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Journal of Chromatography B, 794 (2003) 205–213
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Quantitative trace analysis of estriol in human plasma by negative ion chemical ionization gas chromatography–mass spectrometry using a deuterated internal standard

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Received 14 August 2002; received in revised form 12 May 2003; accepted 22 May 2003

Abstract

A stable isotope dilution gas chromatography–mass spectrometry (GC–MS) assay for the trace level determination of estriol in human plasma is described. Negative ion chemical ionization (NICI) MS is used for highly specific detection. The method involves derivatization of the phenolic hydroxyl to the pentafluorobenzyl ether derivative and subsequent reaction of the remaining hydroxyls with heptafluorobutyric anhydride. This derivative allows detection of the strikingly abundant phenolate ion under NICI conditions. [2,4,17β]-$^{2}H_{3}$-labeled estriol was used as an internal standard. For high-level measurements (>313 ng/l) plasma was directly derivatized by extractive alkylation followed by heptafluorobutylation prior to analysis. A rapid and simple sample work up procedure was elaborated for trace level determinations (>5 ng/l plasma) using solid-phase extraction on C_{18} with an absolute recovery of 92.9%. For low-level measurements, the calibration curve was linear in the range of 5 to 625 ng/l (r=0.99993). Inter-assay analytical precisions (RSDs) were 1.29, 2.30 and 2.89% at 39, 156 and 650 ng/l plasma, respectively. For high-level measurements, calibration curve linearity was observed in the range of 0.313 to 20 μg/l (r=0.99998). Inter-assay analytical precisions (RSDs) were 5.17, 1.92, 2.57 and 2.74% at 0.313, 0.625, 2.5 and 10 μg/l plasma, respectively. Postmenopausal plasma was used for spiked plasma samples. Sensitivity and specificity of the presented method allows adequate determination of estriol in human plasma samples.

Keywords: Trace analysis; Estriol

1. Introduction

The measurement of serum estriol has become a valuable tool in clinical diagnosis and pharmaceutical research. Thus, the use of estriol constitutes an important contribution to the management of postmenopausal syndrome [1,2]. During pregnancy, maternal serum levels of estriol increase in a relatively linear fashion, with a surge in estriol production at late gestation. Among the estrogenic steroids, estriol is unique in that its precursors have an almost exclusively fetal origin. The fetal zone of the fetal adrenal gland synthesizes dehydroepiandrosterone sulfate, which is 16-α-hydroxylated in the fetal liver and metabolized to estriol in placental trophoblasts. Because of its fetal origin, estriol is produced in

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1570-0232/03/$ – see front matter
doi:10.1016/S1570-0232(03)00454-9

much larger quantities in the third trimester of gestation than either estradiol or estrone. Estriol is active in its unconjugated form. Furthermore, this fetal origin renders estriol highly useful as a diagnostic tool for fetal wellbeing. Thus, estriol levels in serum and saliva have been used as an indicator on the induction of preterm labor [3–5]. The simultaneous determination of α-fetoprotein, β-human chorionic gonadotrophin and unconjugated estriol (''triple test'') is now widely used in the first and second trimester maternal serum screening for the prenatal diagnosis of Down's syndrome [6–10], fetal trisomy 18 or Edward's syndrome [11,12], triploidy [13] and Turner Syndrome [14]. Similarily, low maternal estriol levels have been associated with and utilized for the prenatal diagnosis of Smith–Lemli–Opitz syndrome, an autosomal recessive malformation syndrome comprising distinct facial, limb and genital anomalies and mental retardation [15–17].

As circulating levels of estriol are very low [18–20] sensitive analytical tools are necessary to meet the requirements of the assay. Estriol is analyzed in serum largely by radioimmunoassay [21,22], enzyme immunoassays [23,24], high-performance liquid chromatography (HPLC) [25,26] and gas chromatography–mass spectrometry (GC–MS) [27–29]. Most of these procedures, however, are just sensitive enough to measure late pregnancy samples. The reliable and accurate estriol determination at early gestational weeks is a major concern, and thus a method covering this range of expected concentrations is desirable. Together with the well known benefits of stable isotope dilution for internal standardization, mass spectrometry can provide unsurpassed specificity and can hence serve as a reference method to validate results obtained by other methods. In this paper we wish to report the synthesis of a novel derivative of estriol for detection by negative ion chemical ionization (NICI) MS in the low-ng/l range. Furthermore, it was the aim of the study to elaborate a sample work-up procedure that allows the rapid and unattenuated routine analysis of the steroid hormone in human plasma samples.

2. Materials and methods

Estriol and 16α-hydroxyestrone (1,3,5[10]-estratriene-3,16α-diol-17-one) were purchased from Sigma (Vienna, Austria). Pentafluorobenzyl bromide (PFBBr) was from ABCR (Karlsruhe, Germany). Heptafluorobutyric anhydride (HFBA) was supplied by Aldrich. Bond Elut C_{18} extraction cartridges were purchased from Varian (Vienna, Austria). Polypropylene tubes were from Greiner (Kremsmünster, Austria). The estriol enzyme immunoassay (EIA) kit was purchased from Oxford Biomedical Research (Oxford, MI, USA). All other solvents and reagents of analytical grade were from Merck (Darmstadt, Germany).

