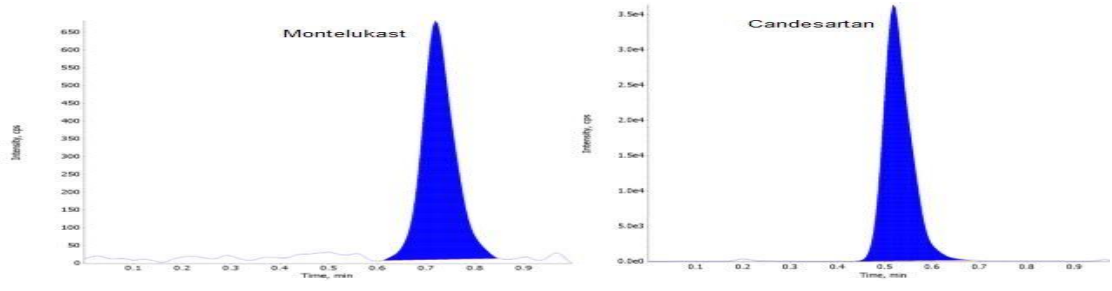

Figure 2a. Montelukast 10ng/mL (LLOQ) with candesartan (IS) chromatograms

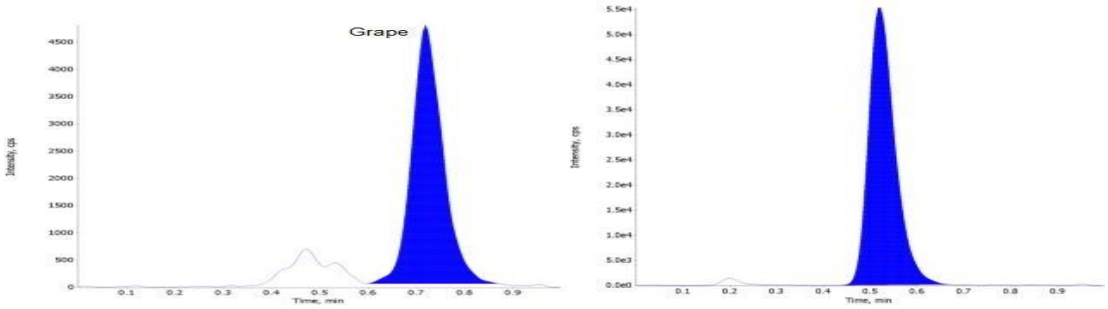

Figure 2b. Montelukast combined with grape with candesartan (IS) chromatograms

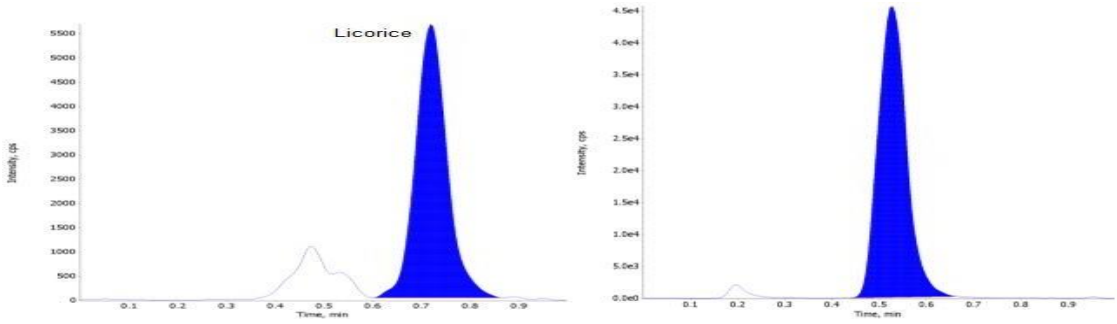

Figure 2c. Montelukast combined with licorice with candesartan (IS) chromatograms

A good separation was achieved between chromatogram peaks between montelukast and candesartan. The peaks were sharp and without tailing or splitting and with good retention time. Chromatograms representing blank rat's serum, montelukast with Low LOQ and IS and combined with grape and licorice (Figure 2a-c).

