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Determination of norgestimate and its metabolites in human serum using high-performance liquid chromatography with tandem mass spectrometric detection

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Abstract

A rapid and reliable analytical method is described for the simultaneous determination of a synthetic progestin norgestimate (NGM), and its metabolites, 17-deacetylnorgestimate (17-DA-NGM), 3-ketonorgestimate (3-keto-NGM) and norgestrel (NGL) in human serum using reversed phase high-performance liquid chromatography (HPLC) with tandem mass spectrometric (MS-MS) detection. The assay was linear over the concentration ranges of 0.1–5.0 ng/ml for 17-DA-NGM and NGL and 0.5–5.0 ng/ml for NGM and 3-keto-NGM. The inter-assay reproducibility was consistently less than 10%. The overall recovery of the analytes ranged from 72 to 92%. Serum profiles following oral administration of norgestimate to female volunteers are presented.

Keywords: Norgestimate; 17-Deacetylnorgestimate; 3-Ketonorgestimate; Norgestrel

1. Introduction

Norgestimate (NGM), currently under development [1–7] at the R.W. Johnson Pharmaceutical Research Institute (RWJPRI, Raritan, NJ, USA), is a novel synthetic progestin. The acute toxicity potential of NGM is low and is consistent with that of comparable steroid hormones. Following oral administration, NGM is rapidly absorbed and metabolized to a number of molecules that are themselves active progestins, most noticeably, 17-deacetylnorgestimate (17-DA-NGM), 3-ketonorgestimate (3-keto-NGM) and norgestrel (NGL) [8,9]. NGM has not been used extensively, but has been shown to be safe and effective for oral contraceptive (OC) use in combination with ethinyl estradiol [10].

The support of clinical pharmacokinetic investigations requires sensitive and specific bioanalytical methodology. This communication describes the

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validation and implementation of an efficient, reliable LC–MS-MS assay for the determination of NGM and its metabolites in human serum.

2. Experimental

2.1. Materials

Norgestimate (A; RWJ-10131-000; 13-ethyl-17-acetyloxy-18,19-dinor-17a-pregn-4-en-20-yn-3-one oxime) and its metabolites, 17-deacetylnorgestimate (B; 17-DA-NGM; RWJ-10553-097; 13-ethyl-17-hydroxy-18,19-dinor-17a-pregn-4-en-20-yn-3-one oxime), (C; NGL; RWJ-9345-999; 13-ethyl-17-hydroxy-18,19-dinor-17a-pregn-4-en-20-yn-3-one), 3-keto-norgestimate (D; 3-keto-NGM; RWJ-19599; 13-ethyl-17-acetyloxy-18,19-dinor-17a-pregn-4-en-20-yn-3-one), norgestrel and the internal standard (E; IS; RWJ-5263-000; 13-ethyl-17-acetyloxy-19-nor-17a-pregn-4-en-20-yn-3-one oxime) were obtained from the RWJPRI. Medicinal Chemistry Department. The chemical structures of compounds A–E are shown in Fig. 1.

All solvents were HPLC grade and all reagents were of the highest grade commercially available. Drug-free human serum and post-menopausal female human serum were obtained from the Intergen Center for Diagnostic Products, Milford, MA, USA. All stock and working solutions were prepared in methanol. Calibration standards were prepared freshly for each assay via serial dilutions of serum to produce concentrations of 0.50, 0.75, 1.50, 2.50, 3.00, and 5.00 ng/ml for NGM and 3-keto-NGM, and 0.10, 0.20, 0.50, 1.00, 2.00, 2.50, and 5.00 ng/ml for 17-DA-NGM and NGL. Serum quality control concentrations (low, medium and high) were prepared separately from the calibration standards to contain 0.90, 1.80, and 2.70 ng/ml NGM and 3-keto-NGM, and 0.30, 0.90, and 1.80 ng/ml 17-DA-NGM and NGL, respectively.

