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Short communication

Automated liquid–liquid extraction based on 96-well plate format in conjunction with ultra-performance liquid chromatography tandem mass spectrometry (UPLC–MS/MS) for the quantitation of methoxsalen in human plasma

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ABSTRACT

A sensitive, specific and high throughput bioanalytical method using automated sample processing via 96-well plate liquid–liquid extraction and ultra-performance liquid chromatography tandem mass spectrometry (UPLC–MS/MS) has been developed for the determination of methoxsalen in human plasma. Plasma samples with ketoconazole as internal standard (IS) were prepared by employing 0.2 mL human plasma in ethyl acetate:dichloromethane (80:20, v/v). The chromatographic separation was achieved on a Waters Acquity UPLC BEH C18 column using isocratic mobile phase, consisting of 10 mM ammonium formate and acetonitrile (60:40, v/v), at a flow rate of 0.5 mL/min. The linear dynamic range was established over the concentration range 1.1–213.1 ng/mL for methoxsalen. The method was rugged and rapid with a total run time of 1.5 min. It was successfully applied to a pivotal bioequivalence study in 12 healthy human subjects after oral administration of 10 mg extended release methoxsalen formulation under fasting condition.

1. Introduction

Methoxsalen (8-methoxypsoralen, 8-MOP), a naturally occurring furanocoumarin is used in the treatment of severe or recalcitrant psoriasis, vitiligo and unresponsive cutaneous T cell lymphoma [1,2]. It belongs to a group of compounds known as psoralens, which are planar, tricyclic compounds consisting of a furan ring fused to a coumarin moiety. Their planar aromatic structure and hydrophobic nature facilitate their intercalation with DNA bases [3]. The combination treatment regimen of psoralen (P) and ultraviolet radiation of 320–400 nm (UVA), known by the acronym, PUVA is a potent modulator of epidermal cell growth and differentiation [4]. Methoxsalen's maximum peak plasma concentration is achieved in about 2 h, after which UV radiation is applied. It is reversibly bound to serum albumin and is also preferentially taken up by epidermal cells [5]. It has been documented in earlier studies that there exist significant inter- and intra-individual variations in the pharmacokinetics of 8-MOP [6]. Thus, monitoring of 8-MOP levels in biological fluids is important for its successful therapy.

Several methods are reported in literature for the determination of 8-methoxsalen in biological matrices based on HPLC [7–12] and GC [13–15]. These published methods either involve cumbersome sample preparation steps or are insufficiently sensitive or precise to perform 8-MOP analysis during photochemotherapy. An automated coupled-column HPLC method for on-line sample processing and determination of 8-MOP in plasma has been developed by Vielhauer et al. [16]. It is based on the use of novel internal-surface reversed-phase pre-column packing material alkyl-diol silica. The linearity covered a wide range of 8-MOP concentration from 1.2 to 3070 ng/mL in a run time of 10 min. A sensitive microdialysis GC–MS method to assess 8-MOP in psoriatic patient dermis is presented by Leveque at al. [17]. Another promising LC–MS/MS method has been proposed by Brautigam et al. [18] to analyze 8-MOP in human plasma and microdialysates after topical application. Plasma samples (0.5 mL) were prepared by liquid–liquid extraction in diisopropylether to achieve a sensitivity of 0.5 ng/mL with a chromatographic run time of 3.0 min.

In recent years, there has been considerable interest in the use of high pressure than what is used in conventional HPLC in order to obtain better separation efficiency at high flow rates from small particle size and narrower column diameter. The ultra-performance liquid chromatography (UPLC–MS/MS) system utilizes high linear velocities with columns packed with porous 1.7 μm particles,

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coupled to a tandem mass spectrometer. This is an extremely powerful approach which dramatically improves peak resolution, sensitivity and speed of analysis [19]. However, with the large amount of samples generated from clinical studies, sample preparation/processing has become the area of concern during high throughput analysis. In this regard, automated 96-well plate format liquid–liquid extraction has significantly reduced manual labour, and time of analysis. Also, it promotes reduction in the use of hazardous solvents and ensures personal safety [20]. Thus, in the present study the objective was to develop a sensitive, reliable and high throughput method for routine determination of methoxsalen in human plasma in support of clinical findings.

