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Selective and sensitive liquid chromatography–tandem mass spectrometry method for the determination of levonorgestrel in human plasma

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Abstract

A selective, sensitive and rapid liquid chromatography–tandem mass spectrometry method for the determination of levonorgestrel in plasma was developed. An Applied Biosystems API 3000 triple quadrupole mass spectrometer set to multiple reaction monitoring (MRM) mode, using atmospheric pressure photospray ionisation (APPI) in the positive mode. Using 17-α-methyltestosterone as internal standard (IS), liquid–liquid extraction was followed by reversed phase liquid chromatography using a phenyl–hexyl column and tandem mass spectrometric detection. The mean recovery for levonorgestrel and 17-α-methyltestosterone was 99.5 and 62.9%, respectively. The method was validated from 0.265 to 130 ng levonorgestrel/ml plasma with the lower limit of quantification (LLOQ) set at 0.265 ng/ml. This assay method makes use of the increased sensitivity and selectivity of tandem mass spectrometric (MS/MS) detection, allowing for a rapid (extraction and chromatography) and selective method for the determination of levonorgestrel in human plasma. The assay method was used in a pharmacokinetic study to quantify levonorgestrel in human plasma samples generated after administrating a single oral dose of 1.5 mg levonorgestrel to healthy female volunteers for up to five half lives. The total chromatographic runtime of this method was 5.0 min per sample, allowing for analysis of a large number of samples per batch.

Keywords: Levonorgestrel; 17-α-Methyltestosterone; Liquid–liquid extraction; Plasma; Liquid chromatography; Mass spectrometry; Atmospheric pressure photospray ionisation (APPI)

1. Introduction

Levonorgestrel, (17-α)-(+/−)-13-ethyl-17-hydroxy-18, 19-dinorpregn-4-en-20-yn-3-one, is a synthetic female contraceptive hormone used in pregnancy prevention in humans. The monitoring of plasma levels of this drug as well as other oral contraceptives has brought about changes in formulations (lowering the expected effective dose) and thus limiting clinical side effects [1]. Johnston [2] described a conventional HPLC method for the determination of oral contraceptives including levonorgestrel. Berzas et al. [3] evaluated the use of capillary electrophoresis versus an HPLC method. These methods are however not sensitive enough for pharmacokinetic analysis of low doses of levonorgestrel. Radio-immuno assay (RIA) methods have been used for determination of levonorgestrel during pharmacokinetic studies [1,4] reaching low detection levels in the pg/ml range. These methods are sensitive, but are expensive, time consuming, hazardous due to radio active labelling and non-specific. Lauritsen and Rose [8], described a method using desorption chemical ionisation membrane inlet mass spectrometry (DCI-MIMS) for identification of steroid hormones including levonorgestrel. LC–MS/MS is becoming the preferred method for quantitative determinations due to rapid, highly selective and sensitive analysis. LC–MS/MS methods using turbo-electrospray ionisation (ESI) for determination of levonorgestrel in human serum [5], environmental water [6] and sewage effluent [7] have been described, but the APPI source used in our laboratory lower the background noise produced by ESI, enabling us to develop a more sensitive method,

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with high sample throughput due to short chromatographic conditions and simple sample preparation. Kushnir et al. [9] used atmospheric pressure photo-ionisation with tandem mass spectrometry to analyse cortisol and cortisone in serum and plasma.

2. Experimental

2.1. Materials and chemicals

A phenomenex Luna® phenyl–hexyl 5 μm, 2.0 mm × 150 mm reverse phase analytical column (Phenomenex, Torrance, CA, USA) was used for chromatographic separation at a flow-rate of 0.3 ml/min. The mobile phase was delivered by an Agilent Series 1100 isocratic pump and the samples injected by an Agilent Series 1100 autosampler (Agilent, Palo Alto, CA, USA). Detection was performed by an Applied Biosystems API-3000 mass spectrometer (Applied Biosystems, Ontario, Canada) fitted with an atmospheric pressure photospray ionisation (APPI) source operating in the positive ion mode.