2.2. Instrumentation and experimental conditions

2.2.1. Chromatographic conditions

The HPLC instrumentation included two Model 4100 Constametric multisolvent pumps fitted with a solvent degasser and a Model AS 3000 autosampler

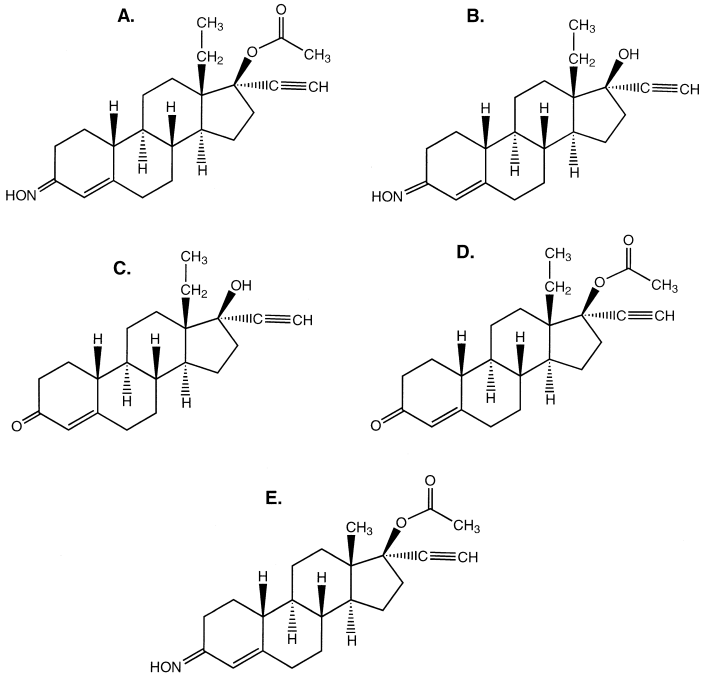

Fig. 1. Chemical Structures of (A) NGM, (B) 17-DA-NGM, (C) NGL, (D) 3-keto-NGM and (E) the internal standard.

(Thermo Separations, Riviera Beach, FL, USA). An Inertsil 5 μm phenyl column (15 cm×4.0 mm I.D.; GL Sciences, Tokyo, Japan) fitted with a phenyl guard column was used to separate NGM, its metabolites and the internal standard from endogenous coextractants. The assay employed a 4 min step-gradient in which the water–acetonitrile composition was changed from 70:30 to 30:70 (v:v). The flow rate was set to 1.0 ml/min and the column was maintained at ambient temperature conditions.

2.2.2. Mass spectral analysis

A PE Sciex API 300 triple quadrupole mass spectrometer, with the associated PE Sciex Macintosh data system, (PE Sciex, Concord, Ontario, Canada) was operated in positive ionization atmospheric pressure chemical ionization (APCI) mode. The APCI probe temperature was maintained at 475°C. The nebulizing, auxiliary and curtain gases were high purity nitrogen delivered from a Dewar liquid nitrogen vessel. The orifice was set at 30 V.

The mass spectrometer was operated in MS-MS mode using selected reaction monitoring (SRM) to detect a specific precursor ion to product ion transition for each analyte. Table 1 lists these transitions. High purity nitrogen was used as the collision gas at a setting of six in the PE Sciex SampleControl software, which produced an overall analyzer pressure of approximately 3.0×10^{-5} Torr.

Instrument tuning parameters were optimized using a 10 μg/ml solution of each analyte prepared in acetonitrile–water (50:50, v/v). Each solution was infused at 10 μl/min into a standard PEEK tee where it was mixed with a co-axial stream of acetonitrile–water (50:50, v/v) supplied at 1.0 ml/min by an HPLC pump. The total effluent of 1.01 ml/min was directed into the APCI interface.

Table 1
Summary of the [MH^+] precursor ion and MS-MS product ion for each analyte

Analyte	Precursor ion (m/z)	Product ion (m/z)
NGM	370.2	124.1
17-DA-NGM	328.3	124.1
NGL	313.2	245.2
3-Keto-NGM	355.1	295.1
Internal Standard	356.2	124.1

2.2.3. Quantitation, data acquisition, and processing

Quantitation, data acquisition, and processing were accomplished using Macintosh Power Mac 8100 and 9500 computers and the proprietary PE Sciex software programs: Sample Control, v. 1.2 and MacQuan, v. 1.4. Calculations were based upon chromatographic peak area ratios of each analyte to the internal standard. Serum concentrations of NGM and its metabolites were determined by using the slope and intercept of the standard curve obtained from a linear least squares regression (weighted as $1/\text{conc}^2$) for the analyte/internal standard peak area ratio vs. the calibration standard concentration.

