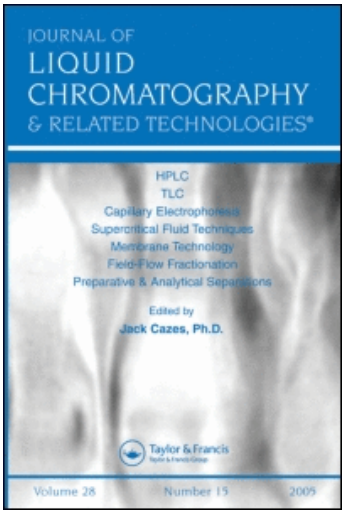

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Development of a Validated Liquid Chromatography Method for the Simultaneous Determination of Ethinyl Estradiol, Cyproterone Acetate, and Norgestrel in Breast Milk Following Solid-Phase Extraction

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Abstract: In the present study, a simple and rapid reversed phase HPLC/diode-array UV spectroscopy method has been developed for the determination of cyproterone acetate, norgestrel, and ethynyl estradiol in human breast milk. The method was based on the use of an isocratic elution system consisting of 0.015 M propane-1-sulfonic acid sodium salt and acetonitrile (60:40 v/v). The analytical column, Hypersil BDS C_{-18} (150 × 4.6 mm) 3 μm, was operating at 25°C with a flow rate 1 mL/min. Good quantitation was obtained in the concentration range of 7.2–115.2 ng/mL for ethynyl estradiol, 4.9–155.6 ng/mL for norgestrel and 4.4–116.2 ng/mL for cyproterone acetate by the use of UV detection at 210, 246, and 284 nm, respectively. The statistical evaluation of the method was examined performing intra- and inter-day validation and was found to be satisfactory, with high accuracy and precision results (R.S.D. < 10.7%). Limits of detection (LOD) and limits of quantitation (LOQ) were 1.0 ng/mL and 4.4 ng/mL for cyproterone acetate, 1.1 ng/mL and 4.9 ng/mL for norgestrel, and 1.8 ng/mL, 7.2 ng/mL for ethynyl estradiol, respectively. The biological fluid (breast milk) was treated using solid phase extraction cartridges, SPE, to remove all endogenous interferences from sample matrix. The solid phase extraction protocol was optimized in terms of

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retention and elution. The mean absolute recoveries were 93.8% for cyproterone acetate, 93.5% for norgestrel, and 99.7% for ethynyl estradiol.

Keywords: Cyproterone acetate, Norgestrel, Ethynyl estradiol, Solid-phase extraction, Breast milk, HPLC

INTRODUCTION

With the increasing popularity of breast feeding, the distribution of drugs and environmental chemicals into human milk has been of clinical interest. Although breast milk is the optimal food for babies, the young breast fed child may be exposed to drugs during maternal drug therapy. It is important to know the extent of drug transfer into human breast milk in order to assess the likely 'dose' received by the infant during the lactation period.

Cyproterone acetate (CYP) because of its antiandrogenic and antigonadotropic properties is used for the control of libido in severe hypersexuality or sexual deviation in adult males. It is also used in males for the palliative treatment of prostatic carcinoma, and may be prescribed jointly with 17α-ethynylestradiol (ETS) in females for the control of severe acne and idiopathic hirsutism.[1,2]

Ethynyl estradiol has been shown to be safe and effective for oral contraceptive use in combination with D(−)-norgestrel (NOR) or cyproterone acetate.[3,4]

The pharmacokinetic properties of CYP, NOR, and ETS in patients, and in healthy volunteers, have been described previously.[5−7] However, nothing is known about the excretion of these substances into breast milk. Generally, it has been recommended that ETS combined with CYP or NOR are to be avoided in breast feeding because their safety has not been established. Therefore, it is essential to have reliable information regarding ETS, CYP, and NOR transfer into breast milk.

