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Quantitative determination of norethisterone acetate in human plasma by capillary gas chromatography with mass-selective detection

F. Pommier*, A. Sioufi, J. Godbillon

Laboratoires Ciba-Geigy, Bioanalytics and Pharmacokinetics, BP 308, 92506 Rueil Malmaison Cedex, France

Abstract

An analytical method for the determination of norethisterone acetate (NETA) in human plasma by capillary gas chromatography–mass-selective detection (GC–MS), with testosterone acetate as internal standard, was developed and validated. After addition of the internal standard, the compounds were extracted from plasma at basic pH into diethyl ether–dichloromethane (3:2, v/v), which was then evaporated to dryness. The compounds were converted into their pentafluoropropionyl derivatives which were determined by gas chromatography using a mass selective detector at *m/z* 486 for NETA and *m/z* 476 for the internal standard. Intra-day and inter-day accuracy and precision were found suitable over the range of concentrations between 0.10 to 10 ng/ml. The method was applied to clinical samples.

Keywords: Norethisterone acetate

1. Introduction

Methods have been published to identify [1] and quantify [2–4] steroid esters in biological fluids.

Norethisterone acetate (NETA) is the precursor of NET (norethisterone or norethindrone), the active hormone at the receptor site. After transdermal application, NETA is hydrolysed enzymatically in skin and blood to NET. It is known [5,6] that steroid esters are readily hydrolysed in tissues by esterases.

In our knowledge, no method was published for quantifying norethisterone acetate in biological media. The objective was to develop and validate a sensitive method for the quantitative determination of NETA in human plasma so as to investigate the presence of NETA in plasma after its transdermal application through patches.

2. Experimental

2.1. Chemicals and reagents

The chemical structures of NETA and testosterone acetate, the internal standard are shown in Fig. 1, and were purchased from Sigma (Saint-Quentin-Fallavier, France).

All the chemicals were of analytical grade: diethyl ether, dichloromethane and toluene (Pestipur SDS) were obtained from Solvants Documentation Synthèse (Pépin, France), pentafluoropropionic anhydride (Ref. 77292) from Fluka (Saint-Quentin Fallavier, France).

*Corresponding author.

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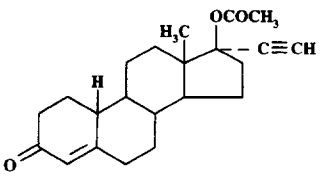
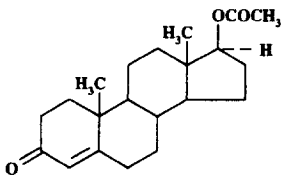


NETA



Testosterone acetate

Fig. 1. Chemical structures of NETA and testosterone acetate, the internal standard.

Buffer (pH 13) was an aqueous solution containing 0.1 mol/l potassium chloride and 0.2 mol/l sodium hydroxide (two ready-to-use ampoules instead of one in the same volume, Titrisol, Merck 9893) (Merck, Darmstadt, Germany).

All the glassware (flasks, glass tubes) was pretreated to prevent adsorption. It was immersed in toluene containing hexamethyldisilazane (Fluka 52619), trimethylchlorosilane (Fluka 92360) and pyridine (Fluka 82704) (1%, v/v, each) for 30 min and rinsed with methanol. The treatment was repeated every month. Between treatments, the glassware was cleaned as usual and rinsed with methanol.

2.2. Standard solutions

The stock solution of NETA was prepared by dissolving 1.02 mg of substance in 10 ml of methanol. Appropriate serial dilutions of the stock solution with methanol were then made in order to prepare the spiking solutions to be used for calibration samples, at concentrations ranging from 0.102–10.2 ng/ml.

Another stock solution of NETA in methanol at the same concentration was prepared from a second weighing and appropriately diluted to give spiking solutions to be used for validation (accuracy and precision assessment) samples.

The I.S. stock solution was prepared by dissolving 1.04 mg of testosterone acetate in 10 ml of methanol. Further dilution of the stock solution with methanol resulted in the internal standard spiking solution (104 ng/ml).

