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Short communication

Expedient liquid chromatographic method with fluorescence detection for montelukast sodium in micro-samples of plasma

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Abstract

This study describes an expedient assay for the analysis of the asthma medication, montelukast sodium (Singulair, MK-0476), in human plasma samples. After a simple extraction of the plasma, the drug and internal standard, quinine bisulfate, were measured by HPLC. The chromatographic system consisted of a single pump, a refrigerated autosampler, a C_8 4-μm particle size radial compression cartridge at 40°C and a fluorescence detector with the excitation and emission wavelengths set at 350 and 400 nm, respectively. The mobile phase which was delivered at 1.0 ml/min, was prepared by adding 200 ml of 0.025 *M* sodium acetate, pH adjusted to 4.0 with acetic acid, to 800 ml of acetonitrile, with 50 μl triethylamine. With a run time of only 10 min per sample, this assay had an overall recovery of >97% with a detection limit of 1 ng/ml. The inter- and intra-run relative standard deviations at 0.05, 0.2 and 1.0 μg/ml were all <9.2%, while the analytical recovery at the same concentrations were within 7.7% of the amount added.

Keywords: Montelukast sodium

1. Introduction

Montelukast sodium (Singulair, MK-0476) is a fast acting and potent cysteinyl leukotriene receptor antagonist which is being used in the treatment of asthma [1]. It can be administered orally once daily thereby increasing compliance over other common asthma treatments, has no known adverse effects or drug interactions, has demonstrated efficacy against allergen or exercise-induced bronchoconstriction (EIB) and is the only leukotriene modifier approved by the US Food and Drug Administration for use by children [2,3] from 2 to 12 years of age. A rapid onset of action is seen after the administration of montelukast sodium, with improvement seen on the first day of treatment [3], and these positive effects may be additive to those of inhaled corticosteroids [4]. It should also be noted that for EIB which affects at least 70% of asthmatic patients, after 4 to 8 weeks of treatment, montelukast sodium has been demonstrated to provide superior protection compared to the long acting inhaled β_2-agonist, sal-

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meterol, due to the progressive loss of protection of salmeterol against EIB [5]. In the study by Leff et al. [6], neither tolerance to the medication nor rebound worsening of lung function after discontinuation of the montelukast sodium were seen. While inhaled β_2-agonists are still considered the first-line therapy for treatment of asthma, montelukast sodium may be given due consideration for use as first line therapy in patients with mild persistent asthma, for additional control in those who remain symptomatic during treatment with inhaled corticosteroids, for patients that are steroid-phobic, or for those who have difficulties with compliance [7,8].

Three previous methods have been reported for the measurement of montelukast sodium using high-performance liquid chromatography (HPLC) with fluorescence detection. Either they did not have a sufficiently low level of detection for the lower doses now being routinely given to patients, or they required the use of column switching with long run times of 30 min or more and use equipment that may not be available in many hospital settings. Additionally, these methods did not report information as to the specificity of their assay and either did not include the incorporation of an internal standard, or used a compound available only from the manufacturer of montelukast sodium [9–11].

In this study, we describe an expedient liquid chromatographic method with fluorescence detection using a rapid sample preparation of 150 μl of plasma. This method does not require time consuming column switching or an evaporation step, has a sufficiently low detection limit to monitor patients and uses a commercially available internal standard. The sensitivity, accuracy, ease and small sample size of this assay make it highly suitable for both therapeutic monitoring and investigating the pharmacokinetics of montelukast sodium in asthma patients.

2. Experimental

2.1. Chemicals

Montelukast sodium was generously given by Merk Frosst Canada (Montreal, Canada). HPLC-grade acetonitrile, triethylamine and dibasic potassium phosphate were purchased from Fisher Scientific (Fairlawn, NJ, USA), quinine bisulfate (internal standard, I.S.) from Kodak (Rochester, NY, USA), and sodium acetate from Fluka (Buchs, Switzerland). HPLC-grade water was prepared by reverse osmosis and further purified by passing through a Milli-Q System (Millipore, Milford, MA, USA).

A stock solution of the montelukast sodium was prepared weekly in methanol at a concentration of 1.0 mg/ml and stored in an amber glass vial at 4°C. The internal standard, quinine bisulfate was first dissolved in water–methanol (50:50) to prepare a solution with a concentration of 1.0 mg/ml, which in turn was diluted with acetonitrile to yield a concentration of 1.0 μg/ml. Quinine bisulfate was chosen as the internal standard as it was the only easily available compound found that yielded a strong fluorescence signal at the same excitation and emission wavelengths as montelukast sodium, had a recovery from the extraction procedure of >95% and had an acceptable retention time.