Acetonitrile, hexane and methanol (Burdick and Jackson, High Purity) were obtained from Baxter chemicals (USA), formic acid from BDH (England) and was used without further purification. Iso-amyl alcohol (pro analysi) was obtained from Merck (Merck, Germany). Water was purified by Millipore Elix 5 reverse osmosis and Milli-Q® (Millipore) Gradient A10 polishing system (Millipore, Bedford, MA, USA). Levonorgestrel ($C_{21}H_{28}O_2$) and 17-α-methyltestosterone ($C_{20}H_{30}O_2$) were obtained from Orgasynth Industries (Paris, France) and FARMOVS-PAREXEL internal chemical reference library, respectively.

2.2. Preparation of standards and quality control samples

Calibration standards (STD) were prepared by dissolving pure reference standard of levonorgestrel powder in methanol to obtain a stock solution, which was used to spike a pool of blank human plasma (stripped of endogenous components). By serial dilution with blank human plasma (1:1, v/v) a calibration standard range between 0.265 and 130 ng/ml was obtained. Similarly, quality control standards (QC) were prepared (using the same methodology, but different stock solutions) spanning a range between 0.340 and 113 ng/ml. Sufficient calibration standards and quality controls were prepared to validate the method and assay all the study samples. Aliquots of the standards and quality controls were stored in polypropylene tubes together with the study samples at −20 °C until processed.

2.3. Extraction procedure

Plasma samples (1 ml) were pipetted into 10 ml amber ampoules, 100 μl internal standard solution (150 ng 17-α-methyltestosterone/ml water) and 5 ml organic solvent (hexane:iso-amyl alcohol, 98:2, v/v) added. The samples were vortex-mixed for 90 s and centrifuged at 1300 × *g* to aid layer separation of the aqueous and organic solvents. The aqueous phase was frozen in an alcohol freezing bath at −25 °C and the organic layer was decanted into a 5 ml amber ampoule.

The organic solvent was evaporated under a stream of nitrogen at 55 °C until dry. The residue was reconstituted in 150 μl, 2% formic acid solution and vortexed for 45 s. The mixture was transferred to 96 deep-well plates, placed onto the autosampler and injected.

2.4. Liquid chromatography

Chromatography was performed at ambient temperature with a mobile phase consisting of acetonitrile, methanol, and 0.1% formic acid (45:35:20, v/v/v) at a flow-rate of 0.3 ml/min. All chromatographic solvents were degassed by sparging helium through the solution for 4 min.

2.5. Mass spectrometry

Atmospheric pressure photospray ionisation was performed in the positive ion mode with nitrogen as the nebulizing and auxiliary gas both set at an optimal value of 9 (arbitrary values) and the temperature of the heated quartz tube was set at 380 °C. The APPI source settings were obtained after conducting flow injection analysis. Toluene was used as a dopant-liquid and delivered via a post column APPI source interface T-piece at a flow rate of 30 μl/min. A turbo electrospray ionisation (ESI) source was used to optimise the triple quadrupole settings of the instrument for detection of levonorgestrel and 17-α-methyltestosterone by infusing a 500 ng/ml solution of each drug dissolved in a formic acid: methanol (1:99, v/v) solution at a constant flow rate of 10 μl/min. The pause time was set at 5 ms and the dwell time at 150 ms. The collision gas (N_2) was set at 9 (arbitrary value).

The Applied Biosystems API 3000 mass spectrometer was operated at unit resolution in the multiple reaction monitoring (MRM) mode, monitoring the transition of the protonated molecular ions *m*/*z* 313.0 and 303.0 to the product ions *m*/*z* 109.1 and 96.9 for levonorgestrel and 17-α-methyltestosterone, respectively. Fig. 1 shows a full scan mass spectrum of pure levonorgestrel showing the M + 1 precursor ion of protonated levonorgestrel (*m*/*z* 313.0, molecular structure given) overlaid by a full-scan mass spectrum of levonorgestrel after collision, showing the most abundant product ions and the principal product ion at *m*/*z* 109.1. Fig. 2 shows a full scan mass spectrum of pure 17-α-methyltestosterone showing the M + 1 precursor ion of protonated 17-α-methyltestosterone (*m*/*z* 303.0, molecular structure given) overlaid by a full scan mass spectrum of 17-α-methyltestosterone after collision, showing the most abundant product ions and the principal product ion at *m*/*z* 96.9.

