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Simultaneous quantitative determination of norgestrel and progesterone in human serum by high-performance liquid chromatography-tandem mass spectrometry with atmospheric pressure chemical ionization

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A selective, reliable and rapid method for the simultaneous determination of progesterone and norgestrel concentrations in human serum after taking oral contraceptive tablet has been developed using high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS-MS) with atmospheric pressure chemical ionization (APCI) interface. The norethisterone was used as the internal standard. Selected transitions of *m/z* 313/245 for norgestrel, *m/z* 315/97 for progesterone and *m/z* 299/109 for norethisterone were monitored using multiple reaction monitoring (MRM) mode for quantitation. The assay was linear over the concentration range of 0.2–50 ng mL^{-1} for norgestrel and progesterone. The lower level of quantitation in human serum was obtained at 0.2 ng mL^{-1} for both norgestrel and progesterone using optimum tuning parameters. The intra-assay precision and inter-assay precision do not exceed 10 and 9%, respectively. The method has been applied to the determination of norgestrel and progesterone in serum of female volunteers.

Introduction

Progesterone (Fig. 1), one of the most important progestogens, is necessary for the development of mammary tissue, and for the maintenance of pregnancy. Norgestrel (Fig. 1), a major active component in many contraceptive preparations, has been extensively used for contraception. A more complete understanding of the relationship between norgestrel and progester-

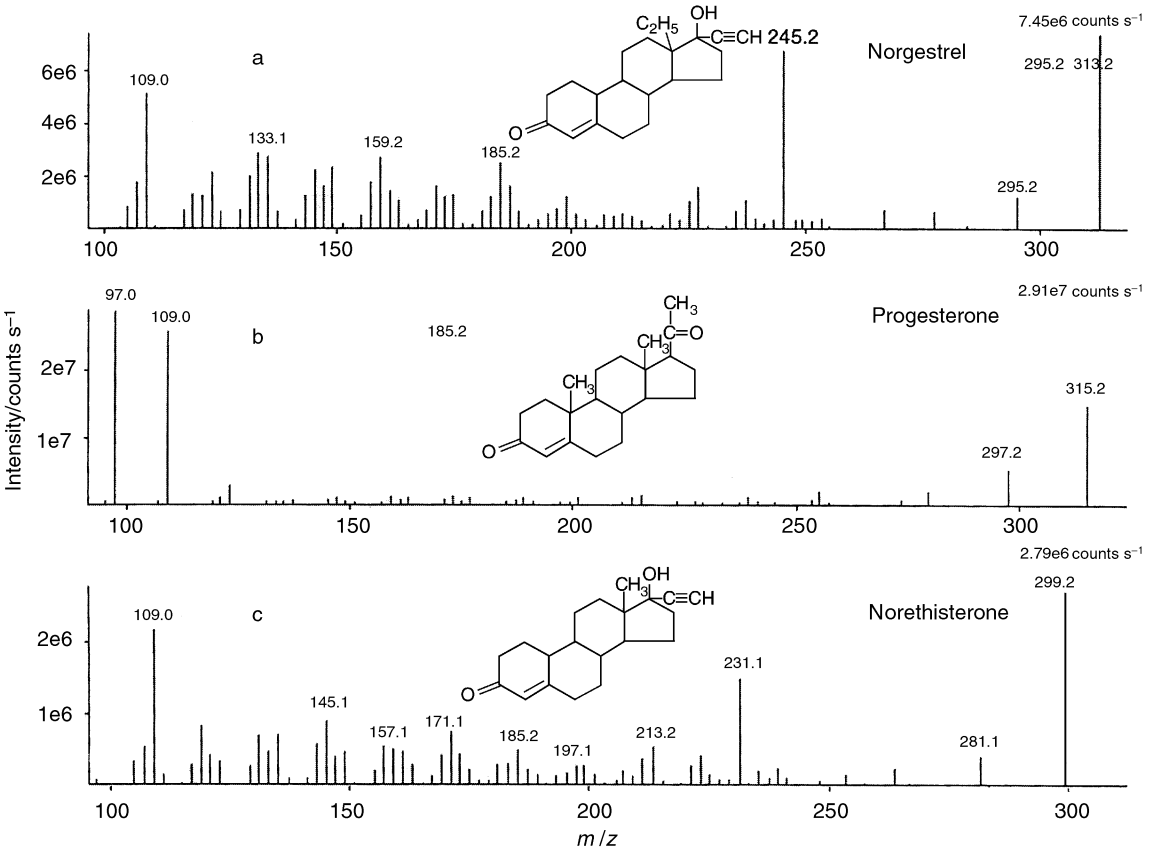

Fig. 1 Positive product ion mass spectra of progesterone, norgestrel and norethisterone: (a) norgestrel; (b) progesterone; (c) norethisterone (internal standard).

one concentration in blood may offer more information for clinical examination and assess the effect of norgestrel. Simultaneous monitoring of concentrations of progesterone and norgestrel in serum following oral administration is of importance.

