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Short communication

Determination of norelgestromin in rabbit plasma by LC–MS/MS and its application to the pharmacokinetic study of ORTHO EVRA®

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ABSTRACT

A simple, sensitive and rapid method is presented for the determination of norelgestromin using LC–MS/MS, interfaced via an electrospray ionization (ESI) probe, operating in the positive ion mode with multiple reaction monitoring (MRM). The method was developed and validated over the concentration range of 0.05–20 ng/ml, and showed excellent linearity. The intra- and inter-assay accuracy error and precision were ranging from −2.3% to 6.0% of nominal values and 2.2% to 7.8% over the three concentration levels evaluated. The concentration of formic acid in mobile phase was optimized to achieve satisfactory injection reproducibility and sensitivity, and sample preparation was optimized, with only 1.6 ml organic solvents used in performing the liquid–liquid extraction. The method has been successfully applied to a pharmacokinetic study of the ORTHO EVRA® patch in rabbits.

1. Introduction

Norelgestromin (NGMN), formerly known as 17-deacetylnoregestimate (17-DA-NGM), is a major active metabolite of norgestimate (NGM), which is a third generation progestin with little androgenic effect, estrogenic or glucocorticoid activity.

Like other contraceptives, the plasma concentration of NGMN is also very low. RIA is sensitive enough to reach detection limits of pg/ml, but susceptible to artifacts. Although LC–MS/MS has become the method of choice for the analysis of contraceptives [1–5], little literature on the determination of NGMN is available. Wong et al. [6] reported an impressive method for simultaneous determination NGM and its metabolites (including NGMN) in human serum by LC–MS/MS using an atmospheric pressure chemical ionization (APCI) source with a low limit of quantification (LLOQ) of 0.1 ng/ml for NGMN. However, 1 ml serum was needed and an aliquot of 70 μl was injected into the LC–MS/MS system to achieve that LLOQ, and a large volume of organic solvents (double extraction with 7 ml of methyl-*t*-butyl ether) was used for liquid–liquid extraction (LLE), making the sample preparation complicated and time-consuming. Abrams et al. [7] used the same sample preparation procedure as Wong et al. [6] but with isocratic elution; however the linear range was 0.1–5.0 ng/ml.

In this paper, a simple, sensitive and relatively high-throughput LC–MS/MS method will be presented for the determination of NGMN in rabbit plasma. Electrospray ion source was applied to perform the determination, achieving a LLOQ of 0.05 ng/ml when 200 μl plasma was used, and a 4.0 min run time for each injection making it possible for high-throughput quantitative bioanalysis. The mobile phase containing different concentrations of formic acid was investigated in order to achieve satisfactory sensitivity and reproducibility. The sample preparation procedure has also been optimized, saving a lot of time in sample preparation.

NGMN is the progestin contained in ORTHO EVRA® patch, hereafter referred to as "the patch", is a once-a-week patch. The method has been successfully applied to the pharmacokinetic study of NGMN delivered via a patch in rabbits following a single application.

2. Experimental

2.1. Chemicals and reagents

HPLC-grade methanol was purchased from Sigma–Aldrich, Inc. (St. Louis, MO, USA). Formic acid was obtained from TEDIA Co. (Fairfield, IA, USA). Analytical grade diethyl ether and hexane were purchased from Sinopharm Chemical Reagent Co. (Shanghai, China). Purified water used throughout the study was commercially available (Wahaha®, Hangzhou Wahaha Co., Ltd., Zhejiang, China). Norelgestromin (purity: 98.3%) was obtained from Shanghai Institute of Pharmaceutical Industry (SIPI, Shanghai, China). Etonogestrel (ENG, used as I.S., purity: 98.8%) was obtained from Beijing Zizhu Pharmaceutical Co. Ltd. (Beijing, China, batch No. 20070113-2). ORTHO EVRA® patch was obtained from Ortho-McNeil Pharmaceutical, Inc. (Raritan, NJ, USA,

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batch No. 6HM 3870), containing 6.00 mg NGMN and 0.75 mg ethiny estradiol (EE) each patch, with a contact surface area of 20 cm^2.

2.2. Animals

Three female New Zealand rabbits (Experimental Animal Center of Shanghai Institute of Pharmaceutical Industry; Shanghai, China; weighting 2.9 ± 0.3 kg) were housed in stainless steel cages at 25 °C, and numbered A, B, and C, respectively. During the experiment, the rabbits were provided free access to food and drinking water. The protocol and any amendments or procedures involving the care or use of animals in this study was approved by SIPI's Institutional Animal Care and Use Committee (IACUC). During the study, the care and use of animals was conducted in accordance with the provisions of Laboratory Animal Administration ordinance issued by State Science and Technology Committee of the Peoples Republic of China (PR China), Act 2 and Laboratory Animal Administration regulations issued by Shanghai Laboratory Animal Administration Office, PR China.

Blank rabbit plasma was taken from other blank rabbits. The blood was taken through auricular vein into heparinized containers, centrifuged and the supernatant plasma was transferred and stored at −20 °C prior to use.