2.2.4. Extraction procedure

Aliquots (100 μl) of methanol and the internal standard solution (20 μg/ml) were added to 1 ml of serum and the mixture was vortexed for 5 s. The serum was extracted twice with 7 ml of methyl-*t*-butyl ether after vortexing for 30 s and centrifuging for 6 min at 1200×*g*. The combined organic layer was evaporated to dryness under nitrogen at 35°C. The residue was reconstituted in 120 μl of an acetonitrile–water mixture (50:50, v:v), vortexed for 30 s and centrifuged for 6 min at 1200×*g*. Thereafter, 90 μl of the top clear layer was transferred to a 1-ml autosampler vial equipped with a limited volume insert. An aliquot (70 μl) was injected into the LC–MS-MS system.

2.2.5. Assay validation

Inter-assay precision and accuracy of the assay were determined from the performance of both the calibration standards over a five-day period and the quality control serum samples (in triplicate) on three separate days. Intra-assay precision and accuracy were determined from the analysis of quality control samples on a single assay day ($N=6$ replicates). Precision data were expressed as percentage coefficient of variation (%CV) and accuracy data were expressed as percentage theoretical value. Specificity was assessed in serum collected from both pre- and post-menopausal female volunteers, and analyte stability (i.e. freeze-thaw, autosampler, and storage conditions) was assessed in serum collected from post-menopausal female volunteers in order to mimic

in vivo conditions. These were periodically assayed together with freshly prepared standards.

Recovery was assessed using the serum quality control samples for NGM, 17-DA-NGM, 3-Keto NGM and NGL. Quadruplicate samples for each quality control concentration were spiked with the internal standard, extracted and analyzed using the procedure outlined above. To determine the recovery, the mean chromatographic peak area responses for each analyte were compared to the peak area responses from analyses of equivalent amounts of reference standard injected from methanol solutions.

3. Results

Although not shown for the purpose of brevity, the Q1 mass spectrum of each analyte was dominated by the intense, protonated molecular ion species