Generally, the concentrations of progesterone and norgestrel in biological fluids are around the ng mL^{-1} level or even lower. The radioimmunoassay (RIA) method has been extensively employed in clinical examination owing to its sensitivity. Although sensitive, RIA is considered to be time consuming and labor-intensive. Moreover, it is not suitable for high throughput analysis.

In recent years electrospray ionization (ESI) has become the most widely applied interface for liquid introduction into the mass spectrometer.[1–5] Also, the advent of the electrospray ionization interface has greatly stimulated the use of another technique, atmospheric-pressure chemical ionization (APCI).[6–12] The improvements in mass spectrometric methodology, such as electrospray ionization and atmospheric pressure chemical ionization, have meant that assay methods with enhanced sensitivity, improved selectivity, robustness and high sample throughput have become possible. Therefore, high-performance liquid chromatography with mass spectrometry (HPLC-MS), especially HPLC-MS-MS, is currently recognized as a powerful tool to characterize complex samples and to analyse biological samples.[13–16]

In this paper, a selective and high-throughput HPLC-MS-MS method for simultaneous measurement of both norgestrel and progesterone concentrations in serum is described. The method is applied to the determination of norgestrel and progesterone in real human serum samples.

Experimental

Chemicals and reagents

Norgestrel and progesterone were purchased from Sigma (St. Louis, MO, USA). Norethisterone (internal standard, Fig. 1) was supplied by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC-grade methanol and acetonitrile was from Fisher Scientific (Fair Lawn, NJ, USA). Other reagents were of the highest grade commercially available. Doubly distilled water was used throughout the experiment.

LC-APCI-MS-MS equipment and conditions

Optimization of parameters of APCI-MS-MS was carried out with the PerkinElmer Sciex API 3000 triple quadrupole instrument (Concord, Ontario, Canada) fitted with an atmospheric pressure chemical ionization source and electrospray ionization interface. The optimization procedure was performed by flow injection analysis on the basis of optimum parameters of ESI-MS-MS (Table 1). The ultra-purity nitrogen gas was used as nebulizer, auxiliary and curtain gases and the settings for the curtain, nebulizer and collision gases were 12, 8 and 6 on the API 3000. The heated nebulizer gas was set at 70 psi. The discharge current was set at 3 μA and the temperature was 450 °C; the orifice potential was set at 50 V. The dwell time was 200 ms for both analytes of interest and internal standard.

Separation was carried out using a Phenomenex Luna 5u C_{18} column (4.6 × 150 mm) with the mobile phase of methanol–H_2O (80 + 20) containing 0.5% formic acid. The flow rate was 1.0 mL min^{-1} and effluent from the column was delivered directly to the APCI-MS interface. The column was maintained at room temperature.

Preparation of standards

Stock standards of norgestrel and progesterone were prepared by dissolving 2 mg of the compounds in 10 mL of methanol to give stock concentrations of 200 μg mL^{-1}. Aliquots (100 μL) of norgestrel and progesterone were mixed and diluted with methanol to give the working stock solution containing 1 μg mL^{-1} of norgestrel and progesterone. The working stock solution was diluted with control serum to obtain a serum spiking solution containing norgestrel and progesterone at 100 ng mL^{-1}. Calibration standards were freshly prepared daily for each assay by serial dilution of control serum to produce concentrations of 0.2 , 0.5, 0.8, 1, 2, 5, 10 and 50 ng mL^{-1}. The internal standard was subsequently diluted to obtain the working solution of 2.5 ng mL^{-1}. A calibration curve was constructed by plotting peak area ratios of analyte to internal standard *versus* nominal concentration using the weighted least ($1/x$) regression mode.

