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Simultaneous determination of norethisterone and six metabolites in human plasma by capillary gas chromatography with mass-selective detection

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Abstract

A method for the simultaneous determination of norethisterone (NET) and six metabolites in human plasma by capillary gas chromatography–mass-selective detection (GC–MS) is described. The compounds are determined in plasma after enzymatic hydrolysis. After addition of norgestrel as the internal standard, the compounds are extracted from plasma at pH 5 using an Extrelut column and elution with dichloromethane. After evaporation, the compounds are converted into bistrimethylsilyl derivatives which are determined by gas chromatography using a mass-selective detector at m/z 429 for the two dihydro-NET (5β-NET and 5α-NET), m/z 431 for the four tetrahydro-NET (3α,5α-NET, 3α,5β-NET, 3β,5β-NET and 3β,5α-NET), m/z 442 for NET and m/z 456 for the internal standard. The reproducibility and accuracy of the method were found suitable over the range of concentrations between 0.50 and 8 ng/ml for NET, and metabolites except for 5α-dihydro-NET (between 1 and 8 ng/ml). The method was applied to clinical samples.

Keywords: Norethisterone; Dihydronorethisterone; Tetrahydronorethisterone

1. Introduction

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, but further metabolic reactions do not occur at these sites. Thus, after transdermal absorption, NET is the major compound in blood, before being further metabolized by the liver.

Gas chromatographic and mass spectrometric methods in biological fluids have been developed for the determination of NET [1] or NET and metabolites after enzymatic hydrolysis [2,3]. These methods were based on the formation of bistrimethylsilyl derivatives [1,2], or of methoxime-dimethylsilyl derivatives [3]. The limit of quantitation (LOQ) of NET was 0.5 ng/ml with a limit of detection (LOD) estimated at 0.02 ng/ml, but no metabolites were measured with this method [1]. The method described by Braselton et al. [2] for the determination of NET and metabolites used approximative quantitation accomplished by

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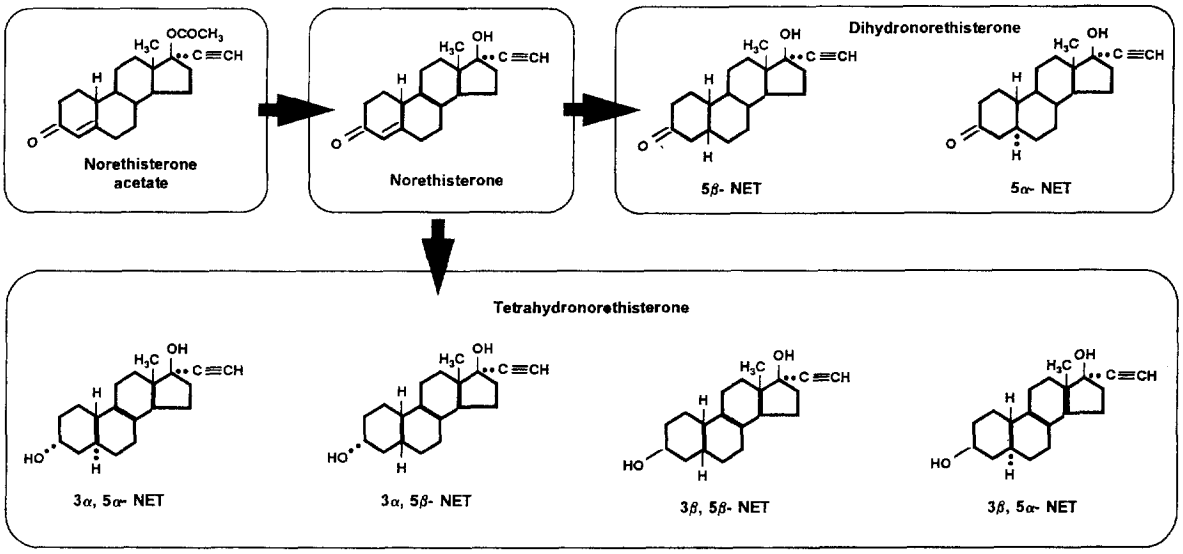

Fig. 1. Chemical structures of NETA, NET and six metabolites.

measurement of peak height and interpolation from a standard curve, without precision and accuracy reported. In the method reported by Rizk et al. [3], the LOQ was estimated to 0.30 ng, but was not related to a volume.

Metabolic profiles of conjugated NET and its metabolites have been determined [4] after administration of oral contraceptives including norethindrone, using laborious methods based on anion and ligand exchange chromatography and gas chromatography–mass spectrometry [5–8]. The amounts of steroids were calculated by comparing peak areas in the samples with peak areas obtained from known amounts.