2.1. Gas chromatography–mass spectrometry

A TRACE GC system coupled to a Finnigan Voyager quadrupole MS system (ThermoQuest, Vienna, Austria) was used. The GC system was equipped with a DB-5MS fused-silica capillary column (15 m×0.25 mm I.D., 0.25 μm film thickness) from ThermoQuest. The injector was operated in the splitless mode at 280 °C. Helium was used as a carrier gas at a constant flow-rate of 1.5 ml/min. The initial column temperature was 140 °C for 1 min, followed by an increase of 40 °C/min to 310 °C and an isothermal hold of 2 min. Split valves were opened 1 min after injection. The mass spectrometer transfer line was kept at 315 °C. NICI was performed with methane as a moderating gas at an electron energy of 70 eV and an emission current of 0.250 A. During single ion monitoring, m/z 679 and m/z 682 were recorded for target and internal standard, respectively, with a dwell time of 50 ms.

2.2. Derivatization

Pentafluorobenzyl (PFB) ethers were formed by extractive alkylation with PFBBr. Thus, the plasma sample or the dry residue after solid-phase extraction (SPE) (and evaporation of the solvent under nitrogen) was treated in a polypropylene tube (70×10 mm) with 250 μl 0.5 *M* NaOH, 125 μl tetrabutylammonium hydrogensulfate (TBAMS) (0.1 *M* in double-distilled water), 0.5 ml of dichloromethane and 10 μl of PFBBr. The vial was closed with a polypropylene stopper and shaken on a reciprocal shaker for 20 min at room temperature. Then, 1.5 ml of *n*-hexane was added, vortexed for 3 s at moderate intensity and the upper organic layer transferred to a

fresh polypropylene tube. The solvent was removed under a stream of nitrogen at 50 °C.

Heptafluorobutyryl esters (HFB) of the estriol PFB ether derivative were synthesized by reaction with 50 μl ethyl acetate and 50 μl HFBA at 75 °C for 30 min. The mixture was dried under a stream of nitrogen and the dry residue redissolved in 100 μl of ethyl acetate. After transfer to conical shaped autosampler vials the samples were stored at −20 °C until analysis.

2.3. Preparation of [2,4,17β]-d_3-estriol

[2,4,17β]-d_3-Estriol was synthesized by a modification of the method of Dehennin [30]. Briefly, 10 mg of 16α-hydroxyestrone was dissolved in 1.2 ml of CH_3OD and treated with 50 μl of each, D_2O and DCl (38% in D_2O) (final concentration: 0.46 *M*). The glass vial is closed under nitrogen and incubated at 75 °C for 45 h. After cooling, 1 ml double-distilled water and 3 ml of ethyl acetate are added, and the organic transferred to a new glass vial and the extract dried under nitrogen. The dry residue is dissolved in 1 ml of CH_3OD and reacted with 50 mg of $NaBD_4$ at room temperature for 30 min. After acidification with 1 ml 1 *M* HCl and extraction with ethyl acetate, the organic layer was transferred to a new vial and dried under nitrogen. The resulting [2,4,17β]-d_3-estriol was dissolved in methanol and stored at −20 °C. Isotopic composition was checked by GC–MS.

2.4. Plasma sample preparation

2.4.1. Direct derivatization

A 50-μl volume of a solution of the internal standard [2,4,17β]-d_3-estriol (1 ng/50 μl methanol) was added to a polypropylene vial and 50 μl of plasma sample (citrate anticoagulant) was added. The agents used for extractive alkylation are subsequently added and derivatization accomplished as described above. After derivatization the dry colored residue was reconstituted in 50 μl of ethyl acetate, transferred to autosampler vials and stored at −20 °C until analysis. Stored samples are stable for at least 4 weeks. A 4-μl volume was subjected to GC–NICI-MS measurement. There was no peak distortion observed comparing injection volumes of 1 and 4 μl.

2.4.2. Solid-phase extraction on BondElut C_{18}

A 50-μl volume of a solution of the internal standard [2,4,17β]-d_3-estriol (1 ng/50 μl methanol) was added to 1 ml of plasma, mixed throughly, equilibrated for 15 min and diluted with 2 ml double-distilled water. The sample was applied to a Bond Elut C_{18} cartridge (200 mg, 3 ml) which had been preconditioned with methanol (1×2 ml) and double-distilled water (3×3 ml). The adsorbed sample was washed with double-distilled water (3×3 ml) and elution was accomplished with 2 ml of ethyl acetate. The solvent was evaporated under nitrogen at 50 °C and derivatives were prepared as described above. The samples were found to be sufficiently pure for analysis by GC–NICI-MS. After derivatization the dry residue was reconstituted in 50 μl of ethyl acetate, transferred to autosampler vials and stored at −20 °C until analysis. A 4-μl volume was subjected to GC–NICI-MS measurement.