There are a number of reports on the analysis of these substances in human plasma or urine and other matrices, using different extraction and determination methods.[8−14]

To our knowledge, no assay for measuring ETS, CYP, and NOR in human breast milk has been reported. The aim of this work was to develop a simple and sensitive HPLC method for measuring these female sex hormones in breast milk; moreover, that which could be used to characterize the excretion of ETS, CYP, and NOR in human milk.

EXPERIMENTAL

Chemicals

Pure standards, D(−)-norgestrel, ciproterone acetate, and 17α-ethynyl estradiol were purchased as crystalline powders from Sigma (St. Louis, MO, USA).

Stock standard solutions for each of the analytes were prepared in methanol and stored in the dark at 4°C.

Acetonitrile (ACN), methanol, and water of HPLC grade were purchased from Merck company (Darmstadt, Germany). Different buffers were used for various chromatographic systems development. These buffers consisting of citric acid monohydrate, tri-sodium citrate-2-hydrate, sodium dihydrogen phosphate monohydrate, sodium acetate trihydrate and diluents 10% v/v of ortho-phosphoric 85%, sulfuric acid 95–97%, acetic acid 100%, and ammonia solution 25% were also obtained from Merck (Darmstadt, Germany). Propane-1-sulfonic acid sodium salt, 1-pentanesulfonic acid sodium salt, and tetraethyl-ammonium hydrogen sulfate were obtained from Sigma Aldrich (Taufkirchen, Germany).

Drug free breast milk samples for assay development were obtained from non smoking volunteer nursing mothers and used for standard curves and validation study. These samples were aliquoted (5 mL) into polypropylene tubes and stored at −20°C.

Analytical Conditions

The apparatus used for HPLC analysis consisted of the following LC-10Avp Shimadzu series: two Model LC-10ADvp pumps for gradient, a Model SCL-10Avp controller, a Model CTO-10ACvp programmable column oven, and a Model SIL-10ADvp programmable auto-sampler with the volume injection set to 100 μL. Detection was via a Model LPD-M10Avp UV/diode array detector operated at 210 nm for ETS, 246 nm, for NOR, and 284 nm for CYP. The chromatographic peaks were recorded by an HP DeskJet 940 c series printer and elaborated automatically by employing a computerized Shimadzu program "CLASS-VP". Separation was achieved on a thermostat at 25°C Hypersil BDS C_{-18} column (150 × 4.6 mm) 3 μm particle size, end capped to minimize unreacted silanol effects. The isocratic elution system consisted of an aqueous 0.015 M propane-1-sulfonic acid sodium salt and acetonitrile (60:40, v/v). The flow rate was 1 mL/min, whereas the mobile phase was degassed by filtering through a Millipore HV 0.45 μm pore membrane filter. Identification of the peaks was accomplished at the convenient wavelength by using diode-array detection.

For sample preparation, a Genie™ K-550-GE vortex mixer, a Hettich EBA22 centrifuge, and a Pierce Reacti-Therm™ heating/stirring module were used.

Standards

The stock methanolic solutions of ETS, NOR, and CYP were prepared every week. These solutions were diluted daily to separately obtain two series of

solutions, in mobile phase and acetone. The first series was used in the concentration range of 0.144–2.304 μg/mL for ETS, 0.088–2.330 μg/mL for CYP, and 0.097–3.110 μg/mL for NOR to obtain the external calibration curve (calibration standards), and the second one to obtain the spiked milk samples (validation standards). Milk standards were freshly prepared for each analytical run by diluting 1 mL of each working standard with 5 mL of blank milk to produce concentrations in the range of 7.2–115.2 ng/mL for ETS, 4.4–116.2 for CYP, and 4.9–155.6 ng/mL for NOR. Milk quality control (QC) concentrations (low, medium and high) were prepared separately to contain 7.2, 43.2, 115.2 ng/mL for ETS, 4.9, 58.3, 155.6 ng/mL for NOR, and 4.4, 43.7, 116.2 ng/mL for CYP, respectively.