The present paper describes a validated GC–MS method for the assay of NET and six metabolites (for structures, see Fig. 1) in human plasma, with precise determination of LOQ and LOD. The compounds were determined in plasma after enzymatic hydrolysis.

2. Experimental

2.1. Chemicals and reagents

NET, the internal standard (I.S.) norgestrel and the metabolites (Fig. 1) were supplied by Ciba (Basle, Switzerland). NETA was obtained from Sigma (St. Louis, MO, USA).

All the chemicals were of analytical grade: dichloromethane (Pestipur SDS) was obtained from Solvants Documentation Synthèse (Pépin, France) and N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA), iodotrimethylsilane (TMIS) from Fluka (Buchs, Switzerland). A pH 5 buffer was prepared with 14.8 ml of 0.2 *M* acetic acid solution and 35.2 ml of 0.2 *M* sodium acetate solution, which were diluted up to 100 ml with water. β-glucuronidase enzyme from Helix Pomatia (Sigma) contained 300 000–400 000 β-glucuronidase and 15 000–40 000 sulfatase units/g solid. The pre-packed columns for extraction were Extrelut 3 obtained from Merck (Merck, Darmstadt, Germany).

2.2. 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 cross-linked 5% phenyl methyl silicone with a film thickness of 0.33 μm (Hewlett-Packard 19091B, Option 101). On-column injection was used with an initial pressure of 100 kPa (15 p.s.i.) and a constant flow-rate in the column controlled by an electronic pressure control. The carrier gas was helium with a constant flow-rate of 1.0 ml/min, a linear velocity of 60 cm/s and a split flow-rate of 64 ml/min. The column was initially held at 180°C for 0.5 min and the temperature was raised at a rate of 20°/min up to 270°C and then increased at a rate of 40°/min up to 300°C.

A Hewlett-Packard 5970B Mass Selective Detector was interfaced with the gas chromatograph, with the capillary column directly inserted into the ion source. The GC–MS interface was maintained at 250°C.

A Hewlett-Packard workstation Model 59940A MS (HP-UX series) was used to control the GC, MS and injector instruments and for data handling.

The MS apparatus was calibrated with the Autotune program at the beginning of each day using perfluorotributylamine (PFTBA). The detector was turned on from 4.5 to 6.5 min after injection. The selected ions monitored on bistrimethylsilyl derivatives were m/z 442 for NET, m/z 431 for the four tetrahydro-NET, m/z 429 for the two dihydro-NET and m/z 456 for norgestrel.

2.3. Calibration and validation samples

Aliquots of working solutions prepared in 0.01 mol/l hydrochloric acid were added to 1 ml human plasma to produce reference samples in the range of concentrations 0.5–10 ng/ml. A constant amount of internal standard (10 ng) was added to each reference sample.

2.4. Enzymatic hydrolysis in plasma

Plasma (1 ml) for calibration and validation (as prepared in Sect. 2.3.) or plasma (1 ml) from actual samples, 20 μl (10 ng) of the I.S. solution, 1 ml of acetate buffer (pH 5) and approximately 10 mg β-glucuronidase/sulfatase were introduced into a 10-ml glass tube and incubated for 24 h at 37°C. The extraction procedure was then performed as described below.

2.5. Extraction from plasma

The mixture was transferred to the top of an Extrelut 3 column and allowed to soak for 10 min. The column was eluted twice with 6 ml of dichloromethane. The eluate was evaporated to dryness under a stream of nitrogen. The residue was dissolved in 1 ml of dichloromethane and transferred into a 2-ml conical tube and evaporated to dryness again.

2.6. Derivatization and chromatography

To the dry residue were added 15 μl of 0.2% TMIS in MSTFA. After 10 min at room temperature, the mixture was immediately transferred into a small conical tube and 1.5 μl were injected into the gas chromatograph.

3. Results and discussion

3.1. Mass spectra

Electron impact spectra of the bistrimethylsilyl derivatives are shown in Fig. 2 for norgestrel (I.S.) and NET, in Fig. 3 for a tetrahydro-NET isomer and a dihydro-NET isomer. Molecular ions were base peaks at m/z 442 for NET and m/z 456 for norgestrel. These ions were selected for quantitative measurements in the SIM mode.

Molecular ions were observed at m/z 446 for the four tetrahydro-NET and m/z 444 for the two dihydro-NET but the fragment ion representing the base peaks was at m/z 431 for the four tetrahydro-NET and at m/z 429 for the two dihydro-NET, i.e. at m/z (M − 15). These fragment ions were selected for quantitative measurements in the SIM mode.