All the solutions were prepared in silanized glass flasks and stored at about +4°C, while not in use, during 1 month.

2.3. Equipment

A Hewlett–Packard 5890 Series II gas chromatograph, equipped with a capillary inlet system and an HP 7673 automatic sampler was used (Hewlett–Packard, Palo Alto, CA, USA). The column was a 12 m×0.2 mm I.D. fused-silica capillary column coated with crosslinked methyl silicone with a film thickness of 0.33 μm (Model HP 19091A, Option 101 supplied by Hewlett–Packard). The carrier gas was helium with an inlet pressure of 48 kPa (7 p.s.i) with a split flow of 50 ml/min and a septum purge of 3.0 ml/min. Sample introduction was performed in the splitless mode at an injection temperature of 250°C with a 2 min splitless-period. The column was initially at 80°C for 0.2 min and the temperature was then raised at a rate of 40°C/min up to 290°C for 3.6 min. A Hewlett–Packard 5970B mass-selective detector (MSD) was interfaced with the gas chromatograph, with the capillary column inserted directly into the ion source.

The MSD was calibrated with the Autotune program at the beginning of each day using perfluorotributylamine (PFTBA). The GC–MSD interface was maintained at 290°C. The detector was turned on from 2–9 min after injection. The selected ions monitored on pentafluoropropionic derivatives were m/z 486 for NETA and m/z 476 for testosterone acetate.

A HP 59940A MS (HP-UX series) ChemStation was used to control GC and injector instruments and for data acquisition and processing.

2.4. Calibration, validation and clinical samples

For calibration and validation, aliquots of working solutions were added to 1 ml of drug-free human plasma to produce reference samples in the range of concentrations 0.102–10.2 ng/ml for NETA. A constant amount of internal standard (2.08 ng/20 μl) was added to each reference sample.

For clinical sample, a constant amount of internal standard (2.08 ng/20 μl) was added to 1 ml of

plasma (obtained from blood collected on solid heparin–lithium and centrifuged).

2.5. Extraction from plasma

To 1 ml plasma in an extraction tube were successively added, an aliquot of the appropriate standard solution (only for calibration and validation), 20 μl of I.S. solution (2.08 ng/20 μl), 1 ml of pH 13 buffer and 5 ml of diethyl ether–dichloromethane (3:2, v/v). The tube was then placed on a rotary mixer for 15 min at a velocity of 300 rpm. Following centrifugation for 10 min at room temperature and about 3000 *g*, the organic layer was transferred into another conical tube. The solvent was evaporated to dryness at room temperature under a stream of nitrogen. The residue was dissolved in 1 ml of diethyl ether, the resulting solution was transferred into a 1 ml reaction vial and evaporated to dryness. 150 μl pentafluoropropionyl anhydride was added to the dry residue. The reaction was run at 30°C in a dry bath for 30 min. The reaction mixture was evaporated to dryness and 30 μl of toluene was added. After vortex mixing, the solution was transferred into an insert introduced in an autosampler vial. A 3 μl aliquot was used for the chromatographic analysis.

3. Results and discussion

3.1. Mass spectra

Electron impact mass spectra of the pentafluoropropionyl derivatives of NETA and testosterone acetate are shown in Fig. 2. Molecular ions were base peaks observed at *m/z* 486 for NETA and *m/z* 476 for testosterone acetate derivatives. These ions were selected for quantitative measurements in the SIM mode.

