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A simple and reliable method for determining plasma concentration of dehydroxymethylepoxyquinomicin by high performance liquid chromatography with mass spectrometry

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ABSTRACT

We have developed a simple and reliable method for determining plasma concentration of dehydroxymethylepoxyquinomicin (DHMEQ), a new low molecular weight NF-κB inhibitor, using high performance liquid chromatography with mass spectrometry (LC–MS). An experiment of mass spectrometry with electrospray ionization in the negative ionization mode was performed to detect ion transitions at m/z 260.05 $[M-H]^-$ for DHMEQ and 240.29 for mefenamic acid as an internal standard. The samples were purified using liquid–liquid extraction with ethyl acetate. The method yielded a standard curve which was linear for the concentration range of 0.1–125 ng/mL when 0.05 mL plasma was used. The correlation coefficients of all standard curves were greater than or equal to 0.999. The limit of detection was 50 pg/mL (signal/noise >3). Daily fluctuation of plasma standard curve was small. The intra- and inter-assay precision ranged from 2.84 to 4.76% ($n=6$) and 2.91 to 7.03% ($n=6$), respectively. The LC–MS technique described provides a simple and reliable liquid chromatographic method for the determination of DHMEQ level and for use in studies involving pharmacokinetics.

1. Introduction

Dehydroxymethylepoxyquinomicin (DHMEQ), synthetically derived from the antibiotic epoxyquinomicin C [1], is a new NF-κB inhibitor with low molecular weight (Fig. 1A). DHMEQ inhibits nuclear translocation of NF-κB p65 and specifically inhibits the signaling pathway for NF-κB activation [2]. Constitutive activation of NF-κB is often involved in human carcinomas and leukemias. DHMEQ inhibits the constitutively activated NF-κB in various carcinoma and leukemia cells, and also the secretion of inflammatory cytokines from cancer cells [3]. Furthermore, DHMEQ was shown to be extremely effective to suppress inflammation and tumors in animal experiments. This compound exhibits anti-inflammatory activities in rheumatoid arthritis [1,4], cancer cachexia [5], and renal inflammation [6]. Furthermore, it shows anticancer activities against prostate carcinoma [7], thyroid carcinoma [8], pancreatic cancer [9], breast carcinoma [3], multiple myeloma [10] and adult T-cell leukemia [11,12] in mice without any toxicity [1,2]. However, data of the effective dose and pharmacokinetic behavior of DHMEQ have been difficult to obtain since a reliable determination method for plasma DHMEQ has not been developed. Therefore, a direct analytical technique for the determination of DHMEQ is desirable to establish recommendations for the optimal dose of DHMEQ.

There is little documented data on the physiochemical properties of DHMEQ, except that it is an acidic and hydrophobic agent. In the present study, we developed an original method of determining DHMEQ in plasma using liquid–liquid extraction with ethyl acetate, which is the most common method of extraction for hydrophobic agent, and high performance liquid chromatography with mass spectrometry (LC–MS) for separation and detection of the analyte. Using this method, we measured the plasma level of DHMEQ in mice.

2. Experimental

2.1. Reagents and materials

DHMEQ was kindly provided by Dr. Umezawa, Department of Applied Chemistry, Faculty of Science and Technology (Keio

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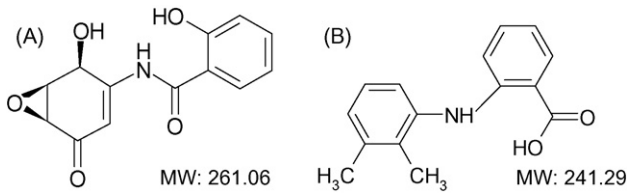

Fig. 1. Chemical structures of DHMEQ (A) and mefenamic acid (B).

University, Yokohama, Japan). Mefenamic acid (internal standard; I.S., Fig. 1B) and the other chemicals were purchased from Wako Pure Chemical Ind. Ltd. (Osaka, Japan). Acetonitrile was of LC–MS grade and other reagents were of analytical grade.