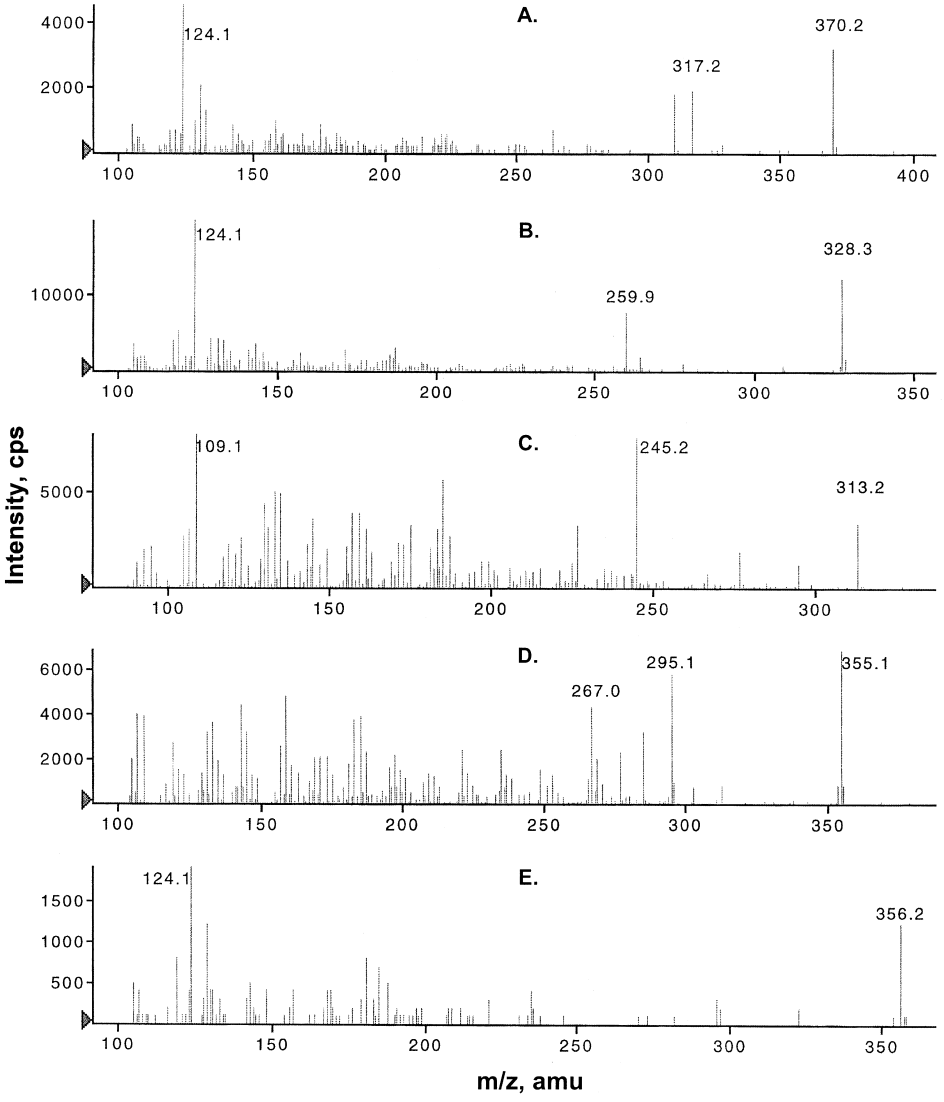

Fig. 2. Mass fragmentation patterns (product ion spectra) of (A) NGM, (B) 17-DA-NGM, (C) NGL, (D) 3-keto-NGM and (E) the internal standard.

(MH^+). The MS-MS product ion spectra resulting from collision activated dissociation (CAD) of each molecular ion are depicted in Fig. 2. The instrument tuning parameters and collision energy were optimized for each analyte to give the most sensitive precursor to product ion transition without regard to the residual intensity of the precursor ion. In the optimized MS-MS spectrum of NGL, two prominent product ions were observed at m/z 245 and 109 (Fig. 2c). After initial scrutiny, the ion at m/z 245 was chosen as the product ion for SRM analysis because interfering peaks from the control serum were ob-