The working solutions and sample extracts are stable for at least 24 h.

Sample Preparation

Working standard solutions (1.0 mL volume) in acetone of concentrations ranging from 36–576 ng/mL for ETS, 22–581.8 ng/mL for CYP, and 24.3–778 ng/mL for NOR were evaporated to dryness, each in a 15 mL glass centrifuge tube under nitrogen. Then, 5 mL of human milk and 5 mL of acetone were added into the tubes, vortexed briefly for 30 s, centrifuged at 5000 rpm for 15 min, left at −20°C for 3 hrs to precipitate the proteins, and evaporated gently for 10 min. The final two steps were repeated three times. After the final centrifugation, supernatant layers were evaporated to remove acetone, filtered through 0.45 μm glass fiber filters, and applied to the preconditioned cartridges (HyperSEP-C_{18} 500 mg, 3 mL) for extraction.

Conditioning of the cartridges was performed with 7 mL of acetonitrile, 5 mL of methanol, and 5 mL of LC-grade water. After loading of the sample and subsequent washing with 2 mL methanol 20%, the cartridges were dried and eluted with 2.0 mL of dichloromethane-isopropanol 9:1. The so-obtained extracts were then blown down to dryness under a stream of nitrogen, and reconstituted in mobile phase to a final volume of 250 μL for further HPLC analysis.

Validation

The linearity of an analytical method is its ability within a definite range to obtain results directly proportional to the concentrations of the analyte in the sample. Consequently, for all series, a regression line was fitted on the introduced concentrations as a function of the estimated peaks area by applying the linear regression model based on the least squares method.

To determine recovery from milk of ETS, NOR, and CYP at five different concentrations, an identical set of reference standards, which have not been subjected to sample extraction, were analyzed. Absolute recovery was

measured by comparing the mean chromatographic peak area response of each analyte with the peak area response to an equivalent amount of reference standard:

$$\text{Absolute Recovery } (\%) = \frac{\text{response of extracted analyte}}{\text{response of reference standard}} \times 100$$

The overall recovery of the drug was calculated by plotting a standard calibration graph of the "added" versus the "found" concentrations in milk samples, which gave a linear relationship. The "found" concentrations of ETS, NOR, and CYP have been calculated by using their "validation standards" curve. Therefore, the slopes of these regression lines were used as estimates of the overall recovery.[15]

The precision of the method based on within-day repeatability was assessed by replicate injections ($n = 5$) of three standard solutions, covering different levels (QC concentrations). Statistical evaluation revealed relative standard deviations, at these different concentration values. The reproducibility (day-to-day variation) of the method was established using the same concentration range as above. A triplicate determination of each concentration was conducted during routine operation of the system over a period of 5 consecutive days.[16]

The limit of quantification (LOQ) was determined as the lowest concentration measured with R.S.D. $<15\%$, while the limit of detection (LOD) was calculated as the concentration with a signal to noise ratio of 3.

RESULTS AND DISCUSSION

Optimization of the LC Conditions

The optimization of the LC conditions was carried out by the study of the influence of two organic solvents (acetonitrile, methanol) with different aqueous buffer solutions on the retention time (in terms of capacity factor, k'), and peaks resolution (in terms of resolution factor, R_s) of the determinants.

The aim of the selection of the appropriate aqueous buffer solution was to obtain a sufficient retention of the peaks corresponding to CYP, ETS, and NOR, and also to reach a good peak symmetry as closely as possible (in terms of symmetry factor, A_s).

These parameters have to be optimized in order to improve the selectivity of the bioanalytical method. The optimization step was performed by directly injecting solutions of each substance, separately, on the analytical column using different mobile phases at a flow rate of 1 mL/min.

Better results with respect to peak symmetry in reasonable retention times, were obtained with acetonitrile. Methanol $<50\%$ gives high k' values for the three active ingredients and produce peak broadening and tailing,

especially for NOR and CYP. At the same time, the presence of this solvent in quantity $>50\%$ decreases, seriously, the R_f factor among the three substances.