3.2. Plasma interferences

Representative selected ion current profiles from extracts of drug-free human plasma and of the same plasma spiked with NETA and I.S. are shown in Figs. 3–5. NETA and I.S. derivatives were eluted

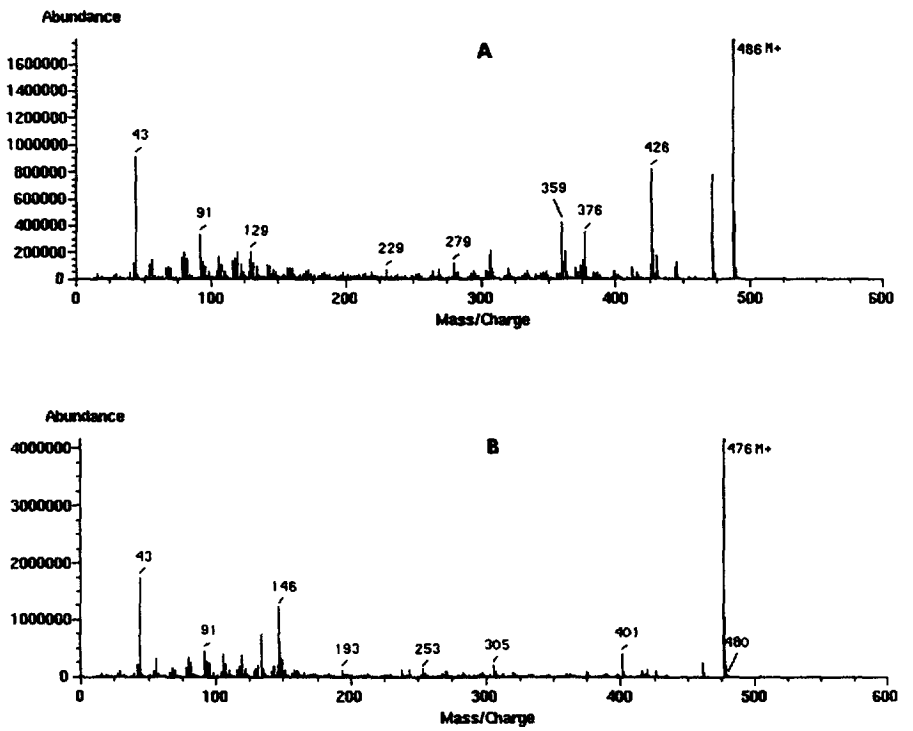


Fig. 2. Electron impact mass spectra of the pentafluoropropionyl derivatives of NETA (A) and testosterone acetate (B).

from the analytical column with retention times of approximately 6.21 min at m/z 486 and 6.03 min at m/z 476, respectively. As shown, the compounds of interest were separated from co-extracted endogenous plasma components. Similar profiles were observed for five different plasma pools from volunteers not given any medication. Two analytical columns demonstrated comparable chromatographic characteristics.

3.3. Calibration curves

Daily calibration standard samples at seven different concentrations in single in the range 0.102 to 10.2 ng/ml were prepared. Calibration curves ($y=mx+b$) were represented by the plots of the peak height ratios (y) of derivatized NETA to derivatized I.S. versus the concentrations (x) of the calibration samples and were generated using weighted ($1/x^2$) linear least-squares regression as the mathematical model [7]. Concentrations in clinical and validation samples were calculated from the resulting peak height ratios and the regression equation of the calibration curve.

The calibration curve data obtained on six days are shown in Table 1. The correlation coefficients were higher than 0.9974. Individual fit of the calibration standards to the curve was assessed from the relative error (RE in %): 100×[(back-calculated concentration from the regression line equation)−(nominal concentration)]/(nominal concentration) [8]. As shown in Table 1, the maximum of mean RE was 5.1% indicating a good fit of the regression model over the range of the calibration curve.

3.4. Accuracy and precision

The accuracy and precision were studied from replicate sets of analyte samples of known concentrations at levels corresponding to the lowest, near the lowest, near the middle and the highest concentration values of the calibration range. Accuracy was determined by calculating the mean recovery for the found concentrations as a percent of the nominal concentrations in standard samples. Precision was assessed from the relative standard deviation (RSD) in % of the mean recoveries. The following validation criteria for accuracy and precision were used to assess the method suitability: mean recoveries should be within 85–115% except at the

