DEVELOPMENT OF A STABILITY-INDICATING HPLC METHOD FOR THE DETERMINATION OF MONTELUKAST IN TABLETS AND HUMAN PLASMA AND ITS APPLICATIONS TO PHARMACOKINETIC AND STABILITY STUDIES

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تم تطوير وإثبات طريقة من طرق كروماتوغرافيا السوائل عالية الكفاءة الدالة على الثبات وذلك لتقدير دواء مونتليوكاست في بلازما الإنسان وفي الشكل الصيدلاني الجرعي. وقد طبقت هذه الطريقة المقترحة أيضاً لتقدير دواء مونتليوكاست في وجود ناتج تحلل هذا الدواء. وقد تم استخدام أسيتونيتريل: فوسفات الصوديوم ثنائي الهيدروجين (0.05 مولار) المعدل لأس هيدروجيني مقداره ± 0.1 3.5 وذلك بحمض الفوسفوريك (70 : 30 ، % حجم/حجم) كطور متحرك وبمعدل سريان مقداره 2 مل/دقيقة باستخدام عمود C18 متناظر. وتمت مراقبة التدفق طيفياً عند 345 نانومتر. وتم استخدام نسبة مساحة منحنى الذروة للدواء والمعاير الداخلي (حمض فلوفيناميك) لتقدير دواء مونتليوكاست في عينات البلازما وكان حد التقدير 10 نغ/مل ، وحد الكشف 1 نغ/مل. وقد أظهرت دقة التحليل المتعدد في اليوم الواحد والتحليل من يوم إلى يوم معاملات تباين تراوحت بين 5.87 % إلى 9.60 % وبين 2.13 % إلى 6.18 % وذلك على التوالي ، عند ثلاثة مستويات تركيز مختلفة وقد وجد أن متوسط الاستعادة (recoveries) التام والنسبي هو 94.7 إلى 98% و 95.5 إلى 97.5% على التوالي. وتم تعيين مؤشرات (بارامترات) حركية دواء مونتيليوكاست بعد إعطاء 10 مغ منه وريدياً لخمسة أرانب. لقد طبقت الطريقة المقترحة بنجاح على دراسة حركية تحلل دواء مونتليوكاست وكانت حركية التحلل الضوئي لدواء مونتليوكاست في المحلول الميثانولي عند تعريضه لضوء النهار المصطنع من الرتبة الأولى وذلك فيما يتعلق بتركيزه.

A stability-indicating high performance liquid chromatographic (HPLC) method has been developed and validated for the determination of montelukast in human plasma and in its pharmaceutical dosage from. The proposed method has been also applied for the determination of montelukast in the presence of its degradation product. Acetonitrile: potassium dihydrogen phosphate (0.05 M) adjusted to pH 3.5 ± 0.1 with phosphoric acid (70:30, % v/v) was used as the mobile phase at a flow rate of 2.0 ml/min using a Symmetry C_{18} column. The effluent was spectrophotometrically monitored at 345 nm. Peak area ratio of the drug to the internal standard (flufenamic acid) was used for the quantification of montelukast in plasma samples and the limit of quantification was 10 ng/ml and the limit of detection was 1.0 ng/ml. The intraday and interday precisions showed coefficients of variation ranged from 5.87% to 9.60% and from 2.13% to 6.18% at three different levels of concentrations. The averages of the absolute and relative recoveries were found to be 94.7 to 98.0% and 95.5 to 97.5%, respectively. The pharmacokinetic parameters of montelukast after intravenous administration of 10 mg solution to five rabbits were determined. The proposed HPLC method was successfully applied to study the degradation kinetics of montelukast. The photodegradation kinetics of montelukast in methanolic solution upon exposure to artificial daylight was first-order with regard to its concentration.

Keywords: Montelukast; pharmacokinetics; HPLC; stability-indicating assay; photodegradation study

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Introduction

Montelukast (MKST) (Fig. 1) is a potent, selective and orally active antagonist of the cysteinyl, $CysTL_1$, leukotriene receptor used for the treatment of asthma in children and adults (1-3). MKST, a leukotriene modifier, has clearly demonstrated the ability to ameliorate bronchoconstriction and indices of airway edema and abnormal mucus production as observed in clinical trials (4).

Liquid chromatography with fluorescence detection (5), stereoselective high performance (HPLC) for MKST and its S-enantiomer (6), column switching HPLC with fluorescence detection (7) and HPLC coupled with fluorescence detection after a semi-automated 96-well protein precipitation methods (8) have been previously reported for the assay of MKST in human plasma.