Changes in percentage of acetonitrile against water in the mobile phase were found to have a profound influence on the retention time and in peak shape of the chromatographic compounds. With a higher percentage of acetonitrile the k' decreased (Fig. 1) and the peak shape improved. However, as seen in Table 1, when using a LC mobile phase consisting of water-acetonitrile (50:50 v/v), the presence of different buffers in concentration of 0.015 M at pH value 4.55, play an important role in improving the asymmetry factor.

Optimum results were achieved with a mobile phase containing 40% acetonitrile and a 0.015 M propane-1-sulfonic acid sodium salt buffer solution adjusted to pH 4.55 with dilute sulfuric acid.

Ionization of the drug enhances the elution ability of mobile phase at low pH value for bases and at high pH value for acidic compounds. Owing to the individual characteristics of the compounds herein analyzed, the effect of the pH variable has been studied.

Studies by using the above mobile phase have proven that changes of pH values between 3 and 7 does not dramatically affect the retention time (decreases <1 min) of CYP, ETS, and NOR. Moreover, the addition of sulfuric acid, in this mobile phase, is not considered important.

Using, as most convenient, a mobile phase consisting of 30% acetonitrile-70% 0.015 M propane-1-sulfonic acid sodium salt, the influence of the samples reconstituted diluent on peak asymmetry factor was investigated. Four different solvents 100% methanol, 100% acetonitrile, 100% water, and "mobile phase" were assessed as the samples diluents. The first two diluents produced significant peak broadening and made the analysis difficult. On the contrary, the two other diluents gave about the same $A_s \approx 1$ value, but the most appropriate reconstituted solvent is the "mobile phase," because it increases the sensitivity of the system, producing greater chromatographic peaks areas.

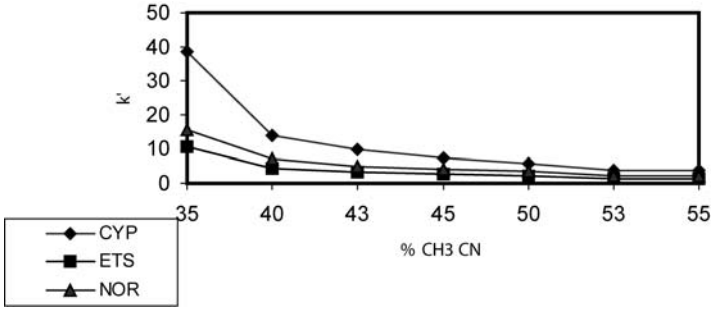

Figure 1. The relationship between the percent acetonitrile concentration in the mobile phase and the capacity ratio k' of CYP, ETS, and NOR.

Table 1. Effect of different buffer solutions on the symmetry and on the capacity factor of the peaks of ETS, NOR and CYP

Composition of 50% buffer solution, 0.015 M	ETS		NOR		CYP	
	k'	A_s	k'	A_s	k'	A_s
Sodium dihydrogen phosphate monohydrate/ortho-phosphoric acid	1.43	1.70	2.49	0.95	4.14	1.44
Sodium acetate trihydrate/acetic acid	1.34	1.15	2.21	1.09	3.69	1.40
Tri-sodium citrate-2-hydrate/citric acid monohydrate	1.38	1.67	2.11	1.19	4.04	1.17
Tetraethylammonium hydrogen sulfate/ammonia solution	1.70	1.05	2.79	1.11	5.06	1.14
Propan-1-sulfonic acid sodium salt/sulfuric acid solution	1.75	1.00	3.26	1.07	5.82	1.05
1-Pentanesulfonic acid sodium salt/sulfuric acid solution	1.77	1.17	2.58	1.19	4.59	1.07
Water for HPLC	1.77	0.91	3.41	0.76	5.74	0.68

Column: Hypersil BDS C_{-18} column (150 × 4.6 mm) 3 μm particle size, mobile phase: buffer solution-acetonitrile 50:50 v/v at pH 4.55, flow rate: 1 mL/min, injection: 100 μL, UV detection at 210 nm, temperature: 25°C.