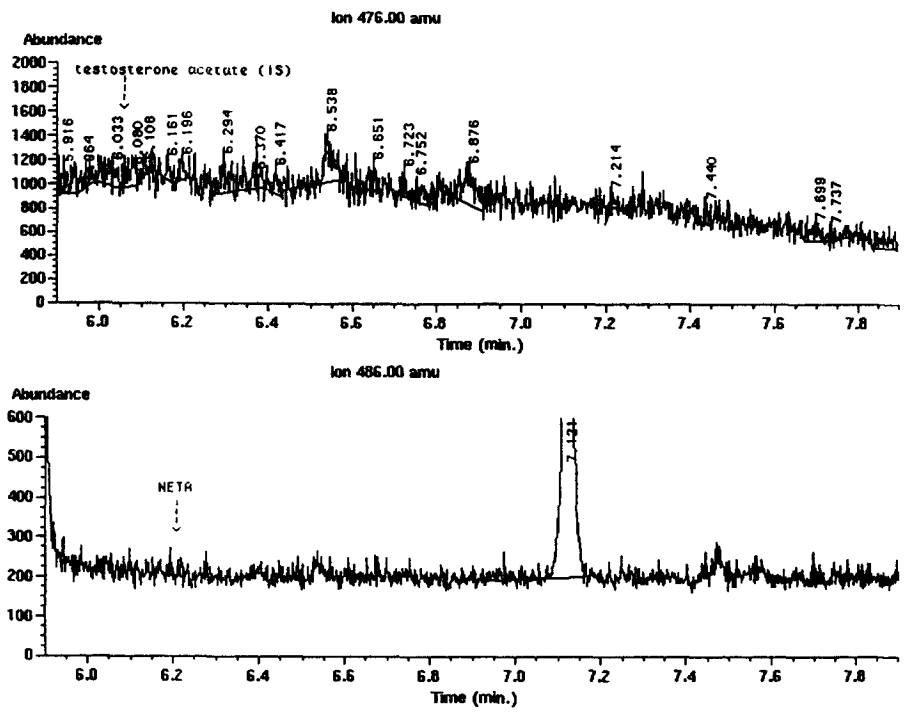


Fig. 3. Examples of selected ion current profiles of an extract of 1 ml drug free human plasma.

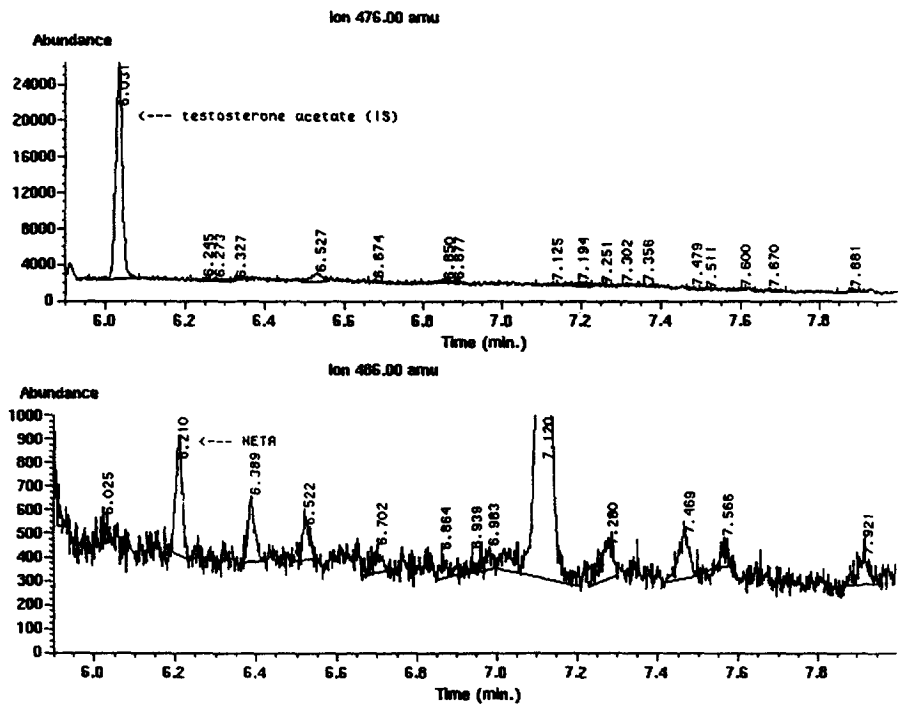


Fig. 4. Examples of selected ion current profiles: an extract of 1 ml plasma spiked with 102 pg of NETA and 2.08 ng of testosterone acetate.