DHMEQ is an acidic and hydrophobic agent, and dissolves well in dimethylsulfoxide (DMSO). However, other basic physicochemical data including solubility of DHMEQ has not been documented. On the other hand, mefenamic acid has a pK_a of 4.2 [13]; is insoluble in water, slightly soluble in alcohol and in dichloromethane, and practically dissolves in dilute solutions of alkali hydroxides [14].

2.2. Preparation of stock solutions, working solutions, calibration standards and quality control samples

A stock solution of DHMEQ was prepared by dissolving in DMSO at a concentration of 10 μg/mL and stored at −20 °C. The stock standards of DHMEQ were diluted with acetonitrile, giving working solutions ranging from 0.05 to 125 ng/mL. Stock and working solutions of DHMEQ were stored separately at −20 °C. A stock solution of mefenamic acid was prepared in acetonitrile at a concentration of 1 μg/mL and stored at −20 °C.

The stability of stock and working solutions of DHMEQ and mefenamic acid, stored separately at −20 °C, was examined by conducting observation and assays at regular intervals. All solutions remained unchanged for at least 1 month, and showed no detectable degradation compared to the freshly prepared solutions.

Calibration standards at concentrations of 0.1, 0.5, 1, 5, 10, 50, 100 and 125 ng/mL were prepared by spiking 50 μL blank plasma with the appropriate working solutions. In the same manner, quality control (QC) samples were prepared at concentrations of 1, 10 and 125 ng/mL.

2.3. HPLC conditions

DHMEQ concentrations were measured using an HPLC system (LC-20AD pump, SHIMADZU, Kyoto, Japan) equipped with autosampler (SIL-20A), column oven (CTO-20A), degasser (DGU-20A3) and system controller (CBM-20A). The analytical column was a Shim-Pack VP-ODS (150 mm × 2.0 mm i.d., 5 μm particle size) with a Shim-Pack GVP-ODS (5 mm × 2.0 mm i.d., 5 μm particle size) as a guard column. The column was heated to 40 °C. Mobile phase consisted of acetonitrile and 0.05% (w/v) ammonium acetate (35:65, v/v). The flow rate was 0.2 mL/min.

2.4. MS conditions

The electrospray mass spectrometer (LCMS-2010EV, SHIMADZU, Kyoto, Japan) was run in the negative ionization mode, focusing on the following masses: m/z 260.05 $[M-H]^-$ for DHMEQ and 240.29 for mefenamic acid (I.S.). Instrument control, date acquisition and processing were performed using the LC–MS solution.

2.5. Extraction procedure

Fifty microliters of sample, 50 μL of I.S. (1 μg/mL in acetonitrile) and 10 μL of 1M HCl were mixed in a 10-mL glass tube. Then 1 mL of ethyl acetate was added, and the extraction was performed by vortexing for 15 min. After centrifugation at 3000 × g for 10 min, the organic phase was transferred to a 10-mL glass tube and evaporated to dryness by a stream of nitrogen at 40 °C. The residue was reconstituted with 50 μL of acetonitrile. The sample was immediately transferred to autosampler vials and 5 μL of sample was injected into the LC–MS system.

2.6. Precision and accuracy

QC samples at three different concentrations (1, 10 and 125 ng/mL), including the lower and upper limits of detection, were used. Six replicates of the three QC samples were analyzed on six different validation days in order to determine the intra- and inter-day accuracy and precision. Precision was assessed by the coefficient of variation (C.V.) and accuracy by the standard deviation (S.D.).

2.7. Matrix effects

To evaluate the absolute matrix effects on electrospray ionization, six different batches of blank plasma were used to prepare QC samples (1, 10 and 125 ng/mL of DHMEQ) and I.S. (1 μg/mL). The corresponding peak areas of extracted QC samples or I.S. (A) were compared with those of DHMEQ or I.S. in acetonitrile (B) at equivalent concentrations. The matrix effect was expressed by the ratio (A/B × 100).

2.8. Recovery

Extraction recovery of DHMEQ and I.S. from plasma was determined by comparing peak areas of DHMEQ and I.S. obtained from injection of extracted QC samples (plasma spiked with 1, 10 and 125 ng/mL of DHMEQ) or I.S. with those obtained from direct injection of the same concentrations of DHMEQ or I.S. dissolved in acetonitrile.