The above reconstituted diluents do not generally affect the retention time of ETS $t_R = 8.35$ min, NOR $t_R = 12.27$ min, and CYP $t_R = 22.62$ min, which remain, in all the cases, about the same.

Moreover, the above study can be used as good guidance for the simultaneous or separate determination of ETS, NOR, and CYP in different matrixes, by using high performance liquid chromatography.

Solid Phase Extraction Procedure

A number of sample clean up techniques, such as ultrafiltration or dialysis,[17,18] protein precipitation,[19] liquid–liquid extraction,[20,21] solid–phase extraction (SPE),[22] and immunoaffinity extraction,[23] have been reported for use with milk prior to liquid chromatographic separation.

SPE is widely used for biological sample preparation before analysis. Compared to the liquid–liquid extraction, SPE is faster and easier to perform, less solvent is used, and provides clean extracts and high recoveries.

Milk is a complex biological matrix that contains nearly as many different components as plasma.[24] When milk samples were applied directly to the preconditioned cartridges, the SPE cartridges were blocked due to the high and variable lipid and protein content of milk.

Besides, the choice for HPLC analysis of a low UV wavelength value 210 nm, with regard to the increase in the sensitivity of both analytes,

makes the purification problem more demanding. The chromatographic peak corresponding to 5 mL milks ingredients, at 210 nm, is huge and covers the ETS peak. This problem can be overcome by using a good clean up procedure, because the more effective the extraction procedure is; the absorption of milk becomes smaller.

Hence, fat globule destruction and a protein precipitation step prior to SPE was applied to the milk sample clean up procedure. One way to achieve this, according to the Laganan and et al. investigation,[22] is by addition of a water miscible solvent to the milk. In this case, the precipitation was accomplished by using acetone (1:1 v/v) because it is more effective and easier to remove than acetonitrile. It was also observed that after the addition of acetone, low temperatures, $-20^{\circ}C$, reduce the molecular mobility and leads to the formation of huge proteins and lipids flocculation, helping their precipitation.

The washing of the cartridge with 2.0 mL of 20% methanolic solution, during the SPE procedure, was considered necessary to remove adequate ingredients of milk without losing ETS, NOR, and CYP substances. The substances were retained on the sorbent and subsequently eluted by passing 2.0 mL dichloromethane-isopropanole 9:1.

Three different elution solvents (dichloromethane-isopropanole 9:1, dichloromethane and acetonitrile) and three solid phase extraction cartridges (C_{18}, C_8 and phenyl) were investigated for the milk sample clean up procedure (Figs. 2, 3).

The choice of a suitable cartridge and elution solvent for the SPE procedure was a compromise between high recovery results of the analytes (Table 2) and clean extracts (Figs. 2, 3). According to Figure 2, Table 2, dichloromethane needs the presence of a more eluting, straight solvent such as isopropanole, in order to produce the desired result. On the other hand, phenyl cartridges (Fig. 3, Table 2) give the cleanest extract but have the disadvantage of giving lower analyte recovery. Moreover, optimum SPE conditions include C_{18} (500 mg, 3 mL) cartridges eluted by passing 2.0 mL of dichloromethane-isopropanole 9:1.

The influence of different sample pH values, before its application to C_{18} (500 mg, 3 mL) cartridges, does not increase the retention of the analytes to the sorbent and, subsequently, is not considered important.

It is usually desirable to use an internal standard when performing HPLC quantitation. An appropriate internal standard can be controlled for extraction and HPLC injection variability. This method did not utilize an internal standard, as it was used as a simple solid phase extraction step, which is not suspected to induce a large variability in the extraction recoveries. In addition, the use of an auto sampler improves the injection volume precision and provides low HPLC injection variability.[25] Intra- and inter-day coefficients of variation for this method (<10.7) showed that this procedure was acceptable without an internal standard.