2.2. Chromatography

The liquid chromatographic system consisted of a single Model 501 HPLC pump, a refrigerated 715 ULTRA WISP with sample compartment set at 4°C, a Guard-Pak pre-column module with a NovaPak C_8 insert, a 10 cm×8 mm NovaPak C_8 4 μm particle size radial compression cartridge in conjunction with an RCM 8×10 radial compression module, an RCM 100 column heater set at 40°C, and a Model 470 fluorescence detector with excitation and emission wavelengths set at 350 nm and 400 nm, respectively. Data were collected with a Pentium III computer using Millennium 32 Chromatography Manager software (all supplied by Waters Associates, Milford, MA, USA).

The mobile phase was prepared by adding 200 ml of 0.025 *M* sodium acetate, pH adjusted to 4.0 with acetic acid to 800 ml of acetonitrile, with 50 μl triethylamine. The triethylamine was used to prevent tailing of the peaks of interest which provided sharper and more symmetrical peaks. The mobile phase was filtered through a 0.45-μm filter and was degassed before use, then delivered at a flow-rate of 1.0 ml/min.

2.3. Standard curves

The concentration of montelukast sodium in the ''unknown'' samples was calculated by use of standard curves constructed by supplementing 150 μl portions of blank plasma with 0.002, 0.005, 0.01, 0.02, 0.1, 0.5, 2.0 μg/ml of montelukast sodium, then processing them identically to the samples as described below.

2.4. Sample preparation

Due to the light sensitivity of montelukast sodium, stock solutions, processed patient samples and standard curve samples were kept in amber glass containers and exposure to light was kept at a minimum during processing by working with them only in a safety cabinet with the light turned off. After collection in heparinized glass tubes, patient blood samples were protected from light by wrapping the tubes in aluminum foil until centrifugation and the plasma was stored in the dark at −80°C until analysis. Into a 500-μl centrifuge tube, 150 μl of plasma sample was added with 30 μl of the 1.0 μg/ml internal standard in acetonitrile solution, 150 μl acetonitrile and 225 μl of aqueous saturated dibasic potassium phosphate. The sample was vortex-mixed for 20 s then centrifuged at 17,000 *g* for 5 min. The clear top layer was collected and a 50-μl aliquot was injected from amber glass vials fitted with 300-μl plastic inserts into the chromatographic system using a 10-min run time.

3. Results and discussion

The retention time of montelukast sodium was 6.1±0.3 min while the quinine bisulfate eluted at 4.2±0.3 min. To demonstrate the utility of the method, the left panel of Fig. 1 depicts three representative chromatograms including an intact blank plasma; and a blank plasma sample supplemented with 0.1 μg/ml of the drug and a plasma sample collected from a patient 3 h after an oral dose of 10 mg montelukast sodium. Similarly, the right panel of Fig. 1 shows three representative chromatograms in a lower range of the method where a patient had received a dose of 10 mg montelukast sodium 18 h prior to sampling.

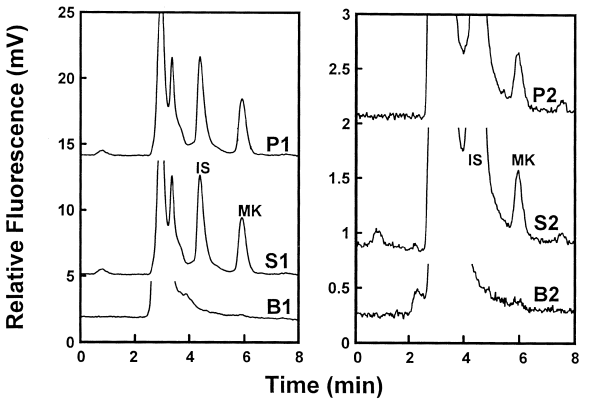

Fig. 1. Left panel, representative chromatograms of (B1) an intact blank plasma; and a plasma sample (P1) from an asthma patient that had been receiving a 10 mg oral dose of montelukast sodium (MK, calculated concentration of 0.103 μg/ml) once daily for 10 days collected 3 h after the last dose and a blank plasma sample supplemented with 0.100 μg/ml of the drug (S1) and 0.20 μg/ml of the internal standard (I.S.), quinine bisulfate. Right panel, representative chromatograms of (B2) an intact blank plasma; and a plasma sample (P2) from an asthma patient that had been receiving a 10 mg dose of the of montelukast sodium (MK, calculated concentration of 0.011 μg/ml) for 6 days collected 18 h after the last dose and a blank plasma sample supplemented with 0.010 μg/ml of the drug (S2) and 0.20 μg/ml of the internal standard (I.S.), quinine bisulfate.

3.1. Linearity, detection limit and recovery

The method demonstrated an excellent linear relationship between concentration of the drug in the standard curve and the peak height ratio of drug to internal standard. The results were calculated by linear regression without weighting, using the formula:

$$\text{Conc.} = a + b(\text{PHR})$$

where Conc. is the concentration of the montelukast sodium, a is the intercept, b is the slope and PHR is peak height of the drug divided by that of the internal standard. For 10 standard curves, the correlation coefficient (r) was >0.998 with the mean $r=0.9993$ [standard deviation (SD)$=0.0005$], the mean $a=0.126$ (SD$=0.033$) and the mean $b=8.271$ (SD$=1.040$). The detection limit of the assay,

defined as three times the baseline noise was 0.001 μg/ml. The total recovery was calculated by multiplying 100 times the peak height of the drug from an extracted plasma that had been supplemented with the drug, divided by the peak height of the drug at the same concentration in water which was put through an identical process. We found average total recoveries at the concentrations of 0.10, 0.50 and 1.0 μg/ml, to be 97.8, 99.5 and 99.1%, respectively ($n=10$).