Precision

The variability of errors (precision) of the inter- and intra- for three days tests showed acceptable lower limit of quantification (LLOQ) values with coefficient of variation (CV%) ranged between 2.37-3.14%. For the quality control samples representing low (QCL), medium (QCM) and high (QCL) concentrations, the CV% was less than 15% and ranged (2.49 – 6.88%), (Table II). The inter-day and intra-day tests of accuracy were within the ±20 % for distinct sample concentrations and within ±15% for the quality control samples (Table II).

Linearity

Standard calibration curves were prepared using sex different concentrations of montelukast ranged between 10.0–600.0ng/mL. The linear regression coefficients (r^2) for montelukast found to be greater than 0.99 when calibration curves were plotted using the peak area and the concentrations. The concentration was found to be proportional to the peak area. Therefore, good linearity was confirmed (Figure 3).

Table II. Results of inter and intra- day precision and accuracy.

Day 1	**LLOQ**	**QC_{Low}**	**QC_{Mid}**	**QC_{High}**
Target conc.	10ng/mL	30ng/mL	225ng/mL	500ng/mL
Calculated conc. ±SD	9.48±0.26	29.96±1.98	222.31±10.37	508.35±29.05
Accuracy ± SD	94.87±2.61	99.87±6.61	98.81±4.61	101.97±5.81
CV%	2.76	6.62	4.67	5.71
		Day 2		
Target conc.	10ng/mL	30 ng/mL	225ng/mL	500 ng/mL
Calculated conc. ±SD	9.96±0.31	30.64±2.11	228.38±5.70	515.77±19.26
Accuracy ± SD	99.96±3.31	102.14±7.03	101.50±2.53	103.15±3.85
CV%	3.14	6.88	2.49	3.74
		Day 3		
Target conc.	10ng/mL	30ng/mL	225ng/mL	500ng/mL
Calculated conc. ±SD	9.65±0.23	29.88±1.67	225.93±8.95	506.29 ± 26.44
Accuracy ± SD	96.45±2.29	99.61±5.57	100.42±3.98	101.26 ± 5.29
CV%	2.37	5.59	3.96	5.22
		Average for the three days		
Target conc.	10ng/mL	30ng/mL	225ng/mL	500ng/mL
Calculated conc. ±SD	9.69 ± 0.33	30.16 ± 1.85	225.54 ± 8.45	510.13 ± 24.09
Accuracy ± SD	96.94 ± 3.97	100.54 ± 2.3	100.24 ± 4.86	102.03 ± 3.18
CV%	3.37	6.12	3.75	4.72

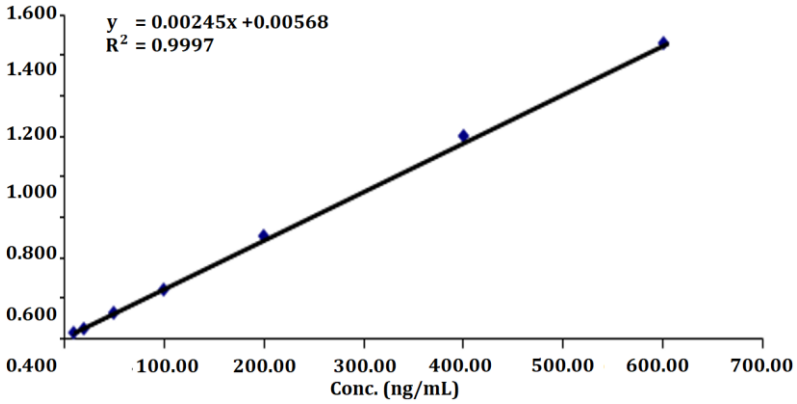

Figure 3. The plot of linearity of calibration curve levels for montelukast quantification against their analytical response and regression linear equation.

Stability

Montelukast levels in QC samples subjected to autosampler stability test, the bench stability test and three freeze-thaws. Results of analysis showed that no significant degradation and stability of the montelukast was confirmed.

Determination of montelukast in plasma subjected to the autosampler stability test and the bench stability test showed that it passed according to the accepted criteria, the accuracy % were less than 15% and RSD's were less than 5.0% (Table III and IV). Regarding the freeze and thaw (−30°C to room temperature) stability, the accuracy for QC low and high after 3 cycles is within the accepted range, which is 85-115% and RSD's were less than 5.11% (Table V).