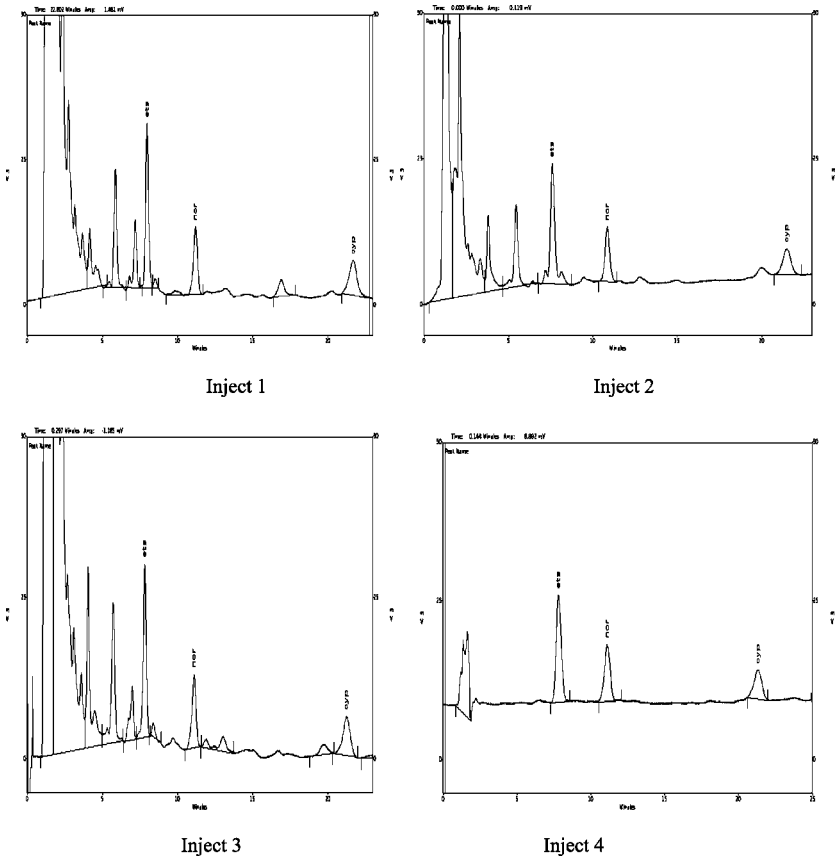

Figure 2. Chromatograms of SPE extraction of human milk spiked with ETS (460.8 ng/mL), NOR (622, 1 ng/mL), CYP (465.9 ng/mL) and eluted with dichloromethane-isopropanole 9:1 ("Inject 1"); dichloromethane ("Inject 2"); acetonitrile ("Inject 3"); pure standard containing the same concentrations of ETS, NOR, and CYP ("Inject 4"). Column: Hypersil BDS C_{-18} column (150 × 4.6 mm) 3 μm particle size, mobile phase: 0.015 M Propane-1-sulfonic acid solution-acetonitrile 60:40 v/v, flow rate: 1 mL/min, injection: 100 μL, UV detection 210 nm, temperature: 25°C.

Linearity

Calibration and validation curves were independently prepared for pure standards of ETS, NOR, and CYP, and human milk samples, which were spiked with the same standards, respectively. Table 3 shows calibration curves, calibration ranges, and detection limits obtained with pure and spiked standards in breast milk. Correlation coefficients in a pure standard curve ranged from 0.9997–0.9999, and in breast milk from 0.9986–0.9995. The intercept with the y-axis was not significantly different from zero.