2. Experimental

2.1. Chemicals and materials

Reference standards of methoxsalen (99.1%) and ketoconazole hydrochloride (internal standard, IS) (99.9%) were procured from Sequent Scientific Ltd. (New Mangalore, India) and Samex Overseas (Surat, India), respectively. Ammonium formate was obtained from Sigma–Aldrich (St. Louis, USA). HPLC grade methanol and acetonitrile were obtained from Mallinckrodt Baker, S.A. de C.V. (Mexico). GR grade formic acid, HPLC grade ethyl acetate and dichloromethane were obtained from Merck Specialties Pvt. Ltd. (Mumbai, India). Water used in the entire analysis was prepared from Milli-Q water purification system procured from Millipore (Bangalore, India). Blank human plasma was obtained from Supratech Micropath (Ahmedabad, India) and was stored at −20 °C until use.

2.2. Instrumentation and conditions

A Waters Acquity UPLC system (USA) consisting of binary solvent manager, sample manager and column manager was used for setting the reverse-phase liquid chromatographic conditions. The separation of methoxsalen and ketoconazole was performed on a Waters analytical column, type UPLC BEH C18, 50 mm × 2.1 mm (length × inner diameter), with 1.7 μm particle size and was maintained at 40 °C in column oven. The mobile phase consisted of 40% acetonitrile and 60% of mobile phase buffer (10 mM ammonium formate, pH 3.00). For isocratic elution, the flow rate of the mobile phase was kept at 0.5 mL/min. The total chromatographic run time was 1.5 min. The sample manager temperature was maintained at 5 °C and the pressure of the system was 8500 psi.

Ionization and detection of analyte and IS was carried out on a triple quadrupole mass spectrometer, MDS SCIEX API-4000 (Toronto, Canada), equipped with electrospray ionization (TIS interface of the API 4000) and operating in positive ion mode. Quantitation was performed using multiple reaction monitoring (MRM) mode to monitor parent → product ion (m/z) transitions for methoxsalen 216.8 → 201.8 and 530.8 → 489.0 for IS, respectively. The source-dependent parameters maintained for both methoxsalen and ketoconazole HCl were—Gas 1 (nebuliser gas): 40 psig; Gas 2 (heater gas flow): 60 psig; ion spray voltage (ISV): 5500 V; turbo heater temperature (TEM): 400 °C; interface heater (Ihe): ON; entrance potential (EP): 10 V; collision activation dissociation (CAD): 7 psig and curtain gas (CUR), nitrogen: 10 psig. The optimum values for compound-dependent parameters like declustering potential (DP), collision energy (CE) and cell exit potential (CXP) set were 75, 38 and 15 V, for methoxsalen and 105, 44 and 13 V for IS, respectively. Quadrupole 1 and 3 were maintained at unit mass resolution and the dwell time was set at 200 ms.

2.3. Standard stock, calibration standards and quality control sample preparation

The standard stock solution of 200 μg/mL of methoxsalen was prepared by dissolving requisite amount in methanol. Calibration standards and quality control (QC) samples were prepared by spiking (2% of total volume of blank plasma) blank plasma with stock solution. Calibration curve standards were made at 1.1, 2.1, 4.3, 10.7, 21.3, 42.6, 106.5 and 213.1 ng/mL concentrations while quality control samples were prepared at three levels, viz. 192.8 ng/mL (HQC, high quality control), 13.5 ng/mL (MQC, middle quality control) and 3.1 ng/mL (LQC, low quality control). Stock solution (200 μg/mL in methanol) of the internal standard was used to prepare the working solution of 100 ng/mL concentration in methanol. All the solutions (standard stock, calibration standards and quality control samples) were stored at 2–8 °C until use.

2.4. Protocol for automated sample preparation

A 200 μL aliquot of the spiked sample was transferred to each well (2.2 mL), 50 μL internal standard was added (excluding blank sample) using Tomtec Quadra 96, sealed with a cap mat and vortexed for 10 s at 200 × g using mixmate. Further, 0.600 mL of extraction solvent (ethyl acetate:dichloromethane, 80:20, v/v) was added and vortexed for another 2 min at 200 × g. Samples were then centrifuged at 800 × g for 2 min at 10 °C. The supernatant organic layer (0.360 mL) was transferred to another 96-well plate. It was evaporated to dryness at 40 °C under a hot stream of nitrogen for about 5.0 min and 150 μL of reconstitution solution (acetonitrile:mobile phase buffer, 90:10, v/v) was added. After vortexing for 10 s at 300 × g, 5 μL was used for injection in the chromatographic system. Plasma samples were also prepared by manual liquid–liquid extraction procedure and the efficiency of both the methodologies was compared.