The modifying effect of combining fruit juices with montelukast

The serum concentrations of montelukast with or without fruit juices were evaluated on rats on a sample size of 4 for each; drug alone, montelukast combined with grapes and montelukast that is combined with licorice. The measurements were repeated at 7 time intervals following drug administration to a maximum of 6h.

Table III. Montelukast QC samples stability at 4°C (auto sampler stability); QCL (30ng/mL) and QCH (500ng/mL).

Time (h)	AUC Drug	AUC IS	Ratio	Measured Concentration (ng/mL)	RSD*	Accuracy %	Stability
			QCH (30ng/mL)				
0	9346±956.1	112144.7±10501.5	0.0833 ± 0.004	30.51±1.52	4.99	101.7±5.08	100
24	9471±1011.2	114561.37±8910.75	0.0823 ± 0.003	30.15±1.24	4.12	100.5±4.15	98.8
			QCH (500ng/mL)				
0	127164.7±8270.9	9500.7±5546.7	1.34 ±0.035	519.12±12.05	2.32	103.8±2.41	100
24	121652.0±2616.3	92715.0±3637.3	1.31±0.040	509.29±17.22	3.38	100.3±2.21	98.1

* RSD= (Standard Deviation/Mean Concentration Measured) *100

Table 4: Montelukast QC samples stability room temperature (Bench stability); QCL (30ng/mL) and QCH (500ng/mL).

Time (h)	AUC Drug	AUC IS	Ratio	Measured Concentration (ng/mL)	RSD*	Accuracy %	Stability
			QCH (30ng/mL)				
0	9346±659.1	112144.7±10501.5	0.0833±0.006	30.51±1.52	4.99	101.7±5.08	100
24	9063.3±858.70	104713.3±10094.1	0.0867±0.006	30.39±1.08	3.54	101.31±3.56	98.8
			QCH (500ng/mL)				
0	127164.7±8270.9	9500.7 ± 5546.7	1.34±0.035	519.12±12.05	2.32	103.8±2.41	100
24	122181.7±6357.1	94856.3±8350.3	1.29±0.060	498.17±25.50	5.11	99.63±5.10	98.1

* RSD= (Standard Deviation/Mean Concentration Measured) *100

Table V. Montelukast QC samples freeze and thaw stability. QCL (30 ng/ml) and QCH (500 ng/ml).

Time (h)	AUC Drug	AUC IS	Ratio	Measured Concentration (ng/mL)	RSD*	Accuracy %	Stability
			QCH (30 ng/mL)				
0	9346.0±659.1	112144.7±10501.5	0.0833±0.006	30.51±1.52	4.99	101.7±5.08	100
72	9604.7±690.14	100938.3±7352.6	0.097±0.006	30.86±0.310	1.01	102.66±1.06	98.8
			QCH (500 ng/mL)				
0	127164.7±8270.9	9500.7±5546.7	1.34±0.035	519.12±12.05	2.32	103.8±2.41	100
72	122181.7±6357.1	94856.3±8350.3	1.29±0.060	498.17 25.50	5.11	99.63±5.10	98.1

***** RSD= (Standard Deviation/Mean Concentration Measured) *100

Table VI. Pharmacokinetic data of montelukast

Drug	C_{max} (ng/mL)	T_{max} (h)	AUC (ng/mL*h)
Montelukast	189.699± 90.7*	0.25	328.96±37.91*
Montelukast with Grape	163.492±113.27*	0.25	384.99±66.12*
Montelukast with Licorice	143.861±54.52*	0.50	408.66±70.58*

P<0.05 (significant), *P>0.05 (insignificant)