Quality control samples of five concentration levels (0.5, 0.8, 2, 5 and 25 ng mL^{-1}) were prepared separately from the calibration standards to assess intra-precision and intra-accuracy in a day ($n = 5$) . The inter-accuracy and inter-precision were examined by using independently prepared QC samples over 4 d. QC samples were spiked with internal standard and extracted and analyzed in the same way as for the analysis of the real samples.

All the stock solutions and working solutions were stored at −20 °C and brought to room temperature before use.

Sample preparation

Human blood samples were collected from healthy childbearing age volunteers who had been administered Norplant (36 mg $rod^{-1} \times 6$) for 1–3 years by venipuncture. Blood samples were allowed to stay at the room temperature for 2 h for clotting. After the clot had broken down, the serum was separated by centrifugation for 15 min at 1000 *g*. All sera were stored at −20 °C until being analysed.

Aliquots of methanol and 25 μL of internal standard (100 ng mL^{-1}) were added to 1 mL of serum and were extracted with 2 mL of diethyl ether. The samples were vortexed for 1 min and

Table 1 Experimental conditions for the HPLC-MS-MS

HPLC conditions—	
C_{18} column	4.6 mm × 150 mm
Mobile phase	Methanol:H_2O (80:20) (0.5% formic acid)
Flow rate	1 mL min^{-1}
Sampling volume	20 μL
Mass spectrometer conditions—	
Instrument	PE Sciex API 3000
Ionization mode	APCI, positive ion mode
Scanning mode	MRM
Transitions	*m/z* 313/245 (norgestrel)
	m/z 315/97 (progesterone)
	m/z 299/109 (norethisterone)
Dwell time	200 ms

State table parameters— Parameter	Value
NEB	8
CAD	6
CUR	12
NC	3 μA
TEM	450 °C
OR	50
RNG	300
RO1	−11
RO2	−39
RO3	−41

frozen in solid CO_2. The ether phase was then decanted into a dry silanised tube. The thawed serum was re-extracted with 2 mL of diethyl ether. Two ether extracts were combined and evaporated to dryness. The residue was reconstituted in 200 μL of mobile phase and an aliquot of 20 μL was injected into the LC-MS-MS.

Results and discussion

Optimization of LC-APCI-MS-MS conditions

During the early stage of method development, both ESI and APCI ionization interfaces coupled to MS-MS were investigated for sensitivity. Significant gain in sensitivity by the use of APCI was found. This indicated that APCI performed better for less polar and non-polar compounds. The APCI interface was therefore selected.

The APCI-MS-MS conditions for the determination of norgestrel and progesterone in human serum were investigated. The parameters such as corona discharge, temperature, orifice potential, flow of the nebulizer and auxiliary gas were optimized (Table 1). The full-scan (Q1 Mode) mass spectra of two compounds of interest and internal standard in the MS positive mode indicated that the most abundant ions were the protonated molecular ions, *i.e.*, *m/z* 313 for norgestrel, *m/z* 315 for progesterone and *m/z* 299 for internal standard. These positive molecular ions were therefore used as the precursor ions in the MS-MS experiment.

Multiple reaction monitoring (MRM) mode was used for the quantitative determination of norgestrel and progesterone in human serum. Using this mode, ions that are not related to the target compound are filtered out, thus minimizing the matrix interference of biosamples. Therefore, selection and optimization of product ions was of great importance in obtaining the best selectivity and sensitivity. As shown in Fig. 1, for norgestrel, $[M + H]^+$ of *m/z* 313 served as the precursor ion, producing two abundant product ions *m/z* 245 and 109. One of the principles of product ion selection is on the basis of best sensitivity with minimum interference from matrix of sample. In addition, for chosen transitions, the chromatographic interference peak had to be avoided when control serum was injected. No interference peak was observed for the transitions of *m/z* 313/245 and 313/109 when the blank was injected because the norgestrel was not an endogenous estrogen in human serum. Therefore, *m/z* 313/245 was selected as the preferred transition. The product mass spectrum of progesterone shown in Fig. 1 illustrated that the predominant fragments were *m/z* 109 and 97. When the control serum was injected, an interference peak was observed for the transition of *m/z* 315/109. So, *m/z* 315/97 was selected as the preferred transition. Similarly, for the internal standard, *m/z* 299/109 was selected as the preferred transition.