2.5. Bioanalytical method validation

The method validation of methoxsalen in human plasma was done following the USFDA guidelines [21].

The linearity of the method was determined by analysis of five standard plots containing eight non-zero concentrations. Peak area ratios of analyte/IS were utilized for the construction of calibration curves, using weighted ($1/x^2$) linear least squares regression of the plasma concentrations and the measured peak area ratios.

The selectivity of the method towards endogenous plasma matrix components was assessed in 12 batches (6 normal, 2 haemolysed, 2 lipemic and 2 heparinised) of blank human plasma.

The extraction efficiency (recovery) of methoxsalen was performed at LQC, MQC and HQC levels. It was evaluated by comparing the mean area response of six replicates of extracted samples (spiked before extraction) to that of unextracted samples (spiked after extraction) at each QC levels. The recovery of IS was similarly estimated.

For determining the intra-day accuracy and precision, replicate analysis of plasma samples of methoxsalen was performed on the same day. The run consisted of a calibration curve and six replicates of LLOQ (lower limit of quantification), LQC, MQC and HQC samples. The inter-day accuracy and precision were assessed by analysis of five precision and accuracy batches on three consecutive validation days.

To study the effect of matrix on analyte quantification 6 samples were prepared from 6 different batches of plasma at LQC and HQC levels (total 12 samples) and checked for the % accuracy and precision (%CV) in both the QC samples. This was assessed by comparing the back calculated value from the QC's nominal concentration.

Short-term and long-term stock solution stability at room temperature was assessed by comparing the area response of stability sample of analyte and IS with the area response of sample prepared from fresh stock solutions. Autosampler stability, bench-top stability, dry extract stability and freeze–thaw stability were performed at LQC and HQC using six replicates at each level.

3. Results and discussions

3.1. Bioanalytical method development

The Q1 MS full scan spectra for methoxsalen and IS showed protonated precursor $[M+H]^+$ ions at m/z 216.8 and 530.8, respectively. The most abundant product ions at m/z 201.8 and 489.0 were found by applying 38.0 and 44.0 eV collision energy for methoxsalen and IS, respectively.

With the use of 96-well plate format, very low volume (0.6 mL) of organic phase was required in the liquid–liquid extraction. This format along with the use of the robotic liquid handling system resulted in a shorter sample preparation time, reducing at the same time the possibility of human error as time-consuming and labour-intensive manual pipetting steps are eliminated. All liquid transfer steps, such as plasma working and plasma standard solution preparation, reagents addition and organic solvent transfer were automatically performed. The best results were obtained with ethyl acetate:dichloromethane (80:20, v/v) mixture as extracting solvent. Compared to manual LLE, the automated procedure based on 96-well plate format gave improved sample handling efficiency. The sample preparation throughput improved three folds compared to manual procedure and much less organic solvent was required with 96-well plates.

For an efficient chromatographic separation of methoxsalen and IS, the mobile phase consisting of 10 mM ammonium formate and acetonitrile (60:40, v/v) ratio and having pH ~ 3.0 was found most suitable for eluting the analyte and IS. The use of Waters Acquity UPLC BEH C18 column with 1.7 μm particle size helped in separation and elution of methoxsalen and IS at 0.61 and 0.63 min, respectively. A general internal standard (ketoconazole) was used to minimize analytical variation due to solvent evaporation, integrity of the column and ionization efficiency.

3.2. Linearity and lower limit of quantification

The least squares regression analysis gave the linear equation $y = 0.0389x - 0.0048$ where y is the peak area ratio of the analyte to the IS and x is the concentration of the analyte. The mean and standard deviation values for slope, intercept and correlation coefficient (r) observed were 0.0398 and 0.0034, 0.0048 and 0.0019, 0.9988 and 0.0005, respectively. The accuracy and precision (%CV) observed for the calibration curve standards ranged from 94.4 to 103.8% and 1.3 to 3.8, respectively. The LLOQ achieved was 1.1 ng/mL for methoxsalen.

3.3. Selectivity, recovery, and accuracy and precision

No endogenous peaks were observed at the retention time of the analyte for any of the batches. Representative MRM ion chromatograms (Fig. 1A–D) of extracted blank human plasma (double blank), blank plasma fortified with IS, methoxsalen at LLOQ and a real patient sample at 1.5 h after oral administration of 10 mg methoxsalen tablet formulation, demonstrates the selectivity of the method.