In dosage forms, MKST has been determined using HPLC and derivative spectroscopy (9) and pressurized liquid extraction followed by HPLC (10).

The reported techniques were tedious, insufficiently sensitive and required highly dedicated instrumentation. Furthermore, none of the previous studies described a stability-indicating method designed to investigate the degradation of MKST in tablet dosage from.

The present study describes a simple, reliable, sensitive and stability-indicating method for the accurate and precise determination of MKST in the presence of its degradation product in both dosage forms and spiked plasma. The method may also be adopted to study the kinetics of degradation of MKST upon exposure to artificial daylight.

Experimental

1. Chemicals and materials:

MKST (MK-0476, batch # L-706.631-001 M045) sample was generously supplied by Merck Frosst (Quebec, Canada). The internal standard (IS), flufenamic acid, was obtained from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Phosphoric acid was purchased from Riedel-de Häen AG, (Sleeze, Hanover, Germany). Acetonitrile and methanol (both HPLC grade) were obtained from BDH Laboratories Supplies (Poole, UK). Tablets containing MKST were obtained from commercial source (Singulair® , batch # 224385) labeled to contain 10 mg. Distilled and deionized water was obtained by passage through ELGA® (Wycombe, Bucks, UK) and was further filtered through a 0.22 μm membrane filter (Millipore, Bedford, MA, USA). Plasma was kindly supplied by blood bank at King Khalid University Hospital (Riyadh, Saudi Arabia) and was kept frozen until use after gentle thawing.

2. Standard stock solutions:

An accurately weighted sample of 10 mg of MKST and 10 mg of flufenamic acid were dissolved in methanol in two separate 100 ml volumetric flasks to give standard stock solution of 100 μl/ml. A series of standard solutions containing both the drug and the internal standard were prepared. The working standard solutions were obtained by diluting the stock solutions to 1.0 μg/ml (for the drug) and 10 μg/ml (for the internal standard) with methanol. All the volumetric flasks containing MKST were wrapped with aluminium foil and stored in the dark.

3. Instrumentation:

The development and validation of the method was performed on a HPLC system consisting of a Waters HPLC pump (Model 515), a Waters autosampler Model 717 plus (Waters Inc., Bedford, MA, USA), a Shimadzu Model SPD-10A variable-wavelength UV detector (Shimadzu Corporation, Kyoto, Japan), and a Waters pump control module with Millennium® version 32 software on a Dell computer. The analytical column used to achieve chromatographic separation was a stainless steel Symmetry C_{18} column (150 x 3.9 mm I.D., 5 μm particle size) purchased from Waters Corporation (Bedford, MA, USA) protected by a guard column of the same material.

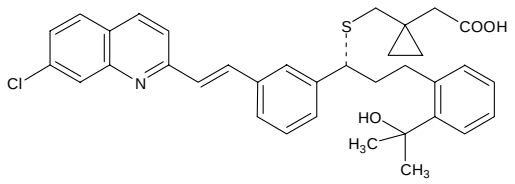

Figure 1. Structure formula of montelukast.

4. Sample preparation:

To a microcentrifuge tube (2.0 ml, Eppendorf), 200 μl plasma sample, 10 μl of the internal standard

(10 μg/ml), 750 μl of acetonitrile were added. The mixture was vortex-mixed on a vortex mixer (Scientific Industries, Inc., NY, USA) for 10 min. The mixture was then centrifuged at 13000 g for 5.0 min (Jouan, GR 412, Saint Mazaire, Cedex, France). After centrifugation, the supernatant was transferred to glass tubes with caps (Millipore, Bedford, MA, USA) and 50 μl was directly injected into the HPLC system.

5. Chromatographic conditions:

Chromatographic analysis was carried out at ambient temperature. The compounds were separated isocratically with a mobile phase consisting of acetonitrile: potassium dihydrogen phosphate (0.05 M) with the pH adjusted to 3.5 ± 0.1 using phosphoric acid (70:30, % v/v). The flow rate was 2.0 ml/min. The effluent was monitored spectrophotometrically at a wavelength of 345 nm. The mobile phase was filtered by passing through a 0.22 μm membrane filter (Millipore, Bedford, MA, USA).