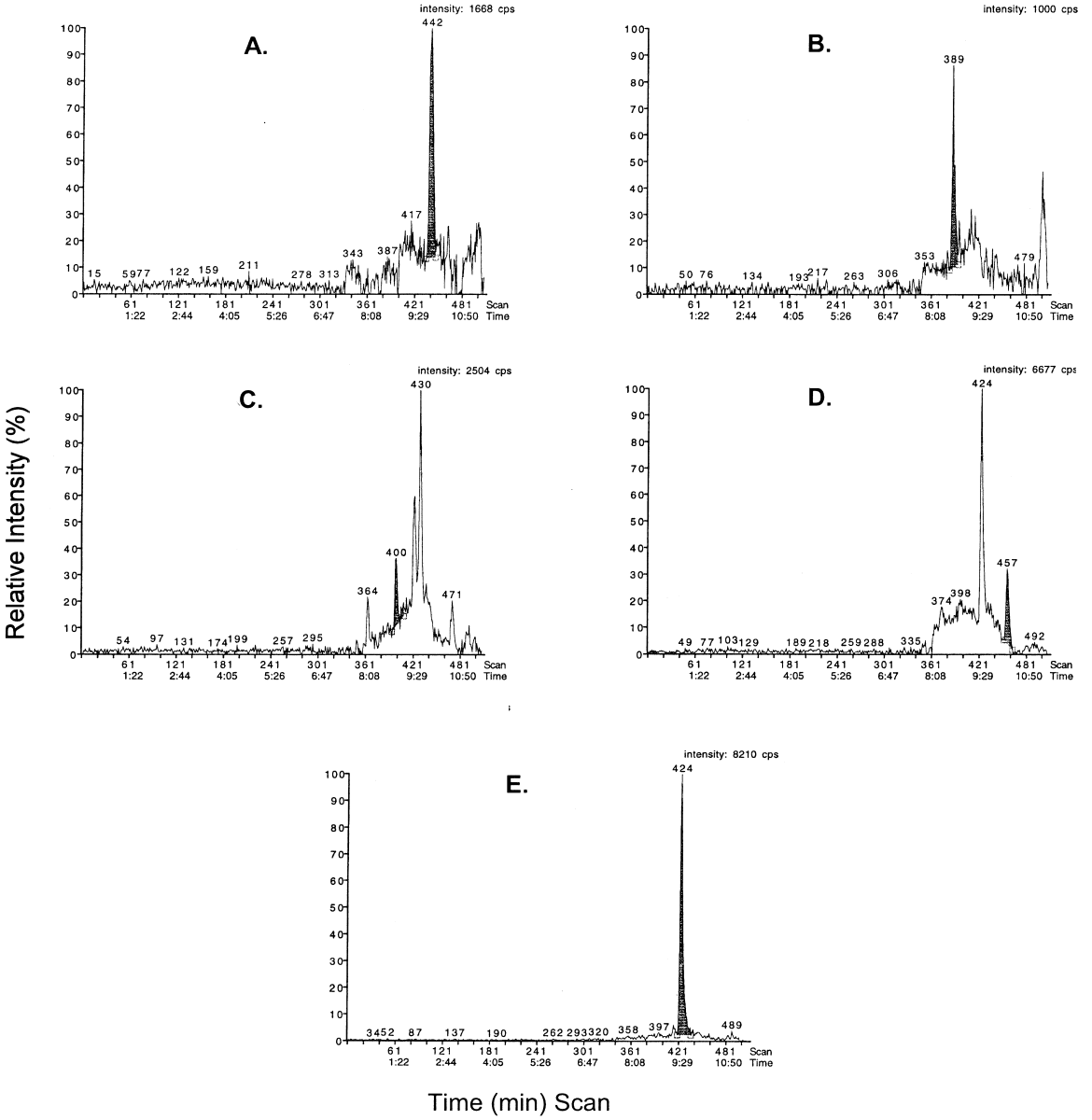

Fig. 3. Representative mass spectral chromatograms of the lowest calibration standard containing (A) 0.5 ng/ml NGM, (B) 0.1 ng/ml 17-DA-NGM, (C) 0.1 ng/ml NGL, (D) 0.5 ng/ml 3-keto-NGM and (E) the internal standard.

served when monitoring the product ion at m/z 109. As seen in Fig. 2d, the optimized product ion spectrum of 3-keto-NGM resulted in the precursor ion at m/z 355 remaining as the base peak. However, the SRM transition from m/z 355 to the product ion at m/z 295 was the most intense under these conditions, which afforded the highest sensitivity. Increasing the collision energy resulted in further fragmentation of the precursor ion, but the sensitivity of SRM transitions to the resulting product ions was lower.

Three of the analytes (NGM, 17-DA-NGM and the internal standard) produced identical product ions at m/z 124. Therefore, it was necessary to avoid ''cross-talk'' during SRM determination of serum samples. Cross-talk arises when product ions stay