The mean recovery for methoxsalen at HQC, MQC and LQC levels was 91.6, 85.5 and 87.6%, respectively. The recovery of internal standard was 100.7%.

Precision (%CV) for intra-batch and inter-batch ranged from 3.0 to 7.7% for all the QC levels. The accuracy results for intra-batch and inter-batch were within 92.2–99.4% at all four QC levels. The detailed results are presented in Table 1.

3.4. Matrix effect and stability results

There was no significant signal enhancement or suppression due to endogenous plasma matrix at the retention times of methoxsalen

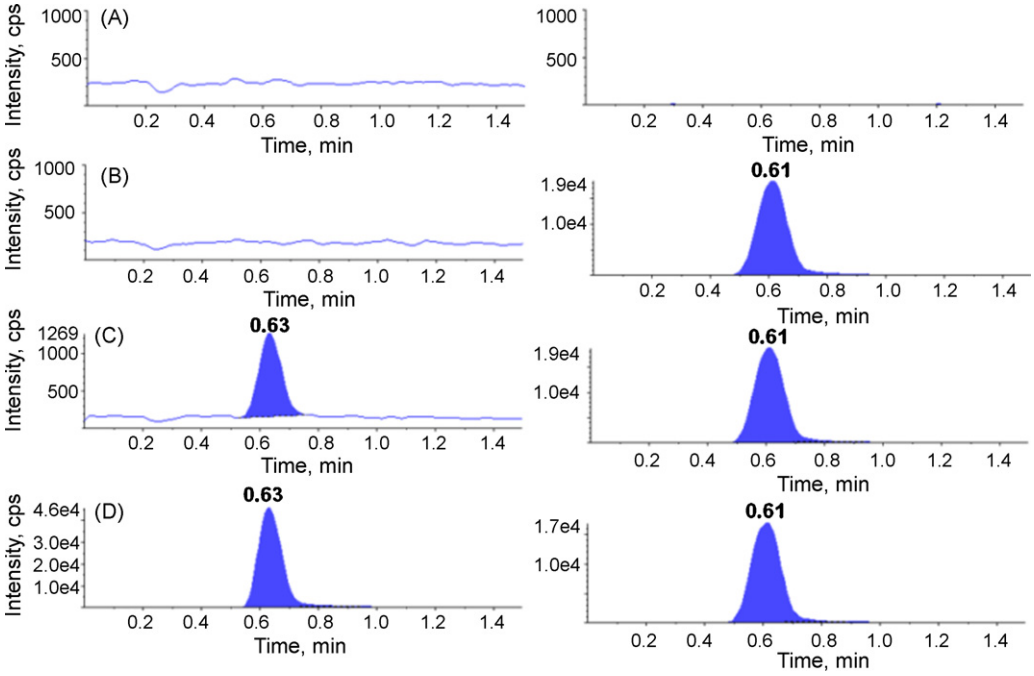

Fig. 1. MRM ion chromatograms of methoxsalen (216.8 → 201.8) in (A) double blank plasma, (B) blank plasma with IS, (C) LLOQ and (D) real subject sample at 1.5 h after administration of 10 mg dose.

Table 1
Comparison of intra-batch and intra-batch precision and accuracy of automated method for methoxsalen

QC ID	Nominal concentration (ng/mL)	Intra-batch				Inter-batch			
		n	Mean concentration observed (ng/mL)[a]	%CV	%Accuracy	*n*	Mean concentration observed (ng/mL)[b]	%CV	%Accuracy
HQC	192.8	6	189.9	3.0	98.5	30	191.7	5.7	99.4
MQC	13.5	6	13.4	3.0	99.1	30	13.2	6.1	97.8
LQC	3.1	6	3.1	4.1	99.3	30	3.0	6.4	97.5
LLOQ	1.1	6	1.0	3.5	95.6	30	1.0	7.7	92.2

CV, coefficient of variance; *n*, total number of observation.
[a] Mean of 6 replicates at each concentration.
[b] Mean of 30 replicates at each concentration.

and IS. The observed precision (%CV) and mean accuracy (%) values for LQC and HQC samples were 2.3 and 2.6% and 95.0 and 95.7%, respectively.

Stock solutions for short-term and long-term stability of the analyte and IS were stable at room temperature for minimum period of 6 h and between 2 and 8 °C for 4 days, respectively. Different stability experiments in plasma and the values for the precision and percent change are shown in Table 2.