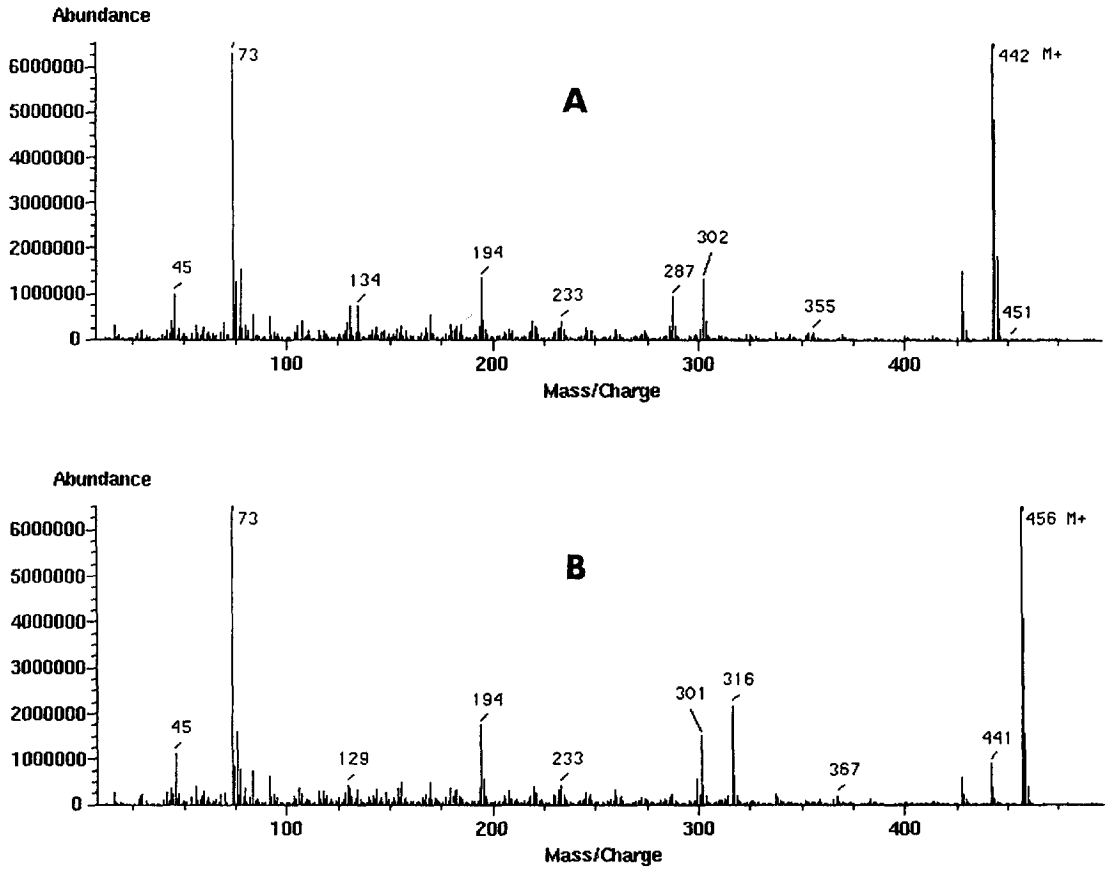

Fig. 2. Electron impact mass spectra of the bistrimethylsilyl derivatives of NET (A) and norgestrel (B).

3.2. Derivatives and chromatography

The bistrimethylsilyl derivatives of NET and the four tetrahydro-NET presented one peak each. Fig. 4 shows the chromatographic separation of the two dihydro-NET metabolites in the SIM mode ($m/z = 429$): the bistrimethylsilyl derivative of each dihydro-NET metabolite produced 2 peaks with different response. These two peaks could be related to the two possible enol ethers of the 3-oxo group. For the 5β-dihydro-NET, the height of the smallest peak (longest t_R) represents about 2/3 of that of the more important peak. For the 5α-dihydro-NET, the height of the smallest peak (shortest t_R) represents about 1/5 of that of the highest peak. Only the highest peak for each dihydro-NET derivative was used for calculations of calibrations and validations.

3.3. Plasma interferences

Typical selected ion current profiles obtained from extracts of drug-free plasma and plasma spiked with NET and six metabolites are shown in Figs. 5 and 6. The extract of drug-free human plasma showed a clean baseline at m/z 431, 442, 456, a plasma component appeared at m/z 429 with a retention time similar to that of the derivative of the 5α-dihydro-NET. Attempts to