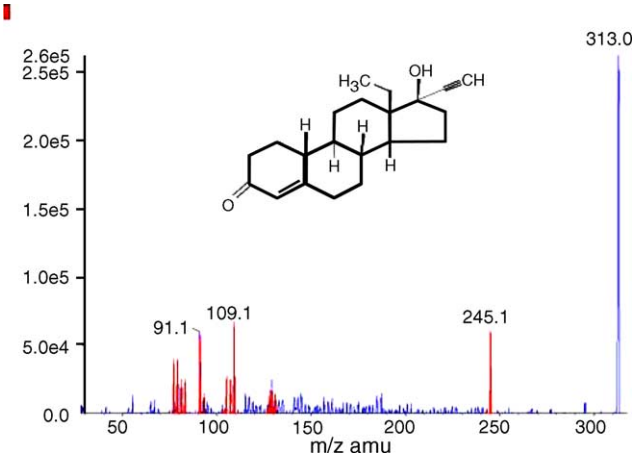

Fig. 1. Full mass spectrum of protonated levonorgestrel showing the (M + 1) ion (*m/z* 313.0, molecular structure given) overlaid by the product ion scan obtained after collision.

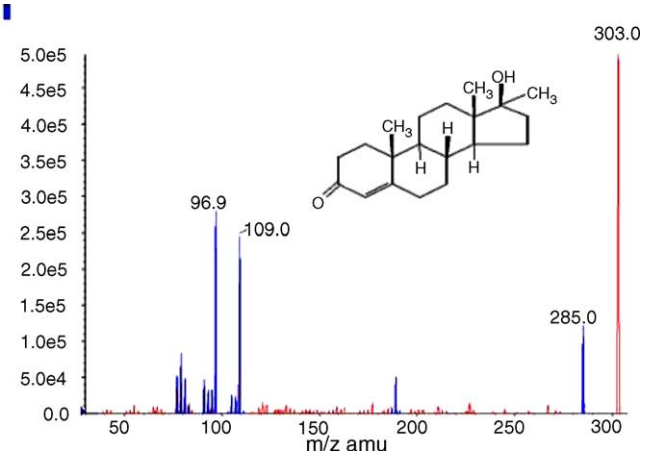

Fig. 2. Full mass spectrum of protonated 17-α-methyltestosterone showing the (M + 1) ion (*m/z* 303.0, molecular structure given) overlaid by the product ion scan obtained after collision.

The instrument was interfaced with a computer running Applied Biosystems Analyst version 1.2 software.

2.6. Validation

The method was validated by analysing quality control samples during three independent validation batches at eight different concentrations of levonorgestrel ranging from 0.340 to 113 ng/ml to determine the accuracy and precision of the method. The quality control values of levonorgestrel were interpolated from a calibration curve containing nine different calibration standards spanning the concentration range of 0.265–130 ng/ml.

A 1/(concentration)^2 linear regression was used to construct the levonorgestrel calibration curve of the drug peak area ratios of the analyte/IS versus nominal drug concentrations.

A system performance verification standard (SPVS) was prepared by extracting drug-free plasma as described previously and reconstituting it with 2% formic acid containing a known concentration of levonorgestrel and internal standard. Absolute recovery of the analyte was determined in triplicate in normal plasma by comparison of the analyte peak areas of the extracted quality control samples with those of the non-extracted system performance verification standard mixtures, representing 100% recovery.