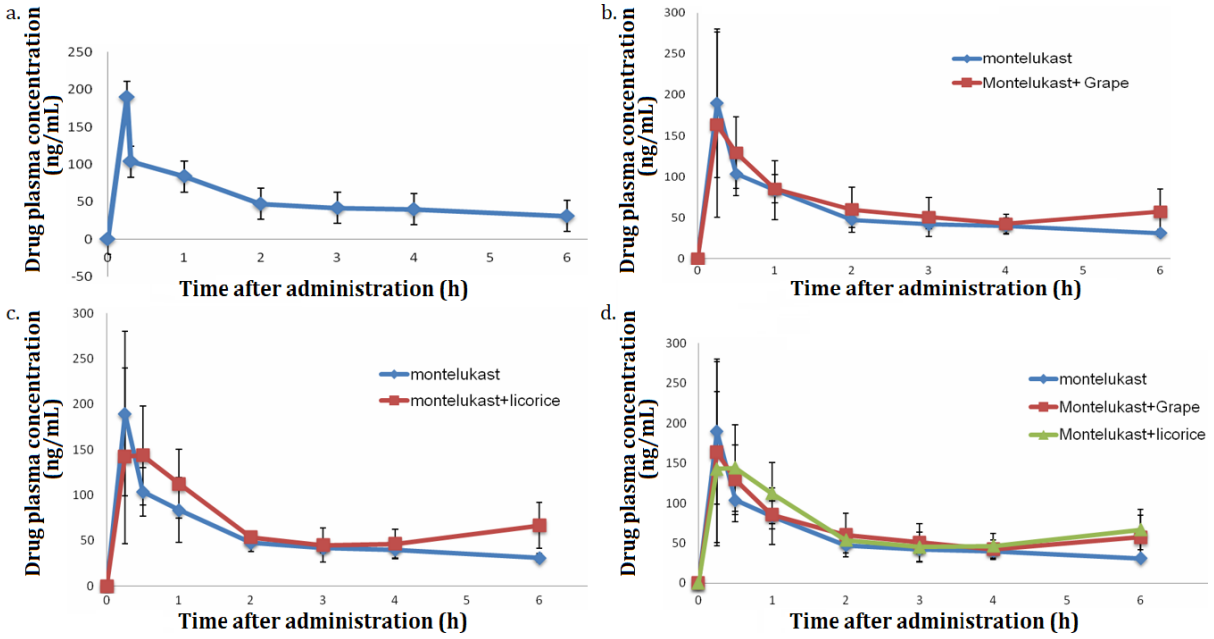

Figure 4. a. Rat plasma profile showing the changes in mean serum montelukast concentration with time after drug administration, each data point represents the mean ± SEM (n=4); b. Rat plasma profile showing the changes in mean serum montelukast concentration with time after drug administration, comparing montelukast with grape juice and solitary drug use, each data point represents the mean ± SEM (n=4); c. Rat plasma profile showing the changes in mean serum Montelukast concentration with time after drug administration, comparing Montelukast with licorice juice and solitary drug use, each data point represents the mean ± SEM (n=4); d. Rat plasma profile showing the changes in mean serum Montelukast concentration with time after drug administration comparing combined and solitary drug use.

Serum levels of montelukast alone was reached its maximum ($C_{max,}$ 189.699ng/mL) after 15min and then gradually declines to reach a minimum concentration of (31.046ng/mL) after 6h from the administration of montelukast (Table VI). Results of C_{max}, AUC, T_{max} showed insignificant correlations (P>0.05). These results suggested that a different site of metabolism and different cytochrome P450 included in the metabolism of Montelukast, grape, and licorice.

The serum level of montelukast when administered combined with grape reached its maximum serum concentration (163.492ng/mL) after 15min and then gradually declines to reach a minimum concentration after 4h (42.46ng/mL), while when combined with licorice the maximum serum level (143.861ng/mL) reached after 30mins, then gradually declines to reach its minimum concentration of (44.953ng/mL) after the third h (Figures 4). Grapefruit metabolized by one of the cytochrome P450 members, the CYP3A4. Cytochrome P450 is essential for oxidizing many drugs and xenobiotics (Bailey *et al*., 1998). Also, licorice juice components found to interact with CYP3A (Li *et al*., 2010).

Our results showed that no significant effect rats taking montelukast with grape fruit and licorice juices, as compared to that without juices. This demonstrates that montelukast and fruit juices may be metabolized by different isozymes of CYP450.

CONCLUSION

The novel HPLC/MS method established is simple, rapid, sensitive, precise, and reproducible for determination of Montelukast in rat plasma in the presence of fruit juices. Also, the method was prepared as per the ICH Guidelines. This method can be performed by the industries and academic institutes for drug estimation.

This study may indicate that there is no possible harmful interaction between Montelukast and grape juice, or between montelukast and licorice. And the montelukast plasma level was not affected by the concomitant administration with grape or licorice.

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