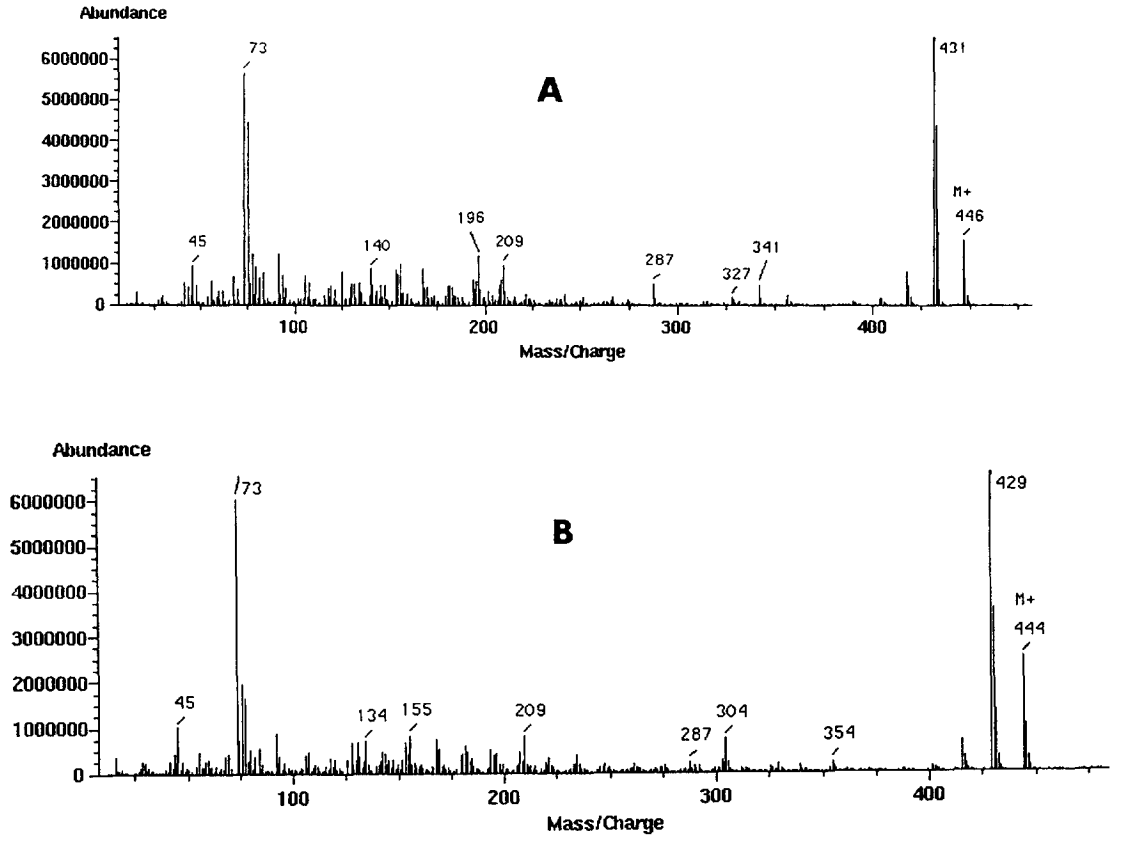

Fig. 3. Electron impact mass spectra of the bistrimethylsilyl derivatives of a tetrahydro-NET isomer (A) and a dihydro-NET isomer (B).

separate this component using different conditions were unsuccessful.

3.4. Calibration curves

The calibration curves were obtained by plotting the peak-height ratio of the derivative of the compound (NET, four tetrahydro-NET and two dihydro-NET) to the internal standard versus the concentration of compound. Their equations were calculated by using weighted linear least-squares regression with a weighting factor of $1/(\text{concentration})^2$. The linear calibration range was 0.5–10 ng/ml for NET and the metabolites. Calibration curves were established on each day of analysis.

3.5. Within-day precision and accuracy

Human plasma samples containing NET, four tetrahydro-NET and two dihydro-NET metabolites at different concentrations (three replicates per concentration) were measured on the same day. The relative standard deviation (R.S.D.) was used as a measure of the precision. The relative difference between found and given (relative error) was a measure of the accuracy. The results obtained are given in Table 1.