The matrix effect (co-eluting, undetected endogenous matrix compounds that may influence the analyte ionisation) was investigated using the procedure described by Matuszewski et al. [10].

3. Results and discussion

The extraction efficiency is displayed by the mean absolute recovery value of 99.5 and 62.9% for levonorgestrel and 17-α-methyltestosterone, respectively (Table 1 summarises the results). Matrix effect was tested at two different concentrations of levonorgestrel, i.e. 25 and 100 ng/ml. Though matrix effect cannot be excluded as an ion suppression factor, it was observed that for 10 different plasma pools tested it had little to no influence on the reproducibility of the method. The peak area ratio of the 10 reconstituted samples had a coefficient of variation of 6.18 and 6.43% at 25 and 100 ng/ml, respectively. Matrix interference also has little effect on the reproducibility of 17-α-methyltestosterone used as internal standard (coefficient of variation of 6.8%).

The much higher selectivity of MS/MS detection allowed the development of a very specific and rapid method for the determination of levonorgestrel in plasma. The LLOQ, defined as that concentration of levonorgestrel, which can still be determined with acceptable precision (CV% < 20) and accuracy (bias < 20%) was found to be 0.265 ng/ml (concentration of the lowest calibration standard) with a signal to noise ratio of more than 5. Results of the intra-batch and inter-batch validation assays presented in Table 2 indicate

Table 1
Summary of absolute recovery data showing the efficiency of levonorgestrel and 17-α-methyltestosterone extraction from plasma

Curve position	Analyte concentration (ng/ml)	Mean of peak areas		Absolute recovery	R.S.D. (%)
		After extraction	Theoretical value		
High	15.0	875,767	903,557	96.9	7.5
Medium	2.70	163,850	162,640	100.7	10.0
Low	0.68	41,170	40,720	101.1	6.3
IS	150	7,321,667	11,641,432	62.9	7.1

Table 2
Summary of intra- and inter-batch quality control results

	Nominal value							
	113 ng/ml	56.4 ng/ml	28.2 ng/ml	14.1 ng/ml	7.05 ng/ml	1.34 ng/ml	0.668 ng/ml	0.340 ng/ml
Mean	113.13	56.43	28.71	14.23	7.16	1.37	0.69	0.33
%Nom	100.1	100.1	101.8	100.9	101.5	102.5	104.0	96.4
R.S.D. (%)	3.4	3.9	3.4	3.0	3.0	3.9	5.2	7.2
n	18	18	18	17	17	18	18	17

a valid calibration range for determination of levonorgestrel of 0.265–130 ng/ml. The quality control values found during the processing of 10 study sample batches over a period of 10 days are summarised in Table 3 and attest to the excellent inter-day performance of the assay method.

Due to the high specificity of MS/MS detection, no interfering peaks were found when chromatographing blank plasma extracts from six different sources.

To optimise chromatographic conditions different analytic columns, mobile phase solutions and injection solvents were tested. Reverse phase C_8 and C_{18} columns retained the analytes for more than 15 min, even when used with mobile phases containing high volumes of acetonitrile and methanol, making it unpractical for high throughput analysis. A mobile phase of hexane:2-propranol (90:10, v/v) was also tested, but the chromatography was unacceptable. Though the retention on cyano columns was between 4 and 5 min, the chromatographic peak shapes were not satisfactory. A phenyl–hexyl column with mobile phase consisting of acetonitrile:methanol:0.1% formic acid (45:35:20, v/v/v) gave excellent chromatography in acceptable run times (5 min) for high throughput analysis.

During the method development stage turbo-electrospray ionisation (ESI+) was initially used. The sensitivity was not sufficient for the expected plasma concentration level of the late elimination phase of the pharmacokinetic study. Different derivatisation reactions were investigated in order to improve sensitivity, which proved to be unsuccessful. Using the atmospheric pressure photospray ionisation (APPI+) source with toluene as dopant the sensitivity was increased by a factor of 4.