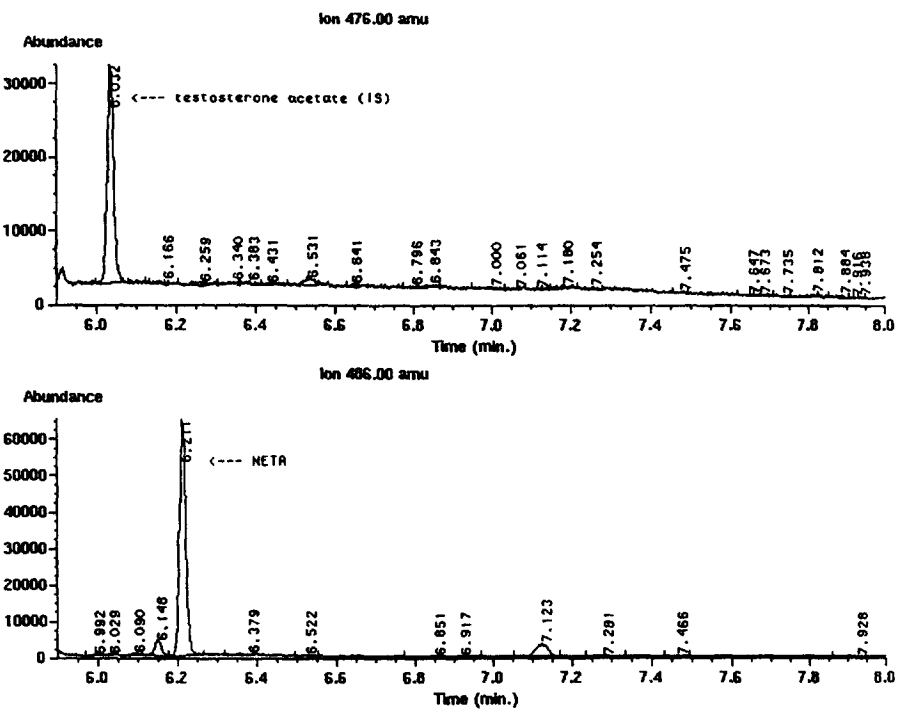


Fig. 5. Examples of selected ion current profiles: an extract of 1 ml plasma spiked with 10.2 ng of NETA and 2.08 ng of testosterone acetate.

Table 1
Statistics, accuracy and precision of calibration curves

Analysis day	Curve Statistics		
	Slope	*y*-Intercept	Correlation coefficient (*r*)
1	0.193	0.0063	0.9978
2	0.190	−0.0009	0.9982
3	0.195	−0.0044	0.9979
4	0.202	−0.0020	0.9974
5	0.227	0.0006	0.9986
6	0.209	0.0001	0.9992
Mean	0.203	0.0000	0.9982
SD	0.014	0.0036	0.0006
RSD (%)[b]	6.8	–	0.06

Accuracy and precision of calibration curves

Analysis day	Concentration (ng/ml)						
	0.102	0.252	0.510	1.02	2.55	5.10	10.2
1	0.102	0.258	0.485	1.12	2.39	5.34	9.79
2	0.106	0.230	0.506	1.03	2.48	5.21	10.8
3	0.102	0.262	0.498	1.01	2.44	4.82	11.3
4	0.103	0.248	0.483	1.11	2.41	5.51	9.75
5	0.099	0.271	0.542	0.993	2.43	5.04	10.1
6	0.103	0.245	0.525	1.00	2.45	5.11	10.7
Mean	0.103	0.252	0.507	1.04	2.43	5.17	10.4
SD	0.002	0.015	0.023	0.06	0.03	0.24	0.6
RSD (%)[b]	2.2	5.7	4.6	5.4	1.3	4.7	6.0
Mean recovery[a] (%)	101	99	99	102	95	101	102
Mean |RE| (%)[c]	1.5	4.5	3.8	4.2	4.3	3.6	5.1