2.9. Determination of plasma DHMEQ concentration in mice

Male C57BL/6J mice (9 week-old, 21–25 g in weight) were fasted overnight before the experiments. Water was allowed *ad libitum*. The experiments were performed in accordance with the "Guide for the Care and Use of Laboratory Animals" (1996) and with the approval of the Ethics Committee in Hokkaido University.

Mice were anesthetized with an inhalation of isoflurane. DHMEQ as a 10 μg/mL solution in DMSO was injected intraperitoneally at a dose of 15 mg/kg body weight. Blood samples were collected from the orbital vein at 0, 2.5, 15, 60 and 120 min after dosing. The blood was immediately centrifuged and the plasma was stored at −80 °C until analysis.

The pharmacokinetics analysis of the data was performed using non-compartmental analysis based on the statistical moment method [15]. The estimated parameters were: area under the plasma concentration–time curve extrapolated to infinity ($AUC_{0-\infty}$) using linear trapezoid method, mean residence time (MRT), total clearance (CL_{tot}), apparent volume of distribution (V_d) and elimination half-life ($t_{1/2}$).

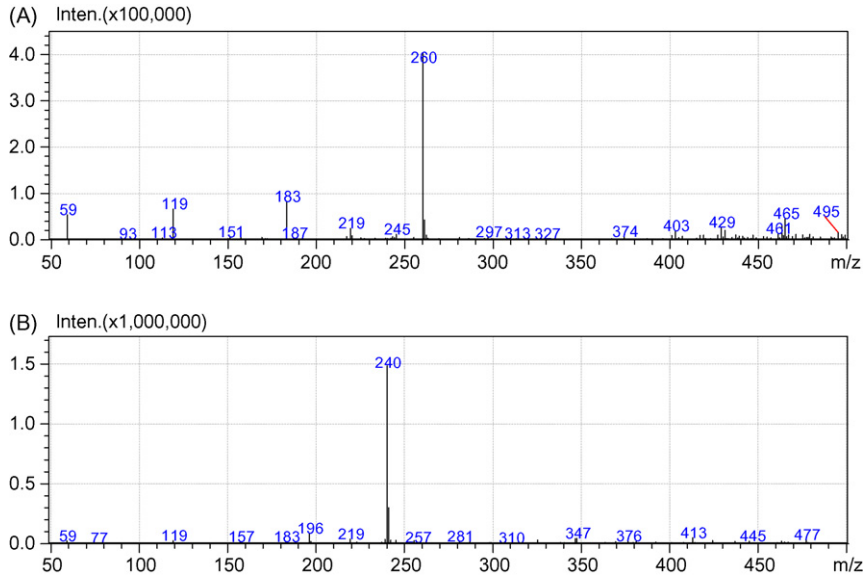

Fig. 2. Representative full-scan product-ion spectra of DHMEQ (A) and the internal standard mefenamic acid (B) .

3. Results and discussion

3.1. Method development

We developed an original method for the measurement of DHMEQ in plasma. The physiochemical properties of DHMEQ are largely unknown, except that it is an acidic and hydrophobic agent. In our method, we first extracted DHMEQ in plasma using liquid–liquid extraction with ethyl acetate, which is the most common method of extraction for hydrophobic agent. Then we used the sensitive LC–MS method for separation and detection.To select an I.S. for quantification, ibuprofen, mefenamic acid, mycophenolate acid, naproxen, and probenecid that have properties similar to DHMEQ were examined. Using an acetonitrile and 0.05% (w/v) ammonium acetate mixture as mobile phase, the peak shape and retention time obtained from these compounds were compared. As a result, mefenamic acid with structural similarity to DHMEQ was considered the appropriate I.S.for the determination of DHMEQ.

3.2. Specificity and selectivity

Fig. 2 shows the mass product-ion spectra of DHMEQ and mefenamic acid (I.S.) in the negative ionization mode. The predominant precursor ions produced for analysis were $[M-H]^-$. The peak areas obtained from selected reaction monitoring for the mass transition of DHMEQ (m/z 260.05) and mefenamic acid (m/z 240.29) were used for quantification.