In the APPI source, the mobile phase and dopant solvent (toluene) were evaporated at 380 °C in the heated quartz tube prior to ionisation excitation obtained from a UV lamp. The toluene is easily ionised in this process. The charged toluene molecules then transfer the charge to the analytes to form the positively charged M + 1 molecule, which can be detected by the MS/MS system. The composition of the mobile phase also contributes to the charge transfer during ionisation.

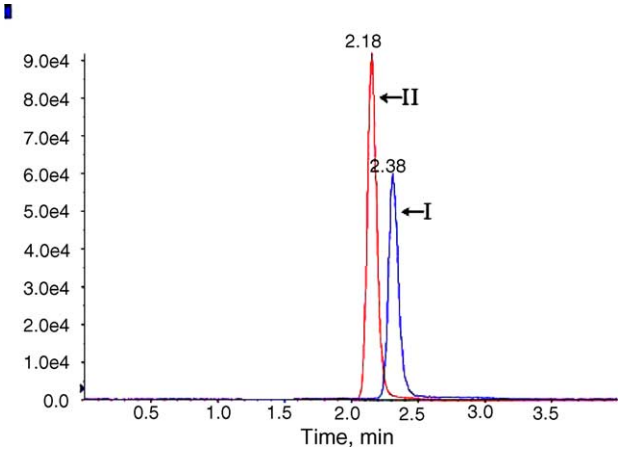

Fig. 3. A representative chromatogram to illustrate the retention times of levonorgestrel (I) and the internal standard, 17-α-methyltestosterone (II).

Various other steroid hormones, including norethistrone, ethinyl estradiol, dehydroisoandosterone and medroxyprogesterone acetate were evaluated as possible internal standards, but 17-α-methyltestosterone compensated best for fluctuations during extraction and ionisation. Hexane, hexane:iso-amyl alcohol (98:2, v/v), pentane and *tert*-butyl-methyl ether were tested during extraction optimisation. Hexane:iso-amyl alcohol (98:2, v/v) gave the best recovery with high reproducibility.

The retention times for levonorgestrel (I) and 17-α-methyltestosterone (II) were 2.38 and 2.18 min, respectively (Fig. 3). The total chromatography run time of 5.0 min made it possible to analyse a large number of samples in a batch. Fig. 4 shows a representative chromatogram obtained of a levonorgestrel calibration standard (I) at a concentration of 0.265 ng/ml in plasma (the LLOQ) and of a study sample

Table 3
Summary of the inter-day quality control results obtained during the processing of 10 batches of levonorgestrel study samples

	Nominal value					
	28.2 ng/ml	14.1 ng/ml	7.05 ng/ml	1.34 ng/ml	0.668 ng/ml	0.340 ng/ml
Mean	29.3	14.6	7.17	1.33	0.655	0.349
%Nom	104.0	103.6	101.7	99.1	98.0	103.0
R.S.D. (%)	5.0	4.4	5.3	5.5	6.6	10.0
n	20	20	20	20	19	19

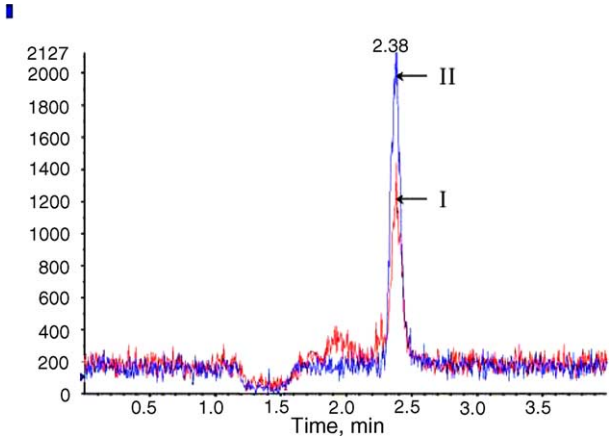

Fig. 4. High-performance liquid chromatograms of the calibration standard at the limit of quantification (I) containing 0.265 ng/ml levonorgestrel and of a study sample (II) at the late elimination phase (96 h after dose) of the pharmacokinetic profile for the analyte.