Table 2
Stability of methoxsalen under various conditions ($n = 6$)

Storage conditions	Mean comparison samples	Calculated concentration (ng/mL)	
		Mean stability samples	%Change[a]
Bench-top stability (7 h, 25 °C)			
HQC	198.6 ± 3.3	192.7 ± 4.5	−3.0
LQC	3.0 ± 0.1	3.0 ± 0.1	0.2
Wet extract stability (39 h, 5 °C)			
HQC	198.6 ± 3.3	210.7 ± 4.4	−6.1
LQC	3.0 ± 0.1	3.3 ± 0.1	−9.2
Dry extract stability (24 h, −20 °C)			
HQC	182.6 ± 7.7	184.1 ± 4.8	−0.8
LQC	2.8 ± 0.1	3.0 ± 0.1	−5.3
Freeze and thaw stability (three cycles, −20 °C)			
HQC	189.9 ± 5.6	202.4 ± 3.7	−6.6
LQC	3.1 ± 0.1	3.2 ± 0.1	−3.8

[a] $\%\text{Change} = \frac{\text{mean stability samples} - \text{mean comparison samples}}{\text{mean comparison samples}} \times 100$

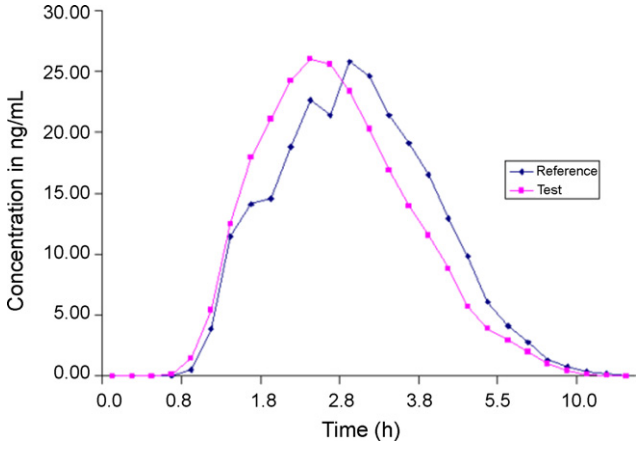

Fig. 2. Mean plasma concentration–time profile of methoxsalen after oral administration of 10 mg (test and reference) tablet formulation to 12 healthy volunteers.

3.5. Ruggedness and dilution integrity

To authenticate the ruggedness of the proposed method, it was done on two precision and accuracy batches. The first batch was analyzed by different analysts while the second batch was examined on two different columns. The precision and accuracy for both the experiments at LLOQ, LQC, MQC and HQC samples ranged from 1.5 to 5.8% and 87.4 to 99.3%, respectively.

The precision for dilution integrity test conducted on a spiked plasma standard solution (two times ULOQ, 426.1 ng/mL) of 1/5th (85.2 ng/mL) and 1/10th (42.6 ng/mL) dilution were 2.9 and 5.5%, while the accuracy results were 88.1 and 97.0%, respectively.

3.6. Application of the method in human subjects

The proposed validated method was successfully applied for the assay of methoxsalen in 12 healthy male volunteers in the age group of 18–45 years. The design of the study comprised of "a randomized, open label, single dose, two treatments and parallel design bioequivalence study of 10 mg methoxsalen capsule formulation in 12 healthy male volunteers under fasting condition". The study was conducted strictly in accordance with guidelines laid down by International Conference on Harmonization and USFDA [22]. Fig. 2 shows the plasma concentration vs. time profile of methoxsalen in human subjects under fasting condition. The values of pharmacokinetic parameters, viz. C_{max}, AUC_{0-t}, AUC_{0-inf}, T_{max} and $t_{1/2}$ for test/reference formulations were 36.0/39.8 ng/mL, 69.3/73.9 h μg/mL, 72.8/75.9 h μg/mL, 2.1/2.3 h and 1.0/1.6 h, respectively. These observations confirm the bioequivalence of the test sample with the reference product in terms of rate and extent of absorption.

4. Conclusion

Liquid–liquid extraction, a commonly used method for extraction of drugs from biological fluids was automated into a 96-well plate format for the UPLC–MS/MS analysis of methoxsalen in human plasma. The sample preparation efficiency was improved by three folds compared to the manual procedure. The overall analysis time is promising compared to other reported procedures for methoxsalen. The validated method showed good intra-bacth and inter-batch accuracy and precision. The established LLOQ is sufficiently low to conduct a pharmacokinetic study with 10 mg test formulation of methoxsalen in healthy human volunteers.

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