6. Procedures:

6.1. Assay of tablets containing the drug:

An average of ten tablets of Singulair® were weighed and ground to fine powder. Accurately weighed powder sample equivalent to 10 mg of MKST was dissolved in methanol in a 100 ml volumetric flask. The flask was placed in an ultrasonic bath at room temperature for 10 min. After sonication, the solution was allowed to stand for 5.0 min. 1.0 ml was transferred into a 100 ml volumetric flask and diluted to the mark with methanol. A sample of 0.5 μl of this solution was directly injected. The average content of the tablets was determined either from the calibration graph or using the corresponding regression equation.

6.2. In vivo pharmacokinetic studies:

In this study, MKST plasma levels in vivo were studied in rabbits. Five healthy New Zealand male rabbits weighing 3.5-4.5 kg were utilized. Sample of 10 mg of the drug was administered intravenously via the ear marginal vein using sterile aqueous solution (prepared in the laboratory using aseptic technique). The study protocol complied with the Institutional Guidelines on Animal Experimentation of King Saud University. Blood samples (2.0 ml) were withdrawn via a cannula from the ear central vein and collected in heparinized tubes at 0, 0.083, 0.3, 0.45, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0 and 8.0 hr. The blood samples were centrifuged immediately to separate plasma and were stored at - 20 °C pending analysis.

6.3. Photodegradation studies:

The effect of artificial light on the degradation of MKST was studied by exposing a methanolic solution of MKST containing 1.0 μg/ml in a 100 ml volumetric flask, placed on a bench, to direct room light. Samples of 0.5 ml were taken at specific time intervals (0, 4.0, 20, 30, 40, 48, 70 and 96 hr) and assayed in triplicate.

6.4. Assay validation:

Various procedures were performed to validate the assay. Calibration standards were prepared in human plasma by spiking a known amount of MKST to control plasma (drug-free) samples to produce standards with final concentrations of 10, 50, 100, 200, 500 and 1000 ng/ml. Calibration curves were generated by measuring the peak-area ratio of the drug and the internal standard. The accuracy and precision were assessed by measuring the intraday and interday coefficient of variation (C.V.) at different quality control (QC) concentrations: low, medium and high (30, 300 and 800 ng/ml). Absolute and relative recoveries were also measured.

Results

1. HPLC study:

The mobile phase consisting of 0.05 M sodium dihydrogen phosphate buffer (pH 3.5) and acetonitrile in the ratio of 30:70 (%, v/v) was found to be an appropriate mobile phase allowing adequate separation of drug and the internal standard using a Symmetry C_{18} column at a flow rate of 2.0 ml/min. The blank chromatogram showed no interference from the endogenous substances. A typical HPLC chromatogram of a pooled blank human plasma sample is shown in Fig. 2A. A chromatogram of an extract of human plasma spiked with 50 ng/ml of MKST and flufenamic acid is illustrated in Fig. 2B. The retention times of MKST and the internal standard were 4.5 min and 1.5 min, respectively.

2. Method validation:

2.1. Quantification and linearity:

The plasma standard curves were prepared over the range of 10 – 1000 ng/ml. Standard curves were analyzed in triplicate and for each standard curve;

five runs were performed over a 6-week period to determine the variability of the slopes and intercepts. The average (drug/internal standard peak-area ratio) for plasma curve and the average area ratio in methanol were plotted against the corresponding concentrations. The calibration curves of MKST were typically described by the following regression analysis equations: $y = 0.0013x (\pm 0.016) - 0.363 (\pm 0.0052)$, $r^2 = 0.9989 \pm 0.005$ (for plasma) and $y = 0.0017x (\pm 0.014) - 0.331 (\pm 0.006)$, $r^2 = 0.9993 \pm 0.007$ (for methanol).

The results showed little day-to-day variability of the slopes and the intercepts. Analysis of variance of the slopes, intercepts and correlation coefficients of standard plots from plasma and methanol indicated non-significant differences ($p > 0.05$). The results confirm the linearity of the standard curves over the range studied and the excellent reproducibility of the assay method. The lower limit of quantification (LLOQ) for this method attained with plasma samples containing was 10 ng/ml of MKST.

2.2. Sensitivity:

The limit of detection (LOD), which is defined as the lowest concentration of the analyte which can be detected but not necessarily quantitated. The LOD was 1.0 ng/ml.

Table 1. Intraday and interday precision of montelukast in human plasma.