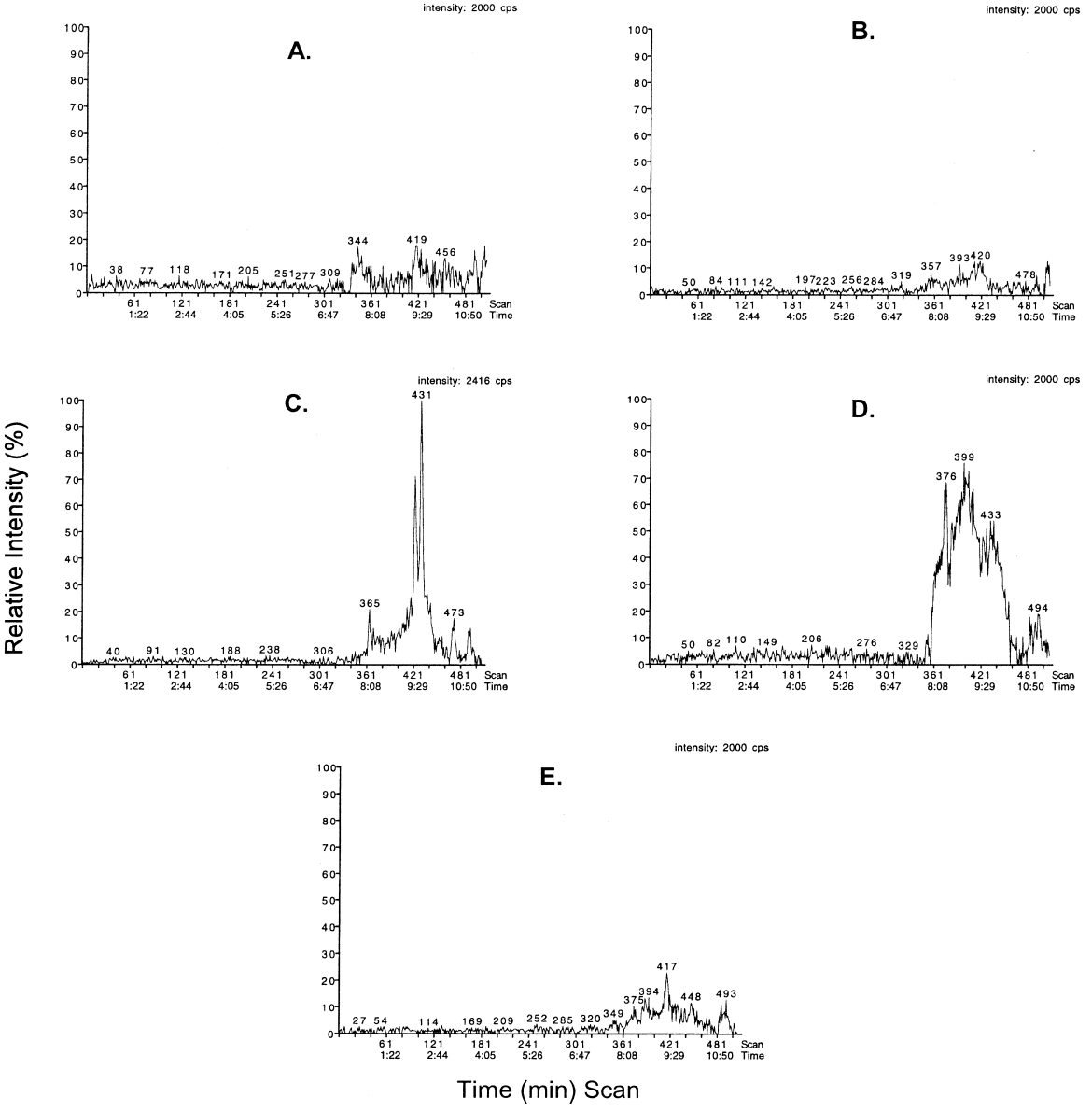

Fig. 4. Representative mass chromatograms obtained from control serum samples from post-menopausal women: (A) NGM, (B) 17-DA-NGM, (C) NGL, (D) 3-keto-NGM and (E) the internal standard.

Table 2
Inter-assay precision and accuracy of NGM, 17-DA-NGM, NGL and 3-keto-NGM in serum calibration standards and serum quality control samples

Analyte	Range			
	Calibration standards		QC samples	
	Precision (%CV)	Accuracy (%)	Precision (%CV)	Accuracy (%)
NGM	2.2–5.7	98.3–102.7	3.7–5.9	89.1–96.4
17-DA-NGM	2.9–6.7	97.8–102.7	3.3–7.0	102.2–104.6
NGL	3.1–6.5	94.2–104.9	4.7–10.3	99.6–107.1
3-Keto-NGM	1.8–7.5	97.4–104.1	3.9–8.9	94.6–112.2

resident in the mass spectrometer collision cell from one analyte and are falsely recorded with the next analyte if its corresponding product ion has the same m/z value. To prevent this, the PE Sciex Sample Control software was set to invoke ''Q2 Settling'' which effectively ''empties'' the collision cell after each SRM transition. This eliminated cross-talk in the assay procedure and ensured integrity of the data.

Representative chromatograms of the low concentration calibration standard containing 0.5 ng/ml NGM and 3-keto- NGM, and 0.1 ng/ml 17-DA-NGM and NGL, are shown in Fig. 3. Also, for comparison, Fig. 4 shows corresponding chromatograms from drug-free serum obtained from three post-menopausal women. Evident in these chromatograms, there are no interfering peaks that co-elute with any of the analytes.

During the development phase of this assay, an isocratic reversed phase HPLC system was used. Although overall run times were similar using either elution mode, the gradient procedure was subsequently adopted as it afforded sharper peaks, which enhanced sensitivity.