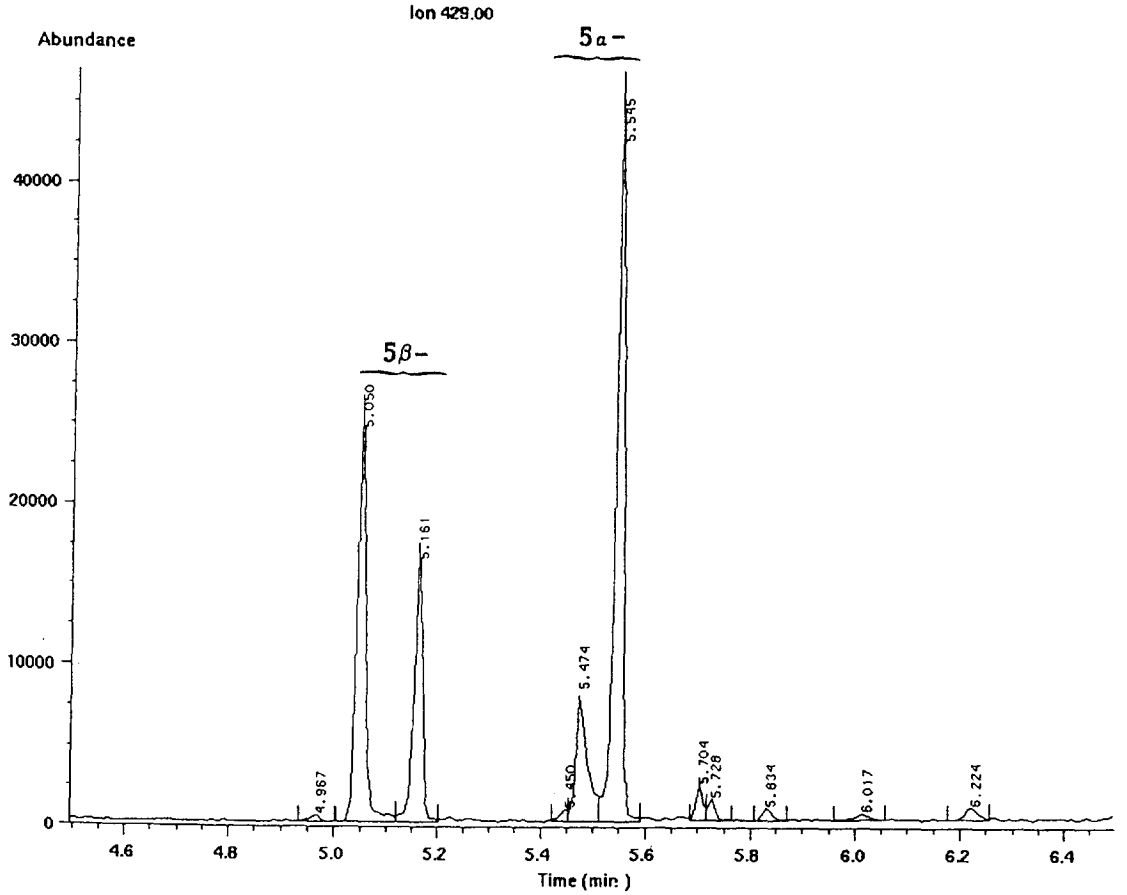

Fig. 4. Example of selected ion current profile of the bistrimethylsilyl derivatives of 5β-dihydro-NET and 5α-dihydro-NET.

3.6. Limits of detection and quantitation

The limit of detection (LOD) was found to be 250 pg/ml for NET and its metabolites, at a signal-to-noise ratio of 5.

The limit of quantitation (LOQ) defined as the lowest concentration measured with a mean recovery between 80 and 120% and a R.S.D. lower than 20% (except for 5α-dihydro-NET) was around 500 pg/ml for NET and its metabolites except for the 5α-dihydro-NET (LOQ = 1 ng/ml) due to the presence of a plasma component at the same retention time. These limits were obtained with a good chromatographic separation of eight compounds.

3.7. Selectivity

NETA was derivatized and injected in SCAN mode under the same conditions as derivatized NET and metabolites. The trimethylsilyl derivative formed with NETA was clearly separated from the silyl derivatives of NET and metabolites. It showed a longer retention time. The electron-impact spectrum showed a molecular ion at m/z 412 and a fragment-ion at m/z 397 (M − 15). Therefore, no interference from NETA was observed in SIM mode with the ions selected for the quantitative measurements of NET, tetrahydro-NET and dihydro-NET (442, 431 and 429, respectively).

