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High-performance liquid chromatographic analysis of mometasone furoate and its degradation products Application to in vitro degradation studies

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Abstract

A method of analysis of mometasone furoate in pharmaceutical formulations and biological fluids is necessary to study the degradation kinetics and determine its stability. A simple high-performance liquid chromatographic method was developed for simultaneous determination of mometasone furoate and its degradation products in human plasma. Plasma (0.5 ml) was extracted with dichloromethane after addition of the internal standard, dexamethasone 21-acetate. Separation was achieved on a Beckman C_8 column with UV detection at 248 nm. The calibration curve was linear ranging from 0.2 to 100 μg/ml. The mean extraction efficiency was $>86\%$. Precision of the assay was $<10\%$ (CV), and was within 10% at the limit of quantitation (0.2 μg/ml). Bias of the assay was lower than 7%. The limit of detection was 50 ng/ml for a 0.5-ml sample. The assay was applied successfully to the in vitro kinetic study of degradation of mometasone furoate in human plasma and simulated biological fluids.

Keywords: Reversed-phase HPLC; UV-detection; Mometasone furoate; Human plasma; Kinetics

1. Introduction

Mometasone furoate (MF, $C_{27}H_{30}Cl_2O_6$, MW 521.4, Fig. 1), 9α,21-dichloro-11β,17α-dihydroxy-16α-methylpregna-1,4-diene-3,20-dione 17-(2-furoate) [1], is a highly potent synthetic chlorinated glucocorticoid with a favorable ratio between local and systemic side-effects [2–5]. Its effectiveness has been shown in the treatment of glucocorticoid responsive dermatological disorders as topical formulations of ointments, creams and lotions (Elocon®) [2], seasonal and perennial allergic rhinitis as an aqueous intranasal spray (Nasonex®) [3,4] and in asthma as a dry powder inhaler (Asmanex®) [5]. In clinical studies, MF exhibits strong anti-inflammatory activity, rapid onset of action and low systemic bioavailability

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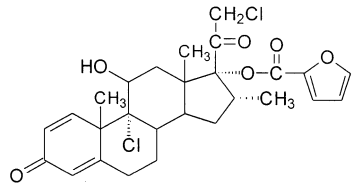

Fig. 1. Structure of mometasone furoate (MF).

[4]. MF does not exhibit significant systemic effects in adults [6], children [7] and infants [8], following topical administration [4,9], after a single intranasal dose up to 4000 μg (20 times the recommended daily dose of 200 μg) [10] or inhalation of 400 μg twice daily [5]. Following oral administration of 1 mg of MF as a solution in six male volunteers, peak plasma concentration of MF of 150 pg/ml appeared at 30 min, and declined rapidly [11]. When MF was given by inhalation at the dose range of 100–400 μg in asthmatic patients, the mean plasma drug concentrations were only slightly above or below 50 pg/ml. This extremely low systemic bioavailability may be therapeutically favourable because of therapeutic concerns of topical glucocorticoids for long-term administration.

Other steroids, such as triamicinolone acetonide, have been shown to decompose at various pHs and be inherently unstable [12]. In order to elucidate the degradation kinetics of MF, knowledge of its degradation pathways in biological fluids is of considerable importance. To the authors' knowledge, no study has been published characterizing the degradation of MF. Before performing a study of in vitro degradation, development of a selective and sensitive assay for MF is necessary. A competitive enzyme immunoassay (EIA) has been reported for the determination of MF in human plasma [11]; however, this assay did not quantify metabolites or degradation products of MF. Cross-reactivity was also shown to occur between parent and metabolites. The present study describes a selective, isocratic reversed-phase high performance liquid chromatography (HPLC) method for the determination of MF and its degradation products in human plasma and its application to in vitro kinetic studies.

2. Experimental

2.1. Chemicals and reagents

MF was kindly supplied by Schering-Plough Pty. (Baulkham hills, NSW, Australia). Dexamethasone 21-acetate, prednisone (PRED), hydrocortisone (HC), beclomethasone dipropionate (BDP) and dichloromethane (99.9%, HPLC grade) were purchased from Sigma (St. Louis, MO). Budesonide (BUD) and fluticasone propionate (FP) were kindly donated by Astra Draco (Lund, Sweden) and Glaxo Group Research (UK), respectively. ChromAR HPLC grade methanol and analytical reagent grade ethanol were purchased from Biolab Scientific (Clayton South, VIC, Australia). Pooled human plasma, using acid citrate dextrose as anticoagulant, pH 7.2 was supplied by the Australian Red Cross Blood Bank (Sydney, NSW, Australia) and stored at −20°C for no longer than 3 months. Simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared following USP (23rd revision, 1995) [13]. Simulated lung fluid (SLF) was prepared as described by Kalkwarf [14]. All salts used for simulated biological fluids were of Analar quality from BDH (Kilsyth, VIC, Australia). Pepsin was from porcine stomach mucosa (Sigma, St. Louis, MO) and pancreatin was from pig pancreas (BDH, UK).