A synthetic analog of norethindrone was chosen as the internal standard because of its chemical similarity to NGM and because it eluted without interference from either NGM, its metabolites, or coextracted endogenous serum components. Using the step-gradient chromatographic conditions described above, the observed retention times were: 8.5, 9.0, 9.4, 10.0, and 10.2 min for 17-DA-NGM, NGL, the internal standard, NGM, and 3-keto-NGM, respectively (Fig. 3).

Inter-assay precision and accuracy were determined as described above and the results are shown in Table 2. For serum calibration standards, the inter-assay precision (%CV) was $\leq$7.5%, and the accuracy values were within 5.8% of their respective theoretical (target) values. Similarly, the serum quality control analyses demonstrated overall inter-assay precision (%CV) of $\leq$10.3% with accuracy values within 12.2% of theoretical values. Additionally, the assay exhibited excellent linearity for all analytes over the defined serum concentration ranges. Linear regression analyses of analyte/internal standard peak area ratios versus concentration consistently afforded mean r^2 (coefficient of determination) values of 0.98 or greater.

Intra-assay precision and accuracy was determined in quality control samples and the results are shown in Table 3. The intra-assay %CV values were $\leq$10.1% and the accuracy values were within 5.3% of theoretical values.

The recovery of NGM and its metabolites from human serum was assessed using the quality control samples at three levels encompassing the general concentration range of the assay. The overall recoveries ranged from 71.8 to 87.7% (NGM), 72.2 to 91.8% (17-DA-NGM), 75.1 to 87.6% (3-keto-NGM), and 77.1 to 91.9% (NGL). For all analytes, the precision (%CV) of recovery at each QC level was consistently $\leq$15%.

There were no observable interferences from coextracted endogenous components of stripped

Table 3
Intra-assay precision and accuracy of NGM, 17-DA-NGM, NGL and 3-keto-NGM in serum quality control samples

Analyte	Precision (%CV)		Accuracy (%)	
	Range	Overall	Range	Overall
NGM	4.6–7.2	5.7	96.5–97.9	96.7
17-DA-NGM	5.4–10.1	7.1	96.1–103.2	100.0
NGL	3.3–8.9	5.8	98.7–105.3	102.8
3-Keto-NGM	4.4–8.0	5.7	96.4–100.5	98.7

serum[1]. The analysis of the standards and quality controls prepared using serum obtained from either pre- or post-menopausal females afforded results that were nearly identical to those obtained using the stripped serum.

Additionally, the stability of NGM and its metabolites under freeze-thaw, autosampler and frozen storage conditions was determined using replicates of the 1.0 and 2.5 ng/ml calibration standards. The results demonstrated little or no observable difference from concentrations obtained upon analysis of freshly prepared samples. All analytes were observed to be stable in serum for at least three freeze-thaw cycles and under frozen storage (−20°C) for at least seven months. Additionally, autosampler stability was demonstrated for all analytes over a period of 32 h at ambient temperature. The analysis of the study sample batches did not exceed 30 h.

4. Discussion

The assay method described in this communication was applied to the measurement of NGM and its metabolites in support of a pharmacokinetic study in which post-menopausal female volunteers received oral tablet doses of NGM (180 μg) in combination with 17-β-estradiol (2 mg). Following drug administration, serum concentrations of both the parent drug and 3-keto-NGM were consistently below assay quantitation limits; only 17-DA-NGM and NGL demonstrated measurable serum concentrations. Fig. 5 shows the chromatograms of 17-DA-NGM [(A) concentration=533 pg/ml], NGL [(B) concentration=188 pg/ml] and the internal standard from a serum sample collected from a healthy post-menopausal female volunteer 4 h after dosing. Fig. 6 shows a representative profile of serum concentration vs. time (0–72 h post dose) for 17-DA-NGM and NGL.