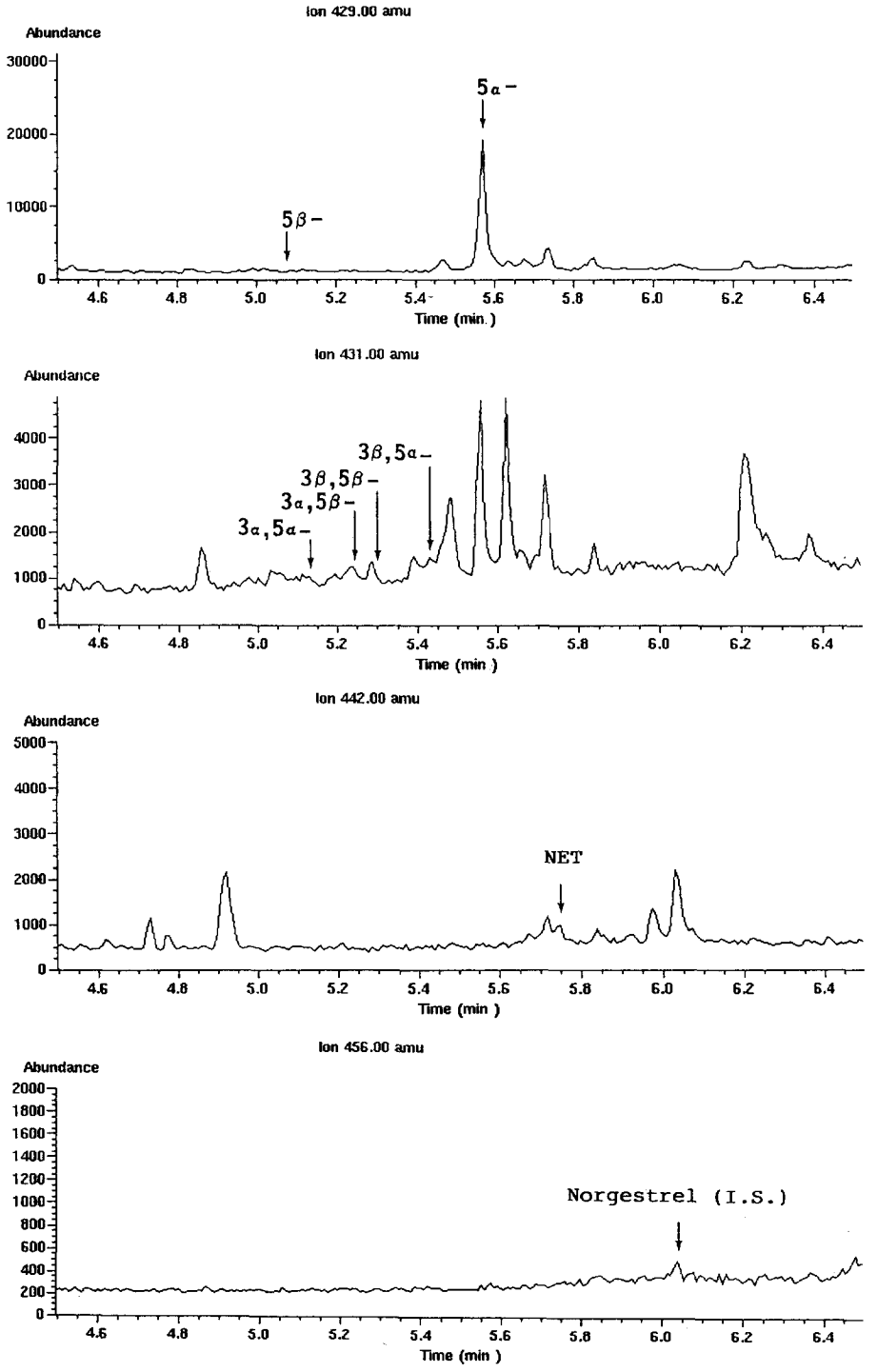

Fig. 5. Example of selected ion current profile of an extract of 1 ml of drug-free human plasma.