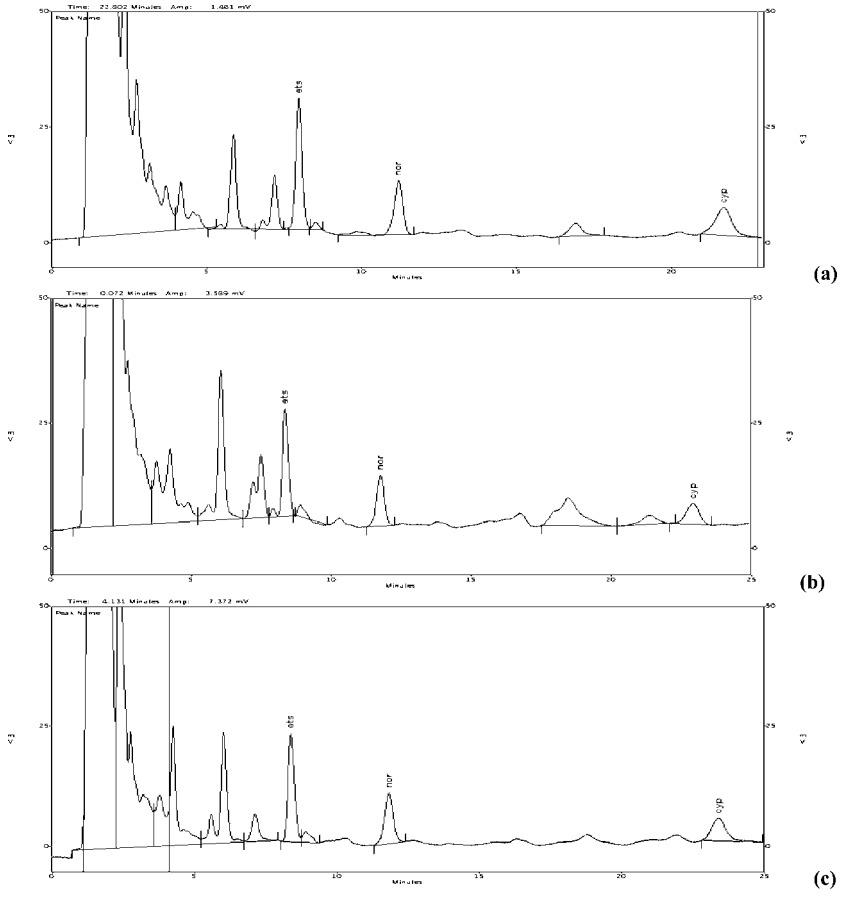

Figure 3. Representative chromatograms obtained from different SPE cartridge (a) C_{18}, (b) C_8 and (c) phenyl.

Moreover, the peak areas obtained for spiked breast milk samples were statistically compared with the peak areas of calibration standards (paired-*t*-test), and there was no significant difference between either ETS, NOR, or CYP areas of spiked milk and calibration standards. ($p \gg 0.01$).

Recoveries

The mean absolute recoveries for ETS, NOR, and CYP in milk samples at five different concentrations are presented in Table 4. The overall recovery was calculated by the linear relationship $y = 0.995\chi + 0.003$ ($r = 0.998$) for ETS, $y = 1.035\chi - 0.082$ ($r = 0.996$) for NOR, and $y = 0.999\chi + 0.001$ ($r = 0.999$) for CYP. The slopes of these regression lines were used as

Table 2. % Recovery of ETS 465.9 ng/mL, NOR 622.1 ng/mL and CYP 465.9 ng/mL by using different cartridges and eluting solvents

	Recovery (%) ($n = 3$)		
	ETS	NOR	CYP
Cartridges			
C18	99.4	89.5	101.4
C8	80.1	82.2	78.4
Phenyl	84.5	91.3	99.0
Elution solvents			
Dichloromethane	98.8	73.9	88.6
Dichlorometh.-isoprop. 9:1	99.4	89.5	101.4
Acetonitrile	98.7	91.4	101.7

estimates of the overall recovery for ETS, 99.5/%, for NOR, 103.5%, and CYP 99.9%.[15]

Accuracy and Precision

These data were determined by intra- and inter-day assay variance in Table 5. Considering the regulatory requirements,[26,27] the precision of the above