Added conc. (ng/ml)	Intraday*		Interday**	
	Found conc. (ng/ml)	Bias*** (%)	Found conc. (ng/ml)	Bias (%)
30				
Mean	31.2	4.0	33.8	12.7
S.D.	2.08		0.72	
C.V. %	6.67		2.13	
300				
Mean	301.3	0.43	304.2	1.40
S.D.	28.9		18.8	
C.V. %	9.60		6.18	
800				
Mean	802.3	0.29	801.5	0.19
S.D.	47.1		44.4	
C.V. %	5.87		5.54	

Mean value represent six different plasma samples for each concentration

** Interday reproducibility was determined from 6 different runs over a 4-week period at the three concentrations.

2.3. Precision and accuracy:

The intraday and interday precision and accuracy of the method are presented in Table 1. Intraday and Interday precision were evaluated by replicate analysis of pooled plasma samples containing MKST at three different levels of QC concentrations in plasma and methanol. The Intraday and interday precision showed a coefficient of variation ranged from 5.87% to 9.60% and from 2.13% to 6.18%, respectively.

The absolute recovery of MKST was determined by comparing the peak area of the drug obtained in plasma with the peak area obtained in methanol of the same concentrations. The relative recovery of the drug was obtained by comparing the concentrations obtained from the drug-supplemented plasma to the actual added concentrations. The results of the recovery studies are shown in Table 2. The averages of the absolute and relative recoveries were found to be 94.7 to 98.0% and 95.5 to 97.5%, respectively.

Table 2. Relative recovery of montelukast from spiked human plasma.

Concen-tration (ng/ml)	Absolute Recovery (%)	Relative Recovery % (mean ± S.D.)	Range of Relative Recovery
30	94.7 ± 6.0	95.7 ± 11.67	91.0-100.3
300	98.0 ± 11.7	97.5 ± 5.55	95.5-99.3
800	96.6 ± 8.9	95.5 ± 7.90	94.3-98.7

Table 3. Retention times of some tested drugs.

Drug	Retention time (min)*
Amitriptyline	0.62
Carbamazepine	0.77
Dihydroergotamine	0.62
Haloperidol	0.62
Itraconazole	0.52
Prazosin	0.60
Propranolol	0.61
Verapamil	0.60
Loratadine	2.60

* From time of injection into the column

Table 4. Mean pharmacokinetic parameters of montelukast following IV administration of montelukast to rabbits (n = 5).

Parameter	Mean ± S.D.
α (hr^{-1})	3.86 ± 0.98
β (hr^{-1})	0.16 ± 0.032
A (ng/ml)	2664.2 ± 355
B (ng/ml)	86.8 ± 13.3
$t_{½}\beta$ (hr)	4.32 ± 1.24
$AUC_{0\rightarrow\infty}$ (ng.hr/ml)	1230.7 ± 213
$AUMC_{0\rightarrow\infty}$ ($ng.hr^2$/ml)	3548 ± 567
MRT (hr)	2.88 ± 0.97

2.4. Stability:

The stability of MKST in human plasma at room temperature was assessed using three QC samples (30, 300 and 800 ng/ml). MKST was found to be stable in plasma for 3.0 hr at room temperature. The mean percent remaining after 3.0 hr ranged from 97 to 102%; which is in agreement with what have been reported previously (9,11). Other stability data found MKST to be stable for at least 19 months in human plasma at - 70 °C and in plasma after three repeated and freezing and thawing cycles (5).

2.5. Selectivity:

Other commonly administered drugs were tested for their possible interference in the aforementioned HPLC assay, namely ibuprofen and phenytoin. Table 3 lists the retention times for some of the drugs tested. No significant chromatographic interference was observed.

3. Applications of the proposed method:

3.1. Clinical applications:

A literature survey has reveled that a number of pharmacokinetic studies of MKST have been previously reported (12-16). In this study, plasma concentrations of MKST after intravenous (IV) administration were analyzed by a linear two-compartment open model with elimination from the central compartment. The model fitting and pharmacokinetic parameters calculations were accomplished using PKAnalyst® software (Micromath Scientists, Inc., Saint Louis, MO, USA). The plasma concentration time course after IV administration of 10 mg of MKST is shown in Fig. 3.