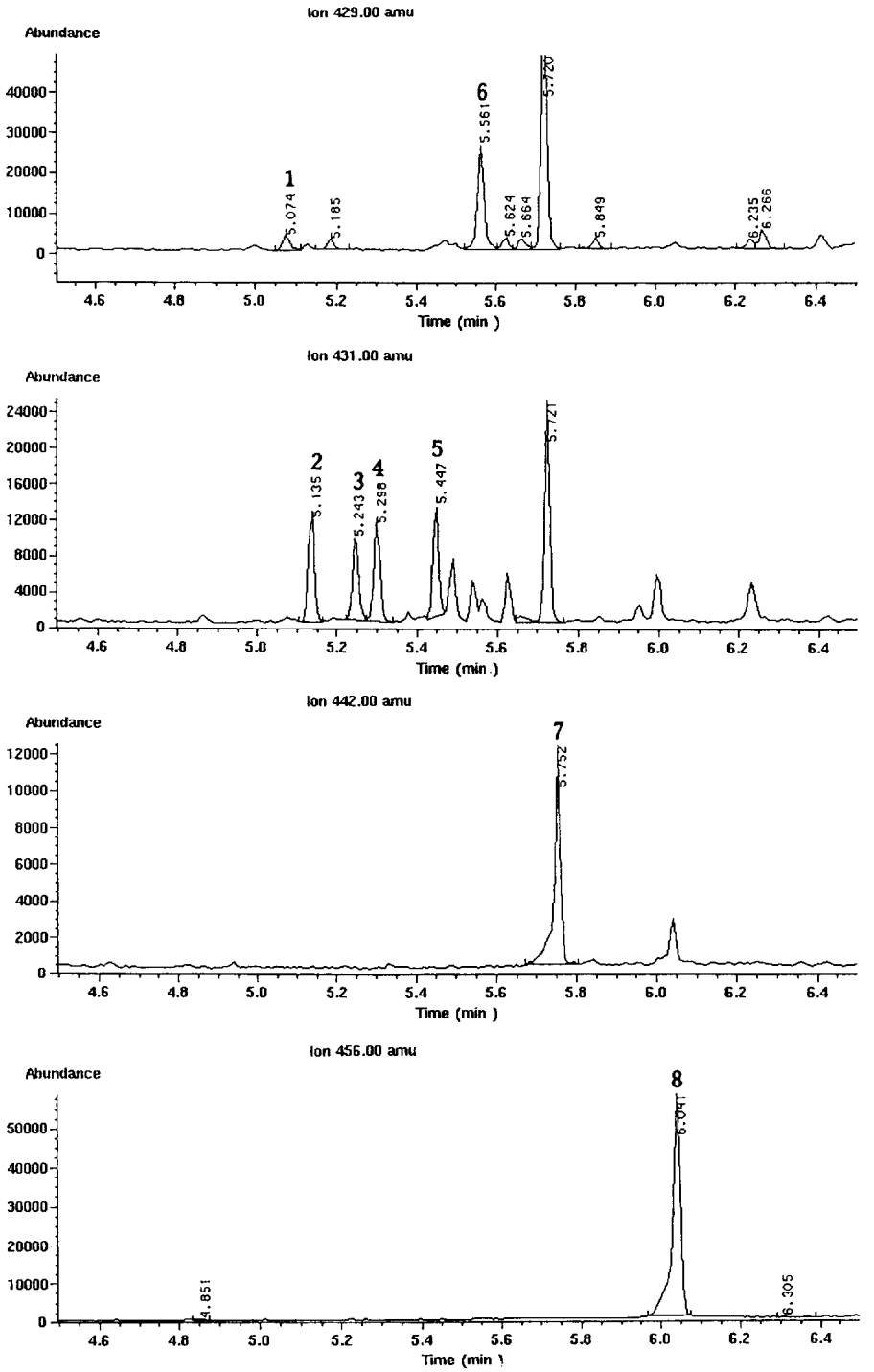

Fig. 6. Example of selected ion current profiles: extract from 1 ml plasma spiked with 1 ng of 5β-dihydro-NET (1), 3α,5α-tetrahydro-NET (2), 3α,5β-tetrahydro-NET (3), 3β,5β-tetrahydro-NET (4), 3β,5α-tetrahydro-NET (5), 5α-dihydro-NET (6), NET (7) and 20 ng norgestrel (8).

Table 1
Within-day precision and accuracy for NET, the tetrahydro-NET and the dihydro-NET in spiked human plasma samples

Compound	Given (ng/ml)	Mean found ($n = 3$) (ng/ml)	R.S.D.[a] (%)	Mean relative error (%)
NET	0.50	0.506	16.5	+1.0
	1.00	1.01	7.2	+1.0
	3.00	3.09	12.6	+3.0
	4.00	4.45	0.80	+11
	8.00	7.84	4.7	−2.1
3α,5α-tetrahydro-NET	0.52	0.522	8.4	+1.0
	1.04	0.977	18.8	+5.9
	3.12	3.59	3.5	+15
	4.16	4.70	8.9	+13
	8.32	8.10	1.7	−2.6
3α,5β-tetrahydro NET	0.50	0.501	18.5	0
	1.00	0.973	15.7	−2.7
	3.00	3.35	4.0	+12
	4.00	4.57	2.9	+14
	8.00	7.71	2.5	−3.7
3β,5β-tetrahydro-NET	0.50	0.485	8.3	−3.3
	1.00	0.976	16.5	−2.4
	3.00	3.51	7.6	+17
	4.00	4.34	4.0	+8.0
	8.00	7.65	1.6	−4.3
3β,5α-tetrahydro-NET	0.52	0.584	7.7	+12
	1.04	1.04	11.2	0
	3.12	3.65	7.9	+17
	4.16	4.80	2.2	+16
	8.32	8.33	6.4	0
5β-dihydro-NET	0.50	0.532	15.5	+7.0
	1.00	0.897	12.1	−1.3
	3.00	3.23	9.2	+8.0
	4.00	4.45	5.9	+11
	8.00	8.05	3.7	+1.0
5α-dihydro-NET	1.00	1.27	4.7	+27
	3.00	3.83	4.7	+28
	4.00	4.80	5.1	+20
	8.00	8.19	4.7	+2.0

[a] R.S.D. = relative standard deviation = (S.D./mean) × 100%.