LC was coupled to MS for the pre-separation of norgestrel, progesterone and internal standard from the sample matrix. Two typical mobile phases used in HPLC, methanol–H_2O (80 : 20) and acetonitrile–H_2O (70 : 30), were investigated. It was found that the intensity by using methanol–H_2O as mobile phase was slightly higher than that obtained by using acetonitrile–H_2O as mobile phase. Although the efficiency of ionization for MS detection could be improved if 100% methanol was used as mobile phase, norgestrel, progesterone and internal standard could be well separated from the matrix by using a mobile phase with 80 : 20 methanol–H_2O. Therefore, a mobile phase composed of 80 : 20 methanol–H_2O was used herein. Flow injection analysis experiments were performed with the mobile phase

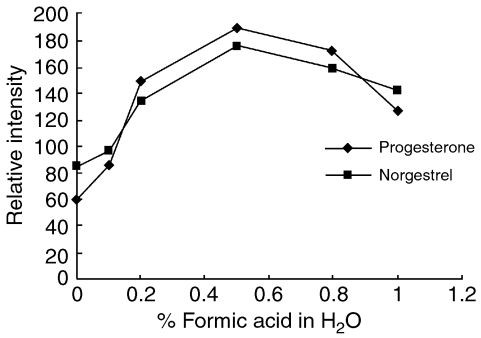

Fig. 2 Effect of formic acid on intensity.

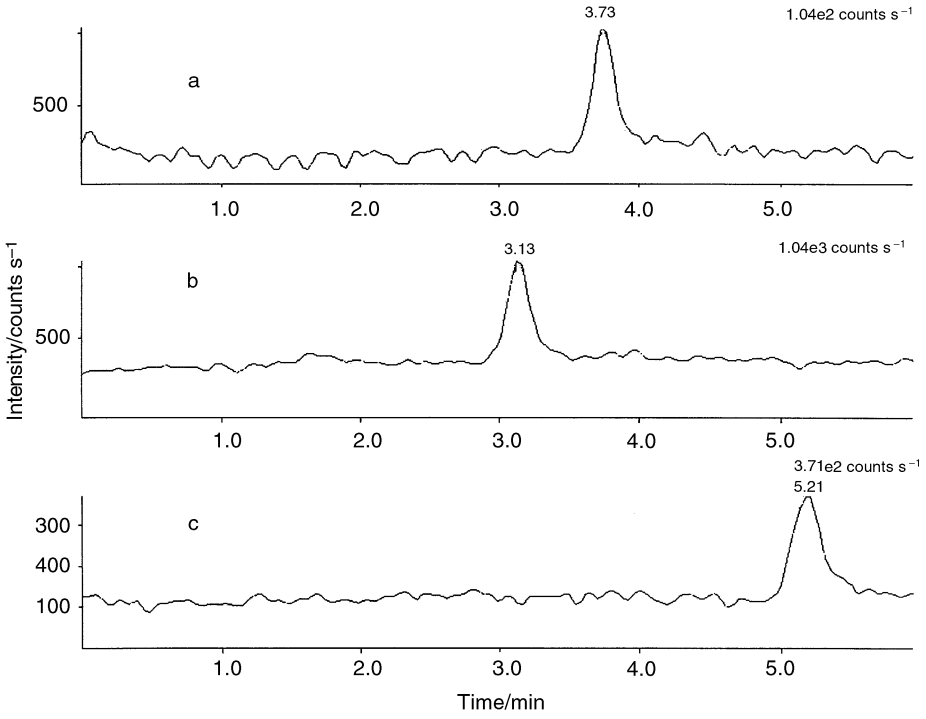

Fig. 3 Representative mass spectral chromatograms of the lowest calibration standard containing: (a) 0.2 ng mL^{-1} norgestrel; (b) 2.5 ng mL^{-1} internal standard; (c) 0.2 ng mL^{-1} progesterone.

composed of 80:20 methanol–H_2O containing different concentrations of formic acid, 0.1–1% (v/v) in H_2O, to determine the optimal concentration added in the mobile phase. It can be seen from Fig. 2 that the intensity was enhanced about two times at 0.5% formic acid when the concentration of formic acid was varied from 0.1 to 1%. In addition, the mobile phase containing 0.5% formic acid gave the best peak shape. Therefore, the optimum mobile phase was methanol–H_2O 80:20 containing 0.5% formic acid.

Quantitative analysis

Representative MRM chromatograms of serum spiked with 0.2 ng mL^{-1} of norgestrel, 0.2 ng $mL^{-1}$of progesterone and 2.5 ng $mL^{-1}$of norethisterone are shown in Fig. 3. The retention times of norgestrel, progesterone and norethisterone were 3.67±0.07 (min), 5.16 ± 0.07 (min) and 3.08 ± 0.09 (min), respectively. No endogenous sources of interference were observed at the retention times of the analytes.