[a] Recovery: found concentration expressed in % of the nominal concentration.
[b] Relative standard deviation.
[c] |RE| (%)=absolute value of RE (%):100× [(back-calculated concentration from the curve)−(nominal concentration)]/(nominal concentration).

limit of quantitation (LOQ) where it should be within 80–120% RSD; should not exceed 15%, except at the LOQ where it should not exceed 20% [9].

3.4.1. Intra-day measurements

Samples were analysed on the same day. Individual, mean recoveries and corresponding RSDs are presented in Table 2. The mean (n=6) recovery ranged from 96–106% over the 0.102–10.2 ng/ml NETA concentration range, with the RSD ranging from 5–13%.

3.4.2. Inter-day measurements

Samples were analysed on six different days over a period of nine days using a daily calibration curve. Individual, mean relative recoveries and RSDs are presented in Table 2. The mean (n=6) recovery ranged from 94–102% and RSD from 4–7% over the 0.102–10.2 ng/ml NETA concentration range.

3.5. Limit of quantitation

The LOQ is defined as the lowest concentration on the standard curve that can be measured with acceptable accuracy, precision and variability. As indicated in the previous section, the mean recovery should be within 80–120% of the expected value with a RSD not exceeding 20%. The lowest concentration value of 0.102 ng/ml, whose accuracy and precision (Table 2) were within the proposed criteria, is quoted as the LOQ.

Table 2
Intra-day and inter-day accuracy and precision for NETA in spiked human plasma samples

Measurements	Given (ng/ml)	Mean found (ng/ml)(n=6)	Accuracy Mean recovery[a] (%)	Precision RSD[b] (%)
Intra-day	0.102	0.103	101	13
	0.918	0.950	103	8
	2.04	1.95	96	8
	4.08	4.02	99	7
	10.2	10.8	106	5
Inter-day	0.102	0.0959	94	7
	0.918	0.872	95	5
	2.04	1.94	95	7
	4.08	4.16	102	4
	10.2	10.4	102	6

[a] Recovery: found concentration expressed in % of the nominal concentration.
[b]RSD: Relative standard deviation on recovery.

3.6. Specificity

NET and 6 metabolites (4 tetrahydro-NET: 3α, 5α-NET, 3α, 5β-NET, 3β, 5β-NET, 3β, 5α-NET and 2 dihydro-NET: 5β-NET, 5α-NET) previously determined [10] were derivatized and injected in SCAN mode under the same conditions as those for NETA. The derivatives presented several peaks with poor resolution. Having shorter retention times, they were clearly separated from the pentafluoropropionyl derivative of NETA. Then, the electron impact mass spectrum showed a molecular ion at m/z 590 for derivatized NET, m/z 592 for the 2 derivatized dihydro-NET and m/z 594 for the 4 derivatized tetrahydro-NET.

No interference from NET and 6 metabolites was observed in SIM mode with the ion selected (m/z 486) for the quantitative measurement of NETA.

3.7. Application

The present method was used to determine the plasma concentrations of NETA achieved after application to four postmenopausal women of one estradiol (E2)/NETA matrix or reservoir patches for 96 h. The patches were designed to deliver about 50 μg E2/day and 250 μg NETA/day.

No detectable NETA plasma concentration was found. This finding confirms that steroid esters are readily hydrolyzed in tissues by esterases [5,6]. NETA is the precursor of NET (norethisterone), the active hormone at the receptor site.

4. Conclusions

The proposed GC–MS technique has been developed and validated for quantifying NETA concentrations in human plasma over the range 0.10–10 ng/ml.

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