2.2. Chromatographic system and conditions

The HPLC system used was a Shimadzu HPLC (Kyoto, Japan), consisting of an LC-10AT pump, an SIL-10AXL auto injector, an SPD-M10A photodiode-array UV/VIS spectrophotometric detector and a CBM-10A system controller. Data collection and integration were accomplished using Shimadzu Class LC-10 computer software version 1.4 (Kyoto, Japan).

The analytical column used was an ultrasphere octyl column (150 × 4.6 mm I.D., 5-μm particle size, Beckman, CA). The mobile phase consisted of methanol and water (59:41, v/v), filtered and degassed under reduced pressure prior to use. Separation was carried out isocratically at ambient temperature (22 ± 1°C) and a flow rate of 1.5 ml/min, with UV detection at 248 nm.

To investigate the possible interference of MF with other glucocorticoids, PRED, HC, BDP, FP and BUD were also analyzed under similar HPLC conditions.

2.3. Stock and working standard solutions

A target amount of 20 mg of MF was accurately weighed on an analytical balance (AG245, Mettler) and dissolved with methanol in a 10-ml volumetric flask to make a stock standard solution in methanol with a target concentration of 2 mg/ml. An ethanolic stock solution of dexamethasone 21-acetate (internal standard, IS) was prepared similarly with the target concentration of 1 mg/ml. This solution was diluted with ethanol to make a working IS solution of 10 μg/ml. These solutions were protected from light and stored at −20°C between use for no longer than 3 months. Calibration standards in plasma were prepared daily from the stock solution of MF by sequential dilution with blank human plasma, yielding a series of concentrations namely 0.2, 0.5, 1.0, 2.0, 5.0, 10.0, 20.0, 30.0, 50.0 and 100.0 μg/ml, in four replicates.

Quality control (QC) samples were prepared from the stock solution of MF by dilution with blank human plasma or simulated biological fluids to yield target concentrations of 0.2, 2.0, 10.0, 50.0 and 100.0 μg/ml. The QC samples were divided into 0.5 ml aliquots in screw-capped test tubes and stored at −20°C before use.

2.4. Sample preparation

A total of 0.5 ml of internal standard solution (10 μg/ml) was added to working standards or samples (0.5 ml). The mixture was extracted with 4.0 ml of dichloromethane for 30 min using a roller mixer. Following centrifugation at 2500 rpm (20°C) for 15 min, the organic layer was collected and evaporated to dryness under a gentle stream of nitrogen at 37°C. The residue was reconstituted using 250 μl of mobile phase and then transferred into a 1.5 ml polypropylene microcentrifuge tube (Eppendorf). Following centrifugation at 15 000 rpm (20°C) for 3 min, 50 μl of the supernatant was injected onto the column.

2.5. Precision and accuracy

The within-run precision and accuracy of the replicate assays ($n = 6$) were tested by using five different concentrations, namely 0.2, 2, 10, 50 and 100 μg/ml. The between-run precision and accuracy of the assays were estimated from the results of six replicate assays of QC samples on six different days within 1 week. The precision was evaluated by calculating the coefficient of variation (CV) using ANOVA. The accuracy was estimated based on the mean percentage error of measured concentration to the actual concentration. The values of CV and bias should be within 15%, at all concentrations tested [15].

2.6. Recovery

Recovery for MF from HP was assessed ($n = 7$) at 0.5, 2, 10, 50 and 100 μg/ml. A known amount of MF was spiked into 0.5 ml human plasma to give the above concentrations. They were extracted with 4.0 ml dichloromethane and analyzed by HPLC. The extraction efficiency was determined by comparing the peak area ratio (PAR) of MF to IS in the plasma matrix to that in the dichloromethane matrix.

2.7. Stability of MF samples

The stability of MF samples were assessed under five different conditions.

The stability of MF in human plasma at room temperature (22 ± 1°C) and in freezer (−20°C) was investigated using QC samples of four concentration levels, 2, 10, 30 and 50 μg/ml in four replicates.

The freeze–thaw stability of MF was evaluated at three concentrations 2, 10 and 50 μg/ml, using QC samples. These samples were analyzed in triplicate without being frozen at first and then stored at −20°C and thawed at room temperature (22 ± 1°C) for three cycles.