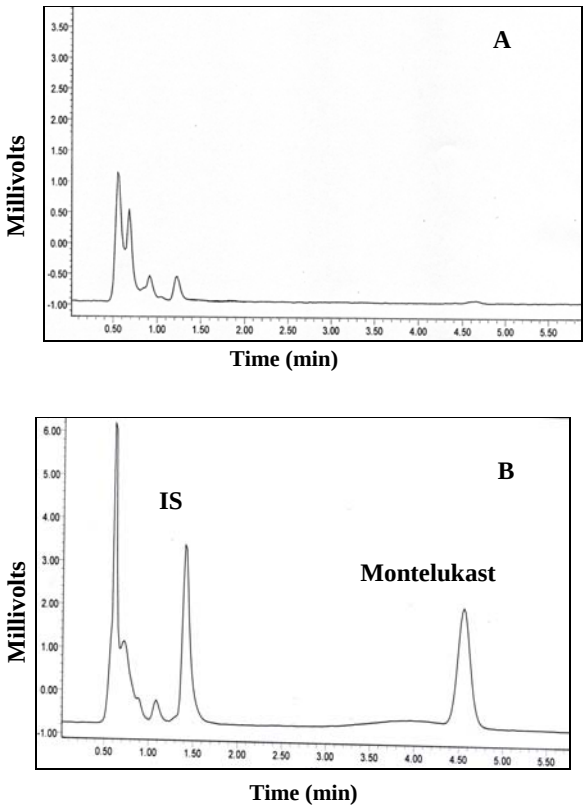

Figure 2. A: Typical HPLC chromatogram of a blank human plasma. B: Chromatogram of an extract of human plasma spiked with 50 ng/ml of montelukast and 50 ng/ml internal standard .

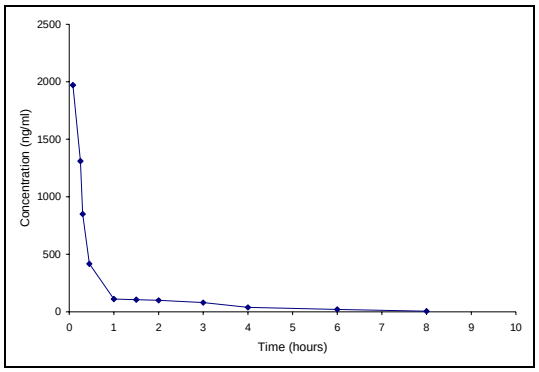

Figure 3. Mean montelukast plasma concentration following intravenous administration of 10 mg dose to rabbits (n = 5).

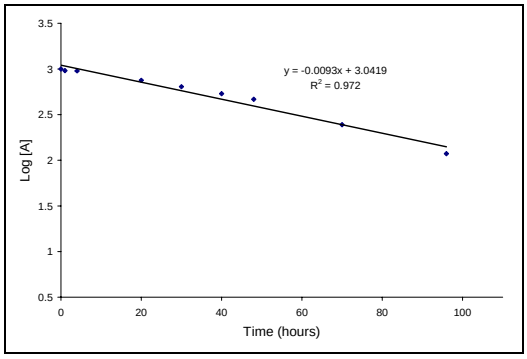

Figure 4. Semilogarithmic plot of montelukast *versus* time of exposure to artificial daylight.

The concentration of MKST in plasma (C_p) was described by the following equation:

$$C_p = Ae^{-\alpha t} + Be^{-\beta t}$$

where *A*, *B*, α and β denote hybrid constants and t is the time. The relevant pharmacokinetic parameters such as the terminal elimination half-life ($t_{½}\ \beta$), the area under the plasma concentration-time curve ($AUC_{0\to\infty}$), the area under the moment curve ($AUMC_{0\to\infty}$) and mean residence time of the drug in the body (MRT) were calculated (Table 4) using the following equations (17):

$$t_{½}\beta = \frac{0.693}{\beta}$$

$$AUC_{0\to\infty} = \frac{A}{\alpha} + \frac{B}{\beta}$$

$$AUMC_{0\to\infty} = \frac{A}{\alpha^2} + \frac{B}{\beta^2}$$

$$MRT = \frac{AUMC_{0\to\infty}}{AUC_{0\to\infty}}$$

3.2. Stability-indication of the method and photodegradation study:

No stability-indicating HPLC method has been reported in the literature for the determination of MKST in the presence of its degradation products, with an exception to the study that was reported by Radhakrishna and his co-workers in which they have reported the acid, basic, thermal and photo-degradations of MKST using accelerated degradation studies (9). It would therefore be beneficial to provide accurate, precise and reliable method for determination of MKST in the presence of its degradation products. To prove that the method is stability-indicating, the drug was assayed in presence of its degradation product obtained under stress condition, e.g. direct exposure to artificial light. The chromatograms obtained revealed that the peak height of MKST was reduced proportionally with time. The semilogarithmic plot of concentration of MKST (log A) versus time for a degraded sample of MKST is depicted in Fig. 4. It indicates a first-order degradation behavior with a rate constant (k) of 0.0214 hr^{-1}, a half-life of 32.4 hr and a correlation coefficient of 0.972. The first-order disappearance rate constant was calculated using the following formula (18):