3.2. Specificity and precision

The specificity of the described assay was investigated by supplementing individual portions of blank plasma separately with 5.0 μg/ml of the other drugs commonly used by asthma patients then processing them identically to the patient and standard curve samples. As may be seen in Table 1 which lists the drugs that were examined for possible interference with the assay, no interfering peaks were seen from any of the compounds. To evaluate the intra-run (same day) precision for plasma specimens, each of three different concentrations, 0.05, 0.2 and 1.0 μg/ml, were analysed 10 times. The same concentrations were used to determine the inter-run (different days) precision from 10 different days, and the results for both precisions are shown in Table 2. The analytical recovery defined as 100 times the amount found divided by the amount added, was within ±7.8% of the amount added, while the relative standard deviation (RSD) was less than 9.2%.

3.3. Light sensitivity

As previous reports indicate that solutions of montelukast sodium are sensitive to light [9–11], extracted samples were made up at three concentrations: 0.002, 0.01 and 0.05 μg/ml. These prepared samples were split into two sets, one kept in clear glass vials exposed to fluorescent lighting at room temperature, while the other set was kept in amber vials in the Ultra WISP at 4°C. The samples were measured by the assay immediately after preparation and 5 days later. Over the 5-day period, the samples kept in clear glass exhibited a decrease in PHR that varied from 53 to 83%, while there was no detectable decrease in the PHR of the samples stored in the amber vials at 4°C. Additionally, the 1 mg/ml stock solution of montelukast sodium which was used to supplement blank plasma for the construction of the standard curve, did not have a measurable decrease in peak height after 1 week of storage in amber glass vials at 4°C. When this 1 mg/ml stock solution was left in amber glass vials inside the darkened safety cabinet at room temperature, it showed no decrease in peak height over a 6-h period whereas the same solution kept in clear glass vials in the darkened

Table 1
Specificity of the assay for drugs which may be concomitantly administered with montelukast sodium

Name of the drug	Retention time (min)	Name of the drug	Retention time (min)
Montelukast sodium	6.1	Gentamicin	ND
Quinine bisulfate (internal standard)	4.2	Ibuprofen	ND
Acetaminophen	ND	Imipramine	ND
Aminosalicylate sodium	ND	Isoniazid	ND
Amitriptyline	ND	Itraconazole	ND
Ampicillin	ND	Methylprednisolone	ND
Aspirin	ND	Metoclopramide	ND
Caffeine	ND	Penicillin potassiumG	ND
Cefazolin	ND	Prednisolone	ND
Ceftriaxone	ND	Prednisone	ND
Clomiphene citrate	ND	Rifampin	ND
Desoxycorticosterone acetate	ND	Sisomicin	ND
Desipramine	ND	Terbutaline sulfate	ND
Dexamethasone	ND	Trimipramine	ND
Dimenhydrinate	ND	Valoproate sodium	ND
Diphenhydramine hydrochloride	ND		

ND = No peaks were detected from plasma samples supplemented with 5.0 μg/ml of the compound which was processed identically to the patient and standard curve samples.

Table 2
Analytical recovery, intra- and inter-run precision of the described assay for montelukast sodium in plasma

	Concentration (μg/ml) added		
	0.05	0.20	1.0
Intra-run precision (within the same day), $n=10$			
Amount found, mean (μg/ml)	0.0506	0.201	0.985
Analytical recovery (%)	101.20	100.5	98.5
RSD (%)	3.28	6.12	9.12
Inter-run precision (between-days), $n=10$			
Amount found, mean (μg/ml)	0.049	0.199	1.077
Analytical recovery (%)	98.8	99.6	107.7
RSD (%)	5.64	5.33	4.03

safety cabinet demonstrated a peak height loss of 20% after 1 h.

It should be noted that overnight washing of the system with 0.3 ml/min water–methanol (50:50, v/v) significantly prolongs the life of the pre-column insert and analytical cartridge. The analytical column can normally be used without decreased performance for more than 2000 samples, whereas changing of the pre-column insert was required after about 500 samples.

In conclusion, this very expedient analytical procedure has been developed to measure montelukast sodium in a hospital laboratory setting using standard HPLC equipment with fluorescence detection. It requires only 150 μl of plasma, does not require multiple pumps or time consuming column switching, uses a rapid sample preparation, incorporates the use of a commercially available internal standard, and has a run time of only 10 min. This procedure is currently being used to measure the drug in patient samples at the King Faisal Specialist Hospital and Research Centre.

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