Table 3. Regression analysis data of ETS, NOR and CYP in "calibration" and "validation" standards curve

Parameter	ETS	NOR	CYP
Calibration curve			
Concentration range[a]	0.29–2.30	0.39–3.11	0.29–2.33
Slope	163993 ± 3122	74392 ± 1562	76160 ± 3455
Intercept	5636 ± 4126	2402 ± 2756	5671 ± 4567
r^2	0.9999	0.9999	0.9997
Validation curve			
Concentration range[b]	7.2–115.2	4.9–155.6	4.4–116.2
LOD (ng/mL milk)	1.8	1.1	1.0
LOQ (ng/mL milk)	7.2	4.9	4.4
Slope	164722 ± 31570	66689 ± 5931	54748 ± 5460
Intercept	5176 ± 28706	1274 ± 10467	25174 ± 8029
r^2	0.9986	0.9988	0.9995

[a]μg/mL Mob. phase.
[b]ng/mL milk.

Table 4. Absolute recovery of ETS, NOR and CYP in human milk

ETS		NOR		CYP	
Concentration spiked (ng/mL) $n = 3$	Absolute recovery (mean ± R.S.D) (%)	Concentration spiked (ng/mL) $n = 3$	Absolute recovery (mean ± R.S.D) (%)	Concentration spiked (ng/mL) $n = 3$	Absolute recovery (mean ± R.S.D) (%)
7.2	95.8 ± 11.8	4.9	82.3 ± 12.12	4.4	88.7 ± 9.2
28.8	102.0 ± 8.3	38.9	92.4 ± 7.6	29.1	88.7 ± 8.9
43.2	99.3 ± 6.3	58.3	100.8 ± 6.5	43.7	95.4 ± 5.1
72.0	101.1 ± 5.7	97.2	98.9 ± 8.5	72.8	100.3 ± 6.8
115.2	100.1 ± 1.5	155.6	92.8 ± 7.2	116.2	96.0 ± 8.6
Mean	99.7 ± 6.7	Mean	93.5 ± 8.4	Mean	93.8 ± 7.7

Table 5. Estimated intra-day and inter-day relative standard deviations (R.S.D) and accuracy of the assay method

	ETS			NOR			CYP		
Type of variance	Conc. spiked (ng/mL)	Conc. found (ng/mL)	RSD (%)	Conc. spiked (ng/mL)	Conc. found (ng/mL)	RSD (%)	Conc. spiked (ng/mL)	Conc. found (ng/mL)	RSD (%)
Intra-day ($n = 5$)	7.2	6.8	8.9	4.9	4.3	8.3	4.4	3.9	7.5
	43.2	42.9	5.4	58.3	55.3	5.7	43.7	40.1	5.8
	115.2	114.0	2.1	155.6	148.7	4.5	116.2	113.8	5.1
Inter-day ($n = 5$)	7.2	6.7	10.7	4.9	4.0	9.5	4.4	3.8	9.6
	43.2	41.8	6.7	58.3	52.6	9.9	43.7	39.7	8.6
	115.2	112.8	2.5	155.6	146.3	5.8	116.2	110.6	6.8

method for the simultaneous determination of ETS, CYP, and NOR was acceptable, since the R.S.D. values did not exceed the value of 15%, irrespective of the concentration level.

At the same concentrations, accuracy ranged from 87.0 to 99.5% intra-day and from 81.6 to 98.0% inter-day.

CONCLUSIONS

The HPLC method described has been validated and is currently being used to analyze ETS, NOR, and CYP in human milk samples. Although, the above substances do not co-exist in the same pharmaceutical preparation, their simultaneous separation and determination reflect the potential of the developed method and make it most suitable for screen testing.

The simplicity of the procedure combined with the good sensitivity, resolution, and the short analysis time, should make this method a useful tool to characterize the excretion of ETS, NOR, and CYP into breast milk.

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