$$\log [A] = \log [A]_\circ - \frac{Kt}{2.303}$$

where $[A]_\circ$ is the initial concentration of MKST at t = 0 and [A] is its concentration at time t. The half-life time was calculated according to the following formula (18):

$$t½ = \frac{0.693}{K}$$

Figure 5 (A, B, C and D) shows the chromatogram of MKST after 0, 30, 48 and 96 hr exposure time to light. It illustrates the reduction of the peak height of MKST and the development of the new peak of its degradation without any interference.

3.3. Analysis of MKST in its tablets:

The proposed method could be successfully applied to the determination of MKST in its tablets. The method was used to determine the content of MKST 10 mg tablets (Singulair®). The average content of ten tablets was found to be 9.65 ± 0.01 mg and ranged from 9.50 – 9.75 mg. The proposed assay was statistically compared with that of previously reported method as well as the manufacturer's data (19) by student's t-test and F-test at 95% confidence level (20). The results indicated that there was no significant difference between the proposed method and the other results regarding accuracy and precision and raveled by the t-test and F-test, respectively.

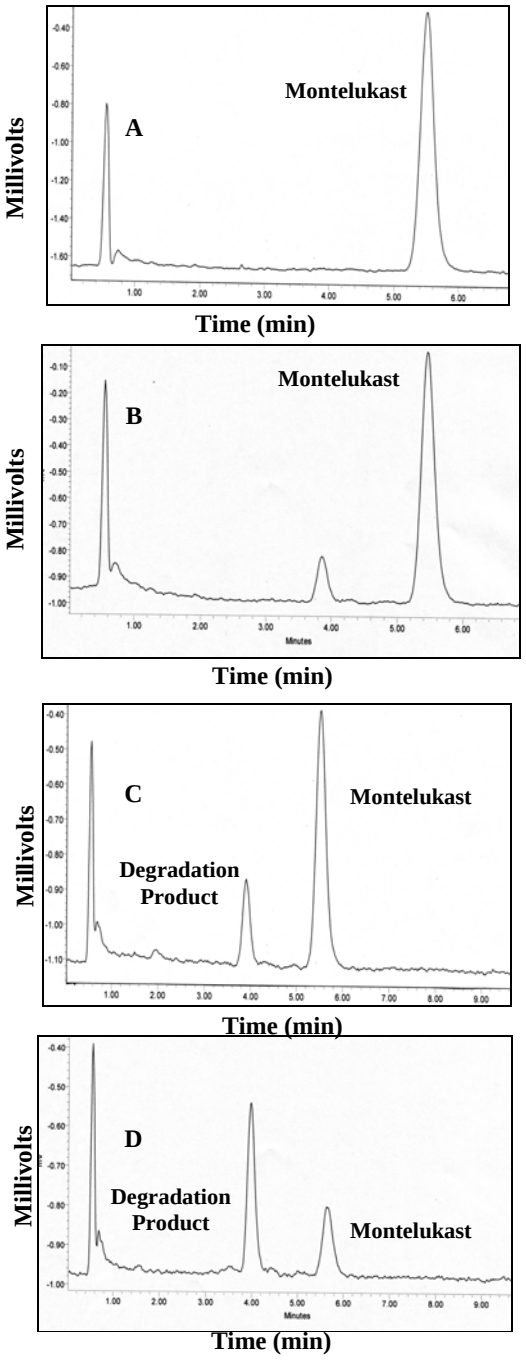

Figure 5. Chromatogram of the degradation product of montelukast upon exposure to daylight. A: Zero exposure time. B: 30 hr exposure time. C: 50 hr exposure time. D: 96 hr exposure time.

Conclusion

The HPLC method developed in this study has the sensitivity, selectivity, reproducibility, and stability which make it versatile and valuable in many applications, specifically in pharmacokinetic studies, kinetics study of MKST in pharmaceutical dosage form and for drug level monitoring. The method can also be readily adapted to routine quality control analysis.

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