The hypothetic presence of tetrahydro-NETA and dihydro-NETA would result in trimethylsilyl derivatives (different from those of tetrahydro-NET and dihydro-NET), with molecular mass equal to 416 and 414, respectively. The electron-impact spectra would present molecular ions at m/z 416 and 414, with fragment ions at m/z 401 and 399 (M − 15). All these ions could not interfere with the ions selected for the quantitative measurements of NET and metabolites.

3.8. Application

Plasma concentrations of NET and six metabolites achieved after application to eight postmenopausal women of one estradiol (E2)/NETA matrix Estragest Transdermal Therapeutic System (TTS) for 96 h were determined. The TTS was designed to deliver about 50 μg E2/day and 250 μg NETA/day. Between 8 and 96 h after application of the TTS, the individual NET

concentrations ranged from 250 to 1020 pg/ml. From 24 to 96 h after application, the mean plasma concentrations showed almost a plateau around 400 pg/ml. After TTS removal (96 h), the mean NET concentration dropped down to 92 pg/ml at 102 h (Fig. 7). The samples originating from this study have been previously analysed for NET by an internal radio-immunoassay (RIA) procedure using specific antiserum, without hydrolysis. The mean NET concentration–time profiles determined by GC–MS were quite similar to those previously obtained by RIA (Fig. 7). The plasma concentrations of NET metabolites were all below the limit of detection (250 pg/ml), except for one postmenopausal woman: the concentration of 3α,5β-tetrahydro-NET was 619 to 920 pg/ml between 32 and 98 h after application of the TTS (Table 2).

Concentrations of NET and its metabolites after transdermal administration of NETA were significantly lower than those achieved by oral dosing [2,3].

3.9. Comparison with previous methods

Siekmann et al. [1] described a GC–MS method for NET, reaching the same LOQ as the method we propose which determines NET and 6 metabolites. Braselton et al. [2] and Rizk et al. [3] published a GC–MS method for NET and metabolites. They used 8 or 6 ml of plasma for extraction, instead of 1 ml in our method. Moreover, in the two methods the LOQ was not well defined, and Rizk et al. did not succeed to separate the two dihydro metabolites.

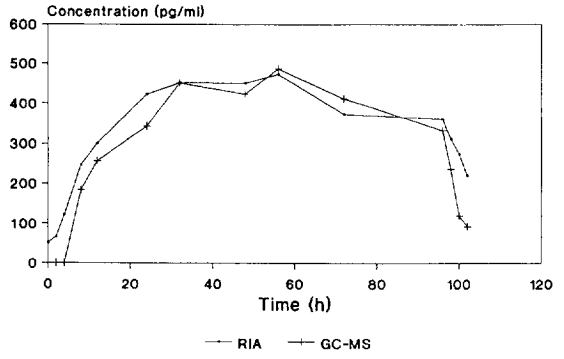

Fig. 7. NET mean plasma concentrations obtained by GC–MS and RIA, from 8 menopausal women which were administered one TTS during 4 days (delivering about 250 μg NETA/day during 4 days).

Table 2
Plasma concentrations (pg/ml) of 3α,5β-tetrahydro-NET obtained by GC–MS for one postmenopausal women after transdermal application of NETA (about 250 μg/day delivered during four days)

Time	Concentration (pg/ml)
Day 0[a]	ND
Day 1[a]	ND
Days 2–6:	
0 h	ND
2 h	ND
4 h	ND
8 h	ND
12 h	ND
24 h	ND
32 h	920
48 h	616
56 h	639
72 h	<LOD
96 h	634
98 h	599
100 h	<LOD
102 h	ND

[a] Blank.
ND = not detected; LOD = limit of detection = 250 pg/ml.

Sahlberg et al. [4] described a complex methodology which limited the studies to a few subjects. In plasma, one sample was obtained every week during 3 weeks as also 24-h urine. In the reported data, mono- and di-sulfate conjugates of NET and metabolites were characterized.

But these findings were obtained using 3 ml of plasma and a very laborious extraction, separation, hydrolysis, solvolysis and derivatization procedures. Our aim was to study the pharmacokinetic profiles of NET and metabolites using a maximum of 1 ml of plasma and several samplings on the same day. The enzyme (β-glucuronidase/sulfatase) used in our laboratory could not cleave the sulfate conjugates. But it should be pointed out that the TTS delivered 250 μg NETA per day and the women studied by Sahlberg et al. [4] received 1 mg NET per day.

The dose differences could explain why in our study almost all metabolites after enzymatic hydrolysis were below detection limit.

4. Conclusions

The proposed GC–MS technique allows the simultaneous determination of NET and six metabolites (four tetrahydro-NET and two dihydro-NET) in plasma, with suitable reproducibility and accuracy.

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