Table 2 Inter-assay precision, accuracy and intra-assay precision, accuracy of norgestrel QC serum samples

Theoretical concentration/ ng mL^{-1}	Intra-assay precision (n = 5) RSD (%)	Intra-assay accuracy (mean ± s, n = 5)	Inter-assay precision (n = 4) RSD (%)	Inter-assay accuracy (mean ±s, n = 4)
0.5	9.2	0.469 ± 0.043	7.2	0.476 ± 0.034
0.8	7.3	0.756 ± 0.056	5.4	0.828 ± 0.045
2	4.8	2.09 ± 0.10	3.1	1.94 ± 0.060
5	1.2	5.16 ± 0.062	3.5	4.94 ± 0.17
25	1.5	24.7 ± 0.37	1.2	25.4 ± 0.30

Table 3 Inter-assay precision, accuracy and intra-assay precision, accuracy of progesterone QC serum samples

Theoretical concentration/ ng mL^{-1}	Intra-assay precision (n = 5) RSD (%)	Intra-assay accuracy (mean ± s, n = 5)	Inter-assay precision (n = 4) RSD (%)	Inter-assay accuracy (mean ± s, n = 4)
0.5	8.5	0.464 ± 0.039	8.1	0.472 ± 0.038
0.8	6.6	0.762 ± 0.050	6.3	0.759 ± 0.048
2	3.2	2.06 ± 0.066	3.4	2.06 ± 0.070
5	2.5	5.18 ± 0.13	2.1	5.14 ± 0.11
25	1.8	24.6 ± 0.44	1.6	25.3 ± 0.40

The data of intra-assay precision and accuracy were obtained from analysis of quality control samples of 0.5, 0.8, 2, 5 and 25 ng mL^{-1}in a single day (n = 5). The inter-assay precision and accuracy were determined by analysing quality control samples of 0.5, 0.8, 2, 5 and 25 ng mL^{-1} on four days. The precision of the method was assessed by analysis of relative standard deviation (RSD) of measured QC samples. The results of precision and accuracy of norgestrel and progesterone are listed in Table 2 and Table 3.

The LOQ was defined as the lowest concentration of quantitation on the calibration curve with both accuracy and precision within 20%.[17] The method has a LOQ of 0.2 ng mL^{-1} for both norgestrel and progesterone in human serum. The recoveries of the analytes were assessed by comparing the peak area ratios of analyte to internal standard with those of equivalent amounts of reference standard of analytes to internal standard. The four replicates of three concentrations of analytes (0.5, 5 and 25 ng mL^{-1}) were determined and the overall recoveries were 78.5–92.1% for norgestrel and 80.4–93.5% for progesterone, respectively. For all analytes, the precision (CV%) of recovery at each QC level was consistently ≤13%.

Additionally, the freeze–thaw stability of 1 ng mL^{-1} norgestrel and progesterone solutions in serum were evaluated. Both norgestrel and progesterone were stable in serum after three freeze–thaw cycles.

Under the optimal conditions described above, the calibration graph of peak area ratios (R) to the norgestrel and progesterone concentration (C) were linear in the range of 0.2–50 ng mL^{-1} (R = 0.359 C + 0.093, r^2 = 0.995, n = 8) for norgestrel and 0.2–50 ng mL^{-1} (R = 1.424C + 0.114, r^2 = 0.995, n = 8) for progesterone.

Sample analysis

The proposed method was applied to the determination of norgestrel and progesterone in human serum from twenty female volunteers. Results showed that sixteen serum samples contained norgestrel concentrations ranging from 0.21–1.16