The analysis of NGM as well as other synthetic steroids in human serum was initially accomplished using radioimmunoassay (RIA) techniques. Although sensitive, the NGM RIA lacked adequate specificity due to a high degree of antibody cross-reactivity with NGM metabolites. In order to ensure assay specificity and maintain adequate sensitivity, an additional clean-up step involving HPLC fractionation was added [11]. However, the overall HPLC–RIA procedure was labor-intensive, resulting in relatively poor sample throughput. The assay described in this communication provided a simplified alternative in

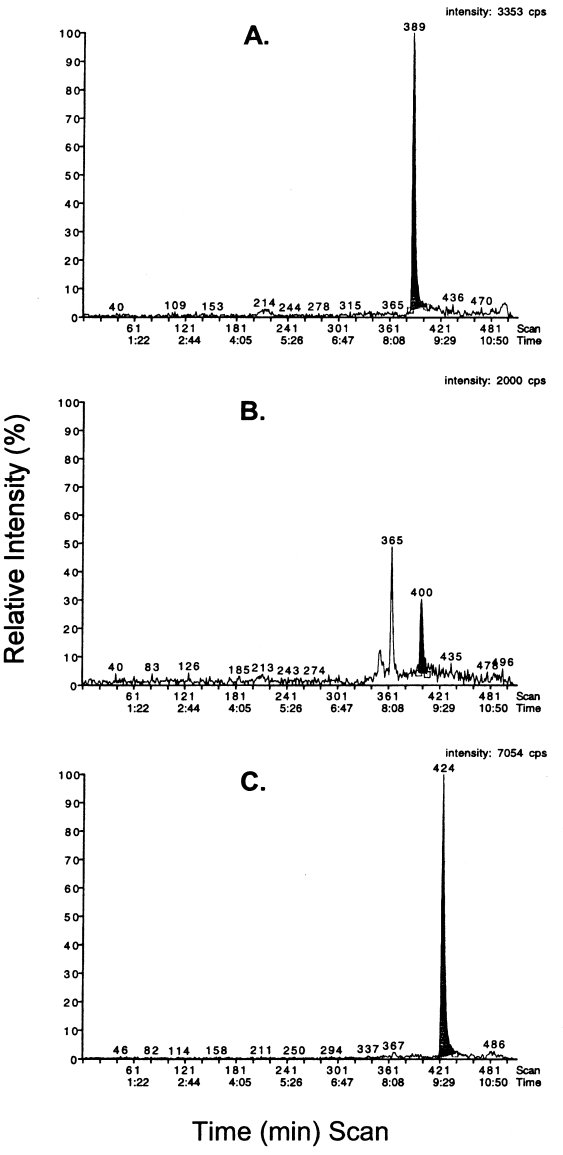

Fig. 5. Representative mass chromatograms of extracted serum collected from a healthy post-menopausal female volunteer 4 h after administration of a single oral 2 mg/180 μg dose of 17-β-estradiol–NGM: (A) estimated concentration of 17-DA-NGM=533 pg/ml, (B) estimated concentration of NGL=188 pg/ml and (C) the internal standard. Concentrations of NGM and 3-keto-NGM were below assay quantitation limits.

[1]Stripped serum was prepared by treating normal serum with charcoal and then removing the charcoal by filtration.

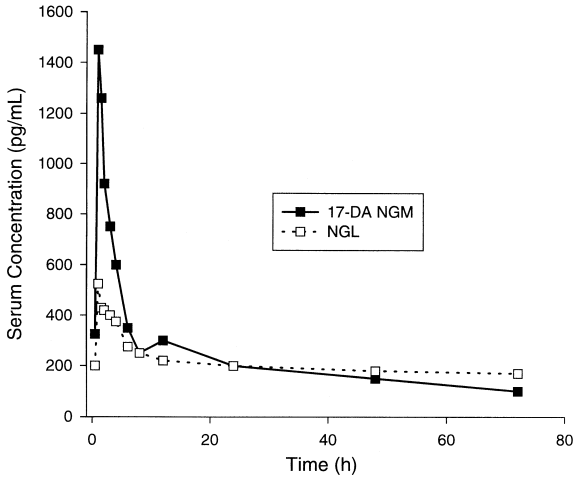

Fig. 6. Representative serum concentration vs. time profile of 17-DA-NGM and NGL in a healthy post-menopausal female subject following administration of a single oral 2 mg/180 μg dose of 17-β-estradiol/NGM.

affording excellent specificity and sensitivity for the simultaneous determination of several analytes. Additionally, as the overall procedure has proven to be rapid, high sample throughput was consistently achieved. Thus, the LC–MS-MS methodology described here should be generally useful for the measurement of serum metabolite concentrations in support of future bioavailability and pharmacokinetic studies. To date the assay has already been used to analyze thousands of clinical samples.

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