The stability of MF in reconstituted extracts during run-time in the HPLC auto-injector was investigated, using pooled extracts from QC samples of three concentration levels, 2, 10 and 50 μg/ml. Samples were kept in the sample rack of

the auto-injector and injected into HPLC system every 6 h, from 0 to 36 h, and at 48 h at the temperature of auto-injector (26 ± 1°C).

The stability of reconstituted extracts was also tested at −20°C for 1 week. The reconstituted extracts of seven concentrations, 0.5, 2, 10, 20, 30, 50 and 100 μg/ml were allocated in injection vials, stored at −20°C and injected onto the column on day 0, 1, 2, 3, 4 and 7.

2.8. Stability of MF in human plasma and simulated biological fluids in vitro

MF was incubated in human plasma or simulated biological fluids, including SLF, SGF and SIF at 37.0 ± 0.1°C, shielded from light in a thermostatically controlled shaking water bath. Prior to the kinetic study, the incubation media were equilibrated to the temperature of the study. Kinetic studies were initiated by the addition of a stock solution of MF to incubation media, yielding an initial concentration of 10 μg/ml. At predetermined time intervals, samples (0.5 ml) were removed and immediately frozen on dry ice/ethanol. Samples were analyzed by HPLC following liquid–liquid extraction using dichloromethane.

2.9. Data analysis

Decomposition products were identified by their relative retention times to the IS on HPLC chromatograms and their UV spectra. Quantification was based on calibration curves constructed using peak area ratio (PAR) of MF to IS, against MF concentration using unweighted least squares linear regression. The percentage of degradation products was estimated as the ratio of PAR of metabolite to PAR of patent drug at time zero. The apparent decomposition rate constants (k_{app}) were estimated from the slope of log-linear phase of declining concentration versus time plots. The half-lives ($t_{1/2}$) were calculated using the following equation: $t_{1/2} = 0.693/k_{app}$ [16]. Data were expressed as the mean ± standard deviation (S.D.) of replicate determinations.

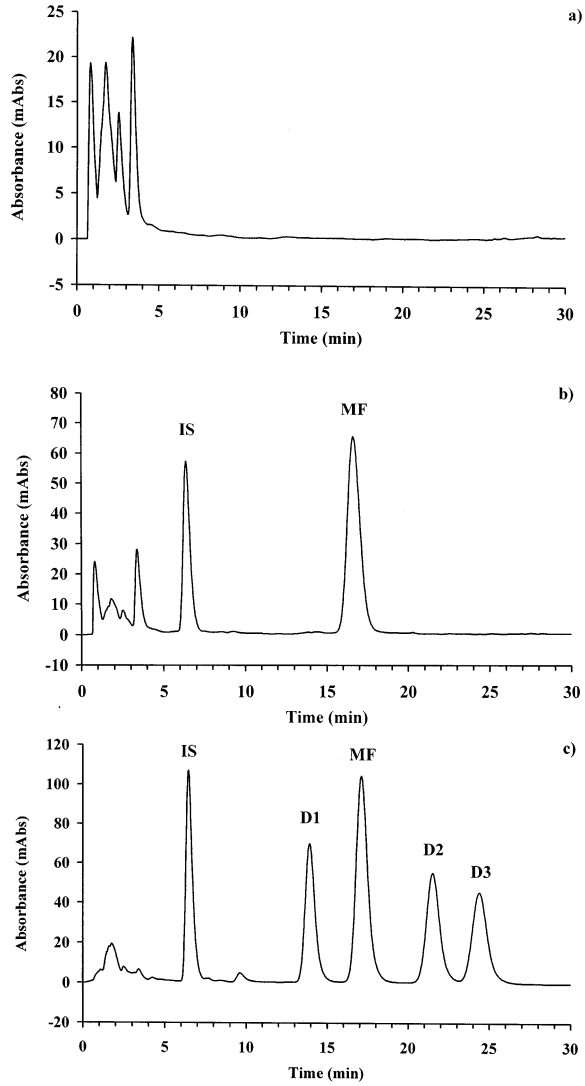

Fig. 2. Representative chromatograms, of (A) drug-free human plasma, (B) plasma containing MF and IS each with concentration of 10 μg/ml, and (C) plasma sample containing parent drug MF and its degradation products, following incubation of MF ($C_0 = 50$ μg/ml) for 24 h.

3. Results and discussion

3.1. Chromatography

Separations of MF and its degradation products as well as the endogenous substances in human plasma were achieved successfully. There were no interfering peaks co-eluted with the com-

pounds of interest (Fig. 2a and b). The retention times of IS and MF were approximately 6.4 and 16.8 min, respectively (Fig. 2b).