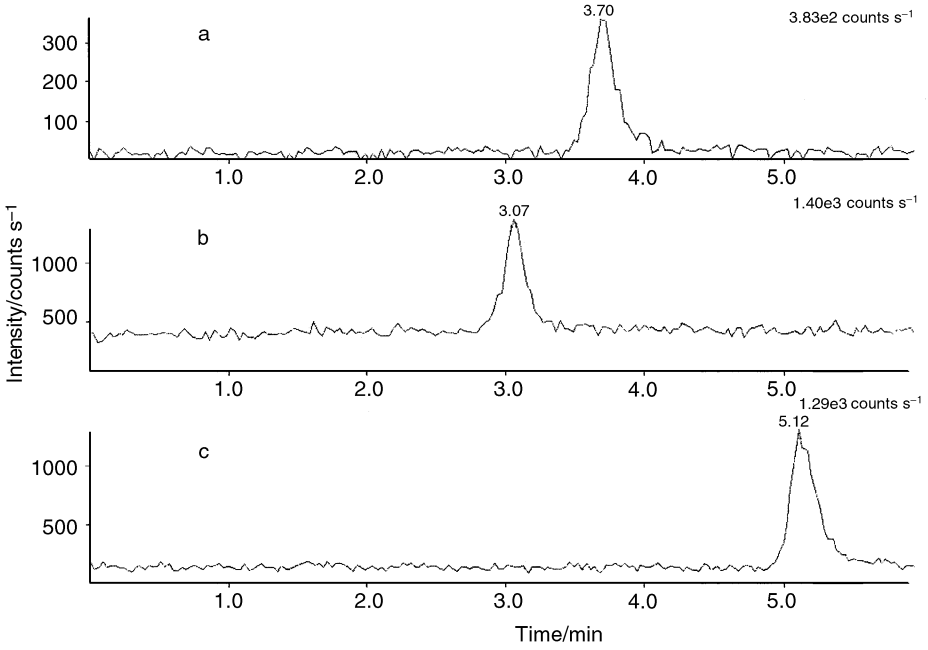

Fig. 4 Representative mass spectral chromatograms of real human sample: (a) estimated concentration of norgestrel 0.617 ng mL^{-1}; (b) 2.5 ng mL^{-1} internal standard; (c) estimated concentration of progesterone 0.573 ng mL^{-1}.

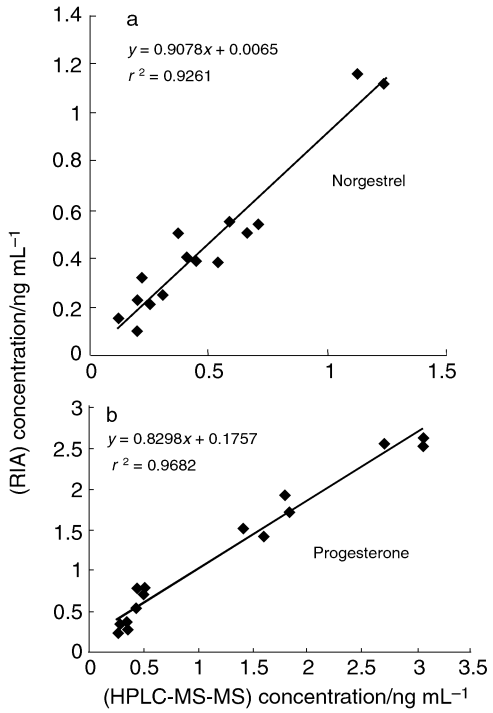

Fig. 5 Correlation of RIA and HPLC-MS-MS methods for norgestrel and progesterone.

ng mL^{-1}, implying that the concentration of norgestrel in human serum varied between individuals. Four samples contained a norgestrel concentration below the detection limit, indicating that the sensitivity of the present method should be further improved. A representative mass spectral chromatogram of real human sample is shown in Fig. 4. The estimated concentrations of norgestrel and progesterone were 0.617 ng mL^{-1} and 0.573 ng mL^{-1}, respectively. It should be noted that the relationship between the concentrations of norgestrel and progesterone seemed complicated for individuals because nine samples showed a high ratio of norgestrel: progesterone in serum but others showed the ratio reversed.

The reliability of the method was evaluated by comparing the results with those obtained by the RIA method. The results are shown in Fig. 5. The correlation factors (r^2) were 0.9261 and 0.9682, respectively, indicating that the present method is very well correlated with the RIA method. Considering that the advantages of the higher performance of the present method for rapid and simultaneous determination of progesterone and norgestrel concentration in human serum and the avoidance of contamination from the radioactive species in the RIA method, the present method is more suitable for routine clinical analysis.

Conclusion

In this paper, a rapid, selective LC-MS-MS method has been presented for simultaneously quantifying progesterone and norgestrel concentrations in human serum. Validation results have shown that the method is reliable and robust. Also, a fast analysis speed and simultaneous multi-component determination makes proposed method more suitable for high throughput clinical analysis involving large amounts of sample.

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