Typical chromatograms of DHMEQ and mefenamic acid (I.S.) are shown in Fig. 3. An assay performed on drug-free plasma showed the absence of interfering peaks at the retention times of DHMEQ (2.5 min.) and mefenamic acid (7.0 min.) (Fig. 3A). A representative chromatogram of blank plasma sample spiked with DHMEQ (100 ng/mL) and mefenamic acid (1 μg/mL) is presented in Fig. 3B.

3.3. Linearity of calibration curves and lower limits of quantification

Calibration curves were obtained by least-squares linear regression analysis. A linear relationship between the DHMEQ concentration (0.1–125 ng/mL plasma) and peak area ratio was obtained. The correlation coefficients were greater than or equal to 0.999; it showed good linear relationship between the peak area ratio and the concentration.

The lowest concentration on the calibration curve of DHMEQ was 0.1 ng/mL. The response obtained from the analyte at this concentration level was >5 times greater than the noise level. The precision and accuracy at this concentration was acceptable, with C.V. < 12.85% and observation concentration of 0.11 ± 0.01 ng/mL. Thus, the lowest concentration on the calibration curve was accepted as the lower limit of quantification, and the limit of detection (signal/noise >3) was 50 pg/mL.

3.4. Precision and accuracy

Intra- and inter-day precision (C.V.) and accuracy (S.D.) were analyzed using the data of DHMEQ concentrations obtained from assaying the QC samples. The results are shown in Table 1. The intra-assay precision for DHMEQ ranged from 2.84 to 4.76% (n = 6), and inter-assay precision ranged from 2.91 to 7.03% (n = 6). The values are within the acceptable range, demonstrating that the method is accurate and precise.

3.5. Matrix effects

The results of matrix effects at QC concentrations of DHMEQ and at working concentration of I.S. showed the presence of matrix effects as indicated by values <100% (DHMEQ 1 ng/mL: mean 94%, range 88.1–98.8%; 10 ng/mL: mean 94%, range 91.2–94.4%; 125 ng/mL: mean 97%, range 95.0–99.4%). This demonstrated an ionization suppression for DHMEQ and I.S. However, such ionization suppression did not affect the slopes and linearity of calibration

Table 1
The intra- and inter-day accuracy and precision results for the assay

Actual concentration (ng/mL)	Observed concentration (ng/mL)			
	Intra-day (n = 6)		Inter-day (n = 6)	
	Mean ± S.D.	C.V. (%)	Mean ± S.D.	C.V. (%)
1	1.02 ± 0.05	4.76	1.09 ± 0.08	7.03
10	10.02 ± 0.32	3.18	9.97 ± 0.44	4.41
125	124.92 ± 3.55	2.84	125.45 ± 3.66	2.91

S.D.: standard deviation; C.V.: coefficient of variation.