2.3. Chromatographic conditions

A HPLC system, consisting of a Shimadzu LC-10ADvp pump, a Shimadzu SIL-HTc auto sampler, and a Shimadzu CTO-10Avp column oven, was used. Chromatographic separations were achieved using a C18 column (100 mm × 2.0 mm, 5 μm, Shiseido Co., Ltd., Chuo-ku, Tokyo, Japan,) maintained at 30 °C with a guard column (C18, 4 mm × 2.0 mm, Phenomenex Co. Ltd., Torrance, CA, USA). Auto sampler was maintained at 15 °C.

Mobile phase was comprised of methanol/water (78/22, containing 0.05% formic acid), and was delivered at constant flow rate of 0.2 ml/min during the isocratic program. Under these conditions, the analytical time was 4.0 min and the retention times for NGMN and ENG were 3.3 ± 0.1 min and 3.1 ± 0.1 min, respectively.

2.4. Mass spectrometric conditions

An API 3000 triple quadrupole mass spectrometer (AB/MDS-Sciex, Ontario, Canada) was interfaced via an electrospray ionization (ESI) probe, operating in the positive ion mode with multiple reaction monitoring (MRM). The characteristic precursor $[M+H]^+$ to product ions transitions were m/z 328.0 → 123.7, and m/z 325.3 → 109.1 for NGMN and I.S., respectively. The instrumental parameters were optimized for maximal generation of the protonated analyte molecules ($[M+H]^+$) and characteristic fragment ions by infusing the diluted stock solutions of NGMN and ENG. Optimal conditions were as the following: heater temperature: 500 °C, ion source voltage: 5500 V, curtain gas: 12.0 L/min, nebulizer gas: 13.0 L/min, respectively. The specific parameters for each transition were: dwell time: 200 ms, FP: 320 V, CXP: 10 V for both NGMN and ENG; DP: 55 and 52 V, CE: 41 and 46 V for NGMN and ENG, respectively. The quadrupoles Q1 and Q3 were set on low resolution.

MS data were acquired and processed (integrated) using the proprietary software application Analyst™ (Version 1.4.1, AB/MDS Sciex, Ontario, Canada).

2.5. Preparation of standards curves and quality control (QC) samples

Stock solution of NGMN was prepared in methanol at 1 mg/ml. A series of standard or QC working solutions at the desired concentrations were prepared by diluting the stock solution with methanol/water (50/50, v/v).

ENG stock solution was prepared at 1 μg/ml in methanol, and diluted with methanol/water (50/50, v/v) to yield an I.S. working solution of 50 ng/ml.

All solutions described above were stored at 4 °C.

The calibration standards were freshly prepared by adding 40 μl of the appropriate standard working solutions to 200 μl blank plasma to provide the final concentrations at 20, 10, 5, 2, 0.5, 0.1, 0.05 ng/ml, respectively. Low, medium and high level of QC samples were at the concentrations of 0.05, 2, 20 ng/ml, respectively.

2.6. Plasma sample preparation

A volume of 200 μl thawed plasma was transferred to a 2 ml eppendorf tube, then 40 μl of methanol/water (50/50, v/v) and 40 μl of I.S. working solution were spiked and briefly vortex-mixed. To each sample, 1.6 ml of diethyl ether/hexane (3:1, v/v) was added, and the tubes were capped and vortexed for 3 min, then centrifuged at 12,750 *g* for 5 min. The upper organic layer was carefully transferred to a glass tube, and evaporated to dryness under a gentle stream of nitrogen. The extract was reconstituted with 200 μl of methanol/water (50/50, v/v), and an aliquot of 20 μl was injected to the LC–MS/MS system for analysis.

2.7. Matrix effect and extraction recovery

Matrix effects were estimated by the post-extraction addition method. Absolute matrix effect (ME %) was used to evaluate the MS signal suppression and enhancement effects. It was calculated by comparing the peak areas of analytes added post-extraction (B) with those of the standards in the reconstitution solvent (A), and expressed as (B/A × 100%). Tests were conducted in five replicates on a single lot of plasma. ME% of 100% indicates that there is no absolute matrix effect, and a signal enhancement if ME% >100%, correspondingly, a signal suppression if ME% <100%.

Relative matrix effect was used to evaluate the variations of different lots of plasma suffered from the matrix effects, and was calculated by the coefficients of variation [CV%] of peak areas of analytes added post-extraction from five different lots of blank plasma. Extraction recovery was calculated by comparing peak areas of QC samples (C) with B, which expressed as (C/B × 100%).

2.8. Method validation

The current LC–MS/MS assay method was validated for selectivity, linearity, intra- and inter-day precision and accuracy, recovery, and stability.

The selectivity was assessed by monitoring ion pairs representing NGMN and ENG for the appearance of peaks in six different lots of blank plasma samples.

Three validation batches, each containing one set of calibration standards and five replicates of QC samples at low, medium and high concentration levels, were assayed to assess the linearity, precision and accuracy of the method. The linearity of each curve was confirmed by plotting the peak area ratio (y) of the analyte to I.S. versus analyte concentration (x). The accuracy of the assay was calculated the deviation observed in analysis of QC samples and expressed by error: [(calculated concentration by the regression equations)/(spiked concentration)] × 100% − 100%, and precision was evaluated by relative standard deviation (RSD).

The stability of NGMN in spiked samples was investigated. The stability experiments aimed at testing the effects of possible conditions that the analyte might experience during collection, storage and analysis, including analyte stability in frozen plasma (−20 °C storage condition), in thawed plasma (room temperature for 4 h),