Considering a degradation product is usually more polar than the parent drug, we investigated if there was any decomposition product of MF co-eluted with IS, using samples of incubated MF prepared with the addition of ethanol instead of IS. There was no extra peak coming at the retention time of IS following the incubation of MF in human plasma up to 72 h.

Under these HPLC conditions, the retention times of other glucocorticoids tested were 2.7 min for PRED, 3.2 min for HC, 12.3 min for BUD, 16.3 min for FP and 33.9 min for BDP.

The performance of the HPLC assay was assessed using the following parameters, namely peak shape and purity, interference from endogenous substances in human plasma and from other glucocorticoids, linearity, limit of quantitation (LOQ), limit of detection (LOD), freeze–thaw stability, stability of reconstituted extracts, precision, accuracy and recovery. Having compared the chromatograms obtained from other glucocorticoids with those from MF, it would be expected that the presence of those glucocorticoids with the exception of FP will not interfere with the determination of MF in human plasma. Various conditions of HPLC were tested to achieve the best resolution between MF and its degradation products. The retention times of analytes were found to be very sensitive to the percentage of methanol in the mobile phase. The optimal separation was achieved when the combination of methanol and water was 59:41 (v/v) and the flow rate was 1.5 ml/min. An Alltima C_{18} column (250×4.6 mm I.D., 5-μm particle size, Alltech, Australia) was also tested, on which the elution time was doubled to achieve similar resolution between peaks of interests.

Based on the wavelength maxima in the mobile phase of MF (251 nm) and IS (242 nm), the UV detector in this study was set at 248 nm.

3.2. Linearity, LOQ and LOD

An excellent linear relationship ($r^2 = 0.9994$) was demonstrated between PAR of MF to IS and the corresponding plasma concentrations of MF over a range of 0.2–100 μg/ml. The mean regression line from the validation runs was described by MF (μg/ml) = PAR × 8.2261 − 0.0214. The LOQ of this assay was 0.2 μg/ml in human plasma with the corresponding relative S.D. and bias of 3.13 and 9.5%, respectively. This calibration curve was cross validated with QC samples of MF in SGF, SIF and SLF. The back-calculated concentration of QC samples in these matrices was within the acceptance criteria. The LOD of MF was estimated to be 0.05 μg/ml in human plasma and 0.02 μg/ml in SGF, SIG and SLF with the signal to noise ratio $\geq 3{:}1$.

3.3. Precision, accuracy and recovery

The within- and between-run CV calculated during replicate assays ($n = 6$) of MF in human plasma were $< 10\%$ over a wide range of MF concentrations (Table 1). The intra- and inter-run bias assessed during replicate assays varied be-

Table 1
Intra- and inter-day precision and accuracy data of MF in human plasma

Actual conc (μg/ml)	Intra-day			Inter-day		
	Measured conc[a] (μg/ml)	CV	Bias (%)	Measured conc[a] (μg/ml)	CV	Bias (%)
0.2	0.20 ± 0.0	6.2	−1.93	0.21 ± 0.01	8.6	6.55
2	1.97 ± 0.04	2.7	−1.14	2.13 ± 0.07	6.0	6.46
10	10.0 ± 0.3	4.4	0.8	10.6 ± 0.3	5.6	5.64
50	49.6 ± 1.6	5.8	−0.8	50.0 ± 1.7	6.0	−0.003
100	99.0 ± 4.8	3.4	−1.0	98.5 ± 1.6	2.9	−1.50

[a] Data were expressed as the mean ± S.D. of six replicates.

Table 2
Recovery (%) of MF extracted from human plasma ($n = 7$)

Concentration (μg/ml)	Mean	Range
0.5	94.1	83.2–103.2
2	91.5	84.2–96.9
10	92.7	85.6–97.9
50	86.8	76.5–96.1
100	92.4	88.4–98.6

tween −3.0 and 6.6% (Table 1). Precision and accuracy studies indicated that the developed HPLC method is reproducible and accurate. The mean extraction efficiency for MF from human plasma varied from 86.8 to 94.1% (Table 2). High recovery of MF from human plasma suggested that there was negligible loss during liquid–liquid extraction process. The efficiencies of extraction of MF and IS were comparable.