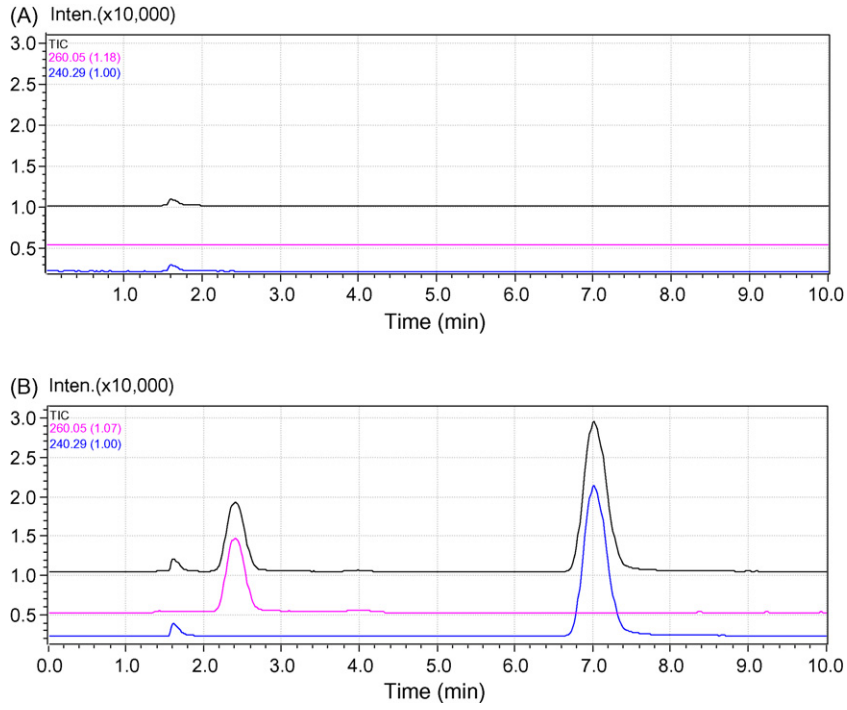

Fig. 3. Typical chromatograms of a blank human plasma (A), and a blank human plasma spiked with DHMEQ (100 ng/mL) and internal standard mefenamic acid (1 μg/mL) (B). Retention times for DHMEQ and mefenamic acid are 2.5 and 7.5 min., respectively.

curves, the ratio of DHMEQ to I.S., and precision and accuracy data. Therefore, the present analytical method was considered reliable with a high sensitivity for DHMEQ determination in plasma, even if matrix effects existed.

3.6. Recovery

Extraction recovery rates of DHMEQ and mefenamic acid (I.S.) are shown in Table 2. Extraction recovery rates for DHMEQ at concentrations of 1, 10 and 125 ng/mL in plasma were found to be 86.4, 92.3 and 86.4%, respectively, and recovery rate for mefenamic acid as 86.9%. These results indicated that the extraction efficacy for DHMEQ and mefenamic acid was acceptable.

3.7. Application of the method

The LC–MS method described was developed for determining plasma DHMEQ concentrations. The plasma concentration–time profile of DHMEQ in mice given an intraperitoneal injection at a dose of 15 mg/kg body weight is shown in Fig. 4.

After intraperitoneal injection, the plasma concentration of DHMEQ reached a peak level of 106.68 ng/mL at 2.5 min. Then the plasma concentration declined, reaching a trough level of 2.2 ng/mL at 120 min after injection. The estimated pharmacokinetics parameters were as follows: $AUC_{0-\infty}$ was 4.1 μg min/mL, MRT was 28.0 min, CL_{tot} was 3.7 L/h/kg, V_d was 102.22 L/kg, and $t_{1/2}$ was 20.6 min.

Table 2
The extraction recovery rates for DHMEQ and mefenamic acid (internal standard) in plasma

Compound	Concentration (ng/mL)	Recovery (%) Mean ± S.D.
DHMEQ	1	86.42 ± 2.89
	10	92.33 ± 2.63
	125	86.44 ± 2.43
Mefenamic acid		86.86 ± 1.18

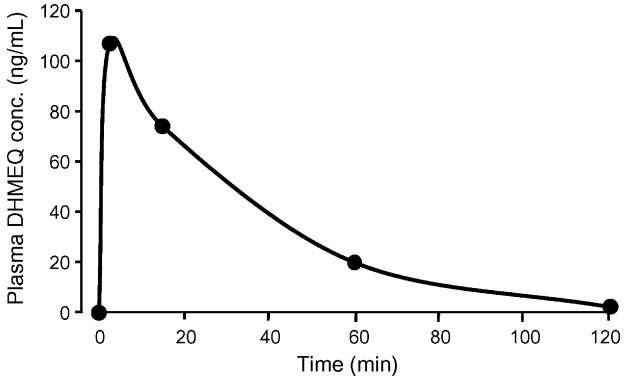

Fig. 4. Plasma DHMEQ concentration versus time profile after an intraperitoneal injection of DHMEQ at a dose of 15 mg/kg body weight.

4. Conclusion

The LC–MS technique described provides a simple and reliable liquid chromatographic method for the determination of DHMEQ level and for use in studies involving pharmacokinetics.

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