(II) taken during the late elimination phase (96 h after drug administration) of the pharmacokinetic profile.

This assay method was employed to analyse plasma samples containing levonorgestrel obtained from 18 healthy postmenopause female volunteers after administrating a single oral dose of 1.5 mg levonorgestrel each. Concentration versus time profiles were constructed for up to 120 h after drug administration (Fig. 5).

3.1. Stability

On-instrument stability was inferred from extracted quality control samples cooled to 5 °C on the autosampler while awaiting injection. By regression analysis of the analyte concentration against cumulative time it was deducted that levonorgestrel tends to decrease by only 0.23% over a period of 31 h and considered to be stable on-instrument for this period.

Plasma samples containing known concentrations of levonorgestrel was subjected to three freeze–thaw cycles to ascertain freeze–thaw stability. The levonorgestrel within these samples were stable during this process yielding concentrations of 95.5 and 96.6% from the nominal at 56.4 and 14.1 ng/ml, respectively. Levonorgestrel is stable in solution (methanol) at 4 °C, −20 °C and room temperature for at least 24 h. No degradation occurred after leaving quality control plasma samples on bench top at room temperature over a period of 16 h.

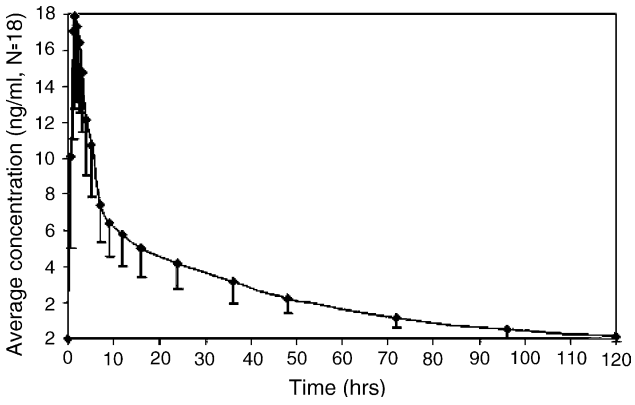

Fig. 5. Levonorgestrel plasma concentrations vs. time profile as obtained after a single 1.5 mg oral dose of levonorgestrel (average of 18 subjects).

Table 4
Matrix stability data showing levonorgestrel to be stable at −20 °C after storage for 267 days

	Nominal concentration	
	2.67 ng/ml	14.1 ng/ml
Mean	2.77	14.29
%Nom	103.7	101.4
R.S.D. (%)	2.28	4.43

Long term matrix stability at −20 °C was assessed over a period of 267 days using two different concentrations of levonorgestrel. Levonorgestrel is stable in plasma when stored at −20 °C in polypropylene tubes for at least 267 days (Table 4).

4. Conclusion

A rapid, sensitive and highly selective method for the determination of levonorgestrel in plasma was developed, using high-performance liquid chromatographic separation with tandem mass spectrometric detection. Atmospheric pressure photospray ionisation (APPI+) improved the sensitivity four times compared to turbo-electrospray ionisation (ESI+). The advantages of using atmospheric pressure photospray ionisation made it possible to use this LC–MS/MS method for analysis of large number of samples with great precision during pharmacokinetic studies. This newly developed assay method was used in a pharmacokinetic study in which 18 healthy post menopause female volunteers were each given a 1.5 mg single oral dose of levonorgestrel. The assay method is more selective than previously described methods (HPLC, TLC and RIA) and allows for a much higher sample throughput due to the short chromatography time (5.0 min) and simple sample preparation. Robust LC–MS/MS instrument performance was observed, with only slight variations in the instrument response within batches. It was not necessary to clean the ion source during the entire study. A single analytical column was used to chromatograph about 1250 extracts and was still in good working condition after the study was completed indicating sufficient sample clean-up. This method is an excellent analytical option for rapid quantification of levonorgestrel in human plasma.

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