3.4. Stability of MF samples

No significant degradation was detected after the samples of MF in human plasma were stored at room temperature for 3 h, or in a freezer at or below −20°C for 4 weeks, or after undergoing one freeze–thaw cycle. Under ambient conditions for 3 h, there were 95.0, 97.5, 98.5 and 101.6% of MF recovered at 2, 10, 30 and 50 μg/ml, respectively. When stored in a freezer at −20°C, recoveries of MF were between 96.2 and 98.0% after 1 week, and 90.9 and 94.6% after 4 weeks, at the four concentrations tested. The recoveries were within 94.4 and 104.6% following three freeze–thaw cycles for 2 and 10 μg/ml, but lower than 90% after two cycles for 50 μg/ml level. There was no significant decomposition observed after the reconstituted extracts of MF were stored in the auto-injector at room temperature for 36 h or in freezer at −20°C for 1 week. The measurements were from 94.1 to 101.9, 97.8 to 100.8, and 91.9 to 95.7% of the initial value for 2, 10 and 50 μg/ml respectively, during the storage in the auto injector at room temperature for 36 h. When stored in a freezer at −20°C, the recovery varied between 95.3 and 104.5% within 1 week at all concentrations investigated.

3.5. Stability of MF in human plasma and simulated biological fluids in vitro

The HPLC method has been applied to the determination of MF and its degradation products in the kinetic studies of MF in human and rat biological matrices and tissues, simulated biological fluids and formulations (unpublished results) in vitro. Following the incubation of MF as parent drug at 37°C in human plasma, three decomposition products, namely D1, D2, and D3, were formed (Fig. 2c and Fig. 3a). During the period up to 30 h, D1 was the major decomposition product, following incubation of MF in human plasma. However D2, rather than D1, was the ultimate major degradation product observed, with PAR of 38.5 ± 0.5% of the parent drug, after longer incubation times (72 h). The degradation

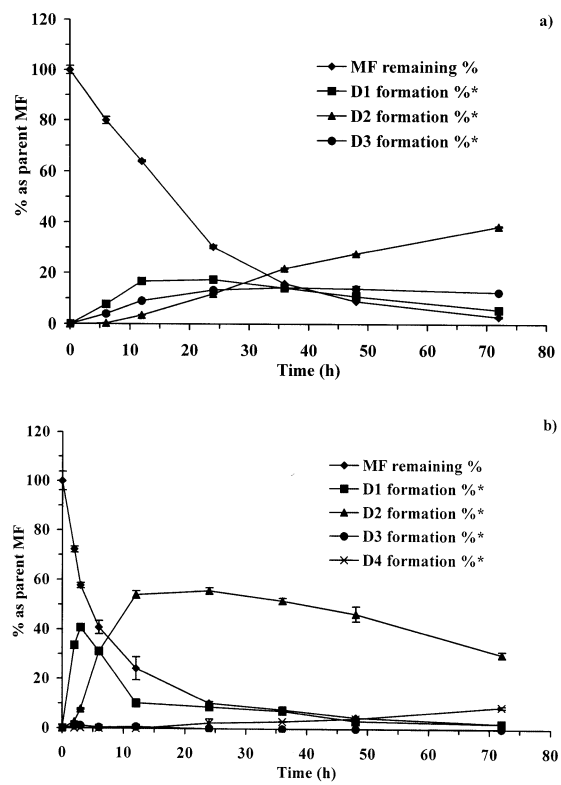

Fig. 3. Concentration time profile of MF ($C_0 = 10$ μg/ml) following the incubation in (A) human plasma and (B) simulated lung fluid at 37.0 ± 0.1°C. * Data were expressed as percentage of initial MF (mean ± S.D., $n = 4$).

products were apparently formed in the sequence of D1, D3 and D2.

MF decomposed into D1, D2, D3 and D4 in SLF. D1 formed rapidly in SLF but declined after 3 h, with the increase of D2 up to 24 h (Fig. 3b). Little of D3 formed in SLF and it became undetectable after 12 h. There was a significant lag time for the formation of D4 which was detected after 24 h.

Products D1, D2, D3 and D4 exhibited UV maxima in the mobile phase at 255, 256, 240 and 256 nm respectively, and have yet to be identified. The retention times of D1, D2, D3 and D4 were about 13.6, 21.2, 24.2 and 8 min, respectively. Thus D1 and D4 were chromatographically more polar than the parent drug, whereas D2 and D3 were less polar than MF. The half-lives of MF ($C_0 = 10$ μg/ml) observed were 18.4 ± 4.3 h in human plasma and 4.5 ± 0.2 h in SLF (Mean $\pm$ S.D., $n = 4$).

In summary, the developed HPLC assay is selective, reproducible and accurate. It has been successfully applied to the study of degradation of this drug in human and rat biological matrices and tissues, simulated biological fluids and formulations in vitro.

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