2.5. Recovery of estriol on Bond Elut C_{18}

To estimate analyte recovery during SPE, estriol-free postmenopausal plasma was spiked with 500 pg of estriol and SPE carried out as described above. After elution, 1 ng of the internal standard was added to the eluate, and the sample derivatized as described. To estimate the maximal value (100%), 500 pg of estriol was mixed with 1 ng of the standard and derivatized directly. Sixfold determinations were carried out. Estriol-free plasma showed no estriol levels above the limit of detection.

2.6. Analytical method validation

Calibration graphs were established in the range of 5 to 650 ng/l plasma (low-level measurements) and in the range of 0.313 to 20 μg/l plasma (high-level measurements). For this purpose, plasma from postmenopausal female volunteers was spiked with the appropriate amounts of estriol by adding 50 μl of the corresponding methanolic solution. Standard solutions of estriol were prepared by serial dilution in methanol to yield concentrations of 650, 325, 163, 81, 41, 20, 10 and 5 pg/50 μl (low level), and of 20, 10, 5, 2.5, 1.25, 0.615 and 0.313 ng/50 μl (high level), respectively. Standard solutions were stored at −20 °C. No decomposition was measurable after 2

months of storage. Postmenopausal plasma was checked for possible estriol content before use.

The instrumental detection limit was estimated by derivatizing 1 ng of pure estriol sample and injecting diluted aliquots into the GC–MS system.

Inter-assay precision was determined at 39, 156 and 650 ng/l plasma (low-level determinations) and at 0.313, 0.625, 2.5 and 10 μg/l plasma (high-level measurements) by carrying identical samples throughout the analytical sequence at one single day. Spiked samples were prepared from postmenopausal plasma. Sixfold determinations were carried out.

Intra-assay precision was determined at 39, 156 and 650 ng/l plasma (low-level determinations) and at 0.625, 2.5 and 20 μg/l plasma (high-level measurements) by multiple GC–MS analysis of one prepared sample. Spiked samples were prepared from postmenopausal plasma. Sixfold determinations were carried out.

Accuracy of the methods was also tested at the abovementioned concentrations after fivefold determinations. Thus, the data from inter-assay precision measurements were used to calculate the deviation of the values measured from the actual spiked values.

3. Results

3.1. Sample preparation and gas chromatography–mass spectrometry

The NICI mass spectrum of the estriol PFB ether HFB derivative is shown in Fig. 1A. The phenolate anion at *m/z* 679 is strikingly predominant. In Fig. 1B, the NICI mass spectrum of d_3-labeled estriol PFB ether HFB derivative is presented. The product is isotopically pure in terms of absence of any unlabeled estriol. There was no improvement in baseline noise when a more shallow temperature gradient on the GC was used (15 °C/min).

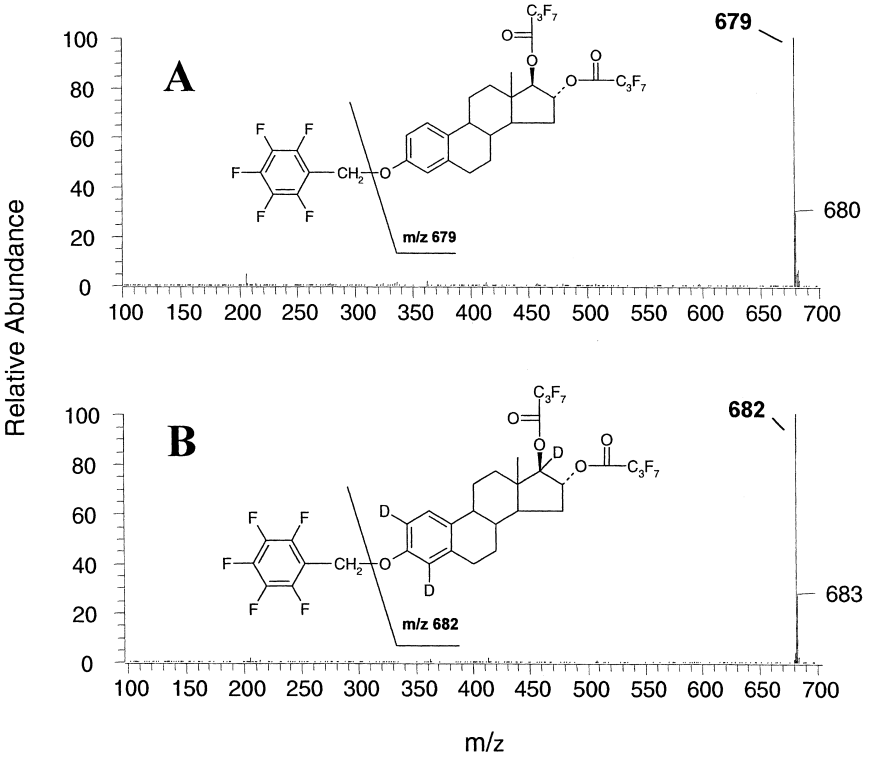

Fig. 1. NICI mass spectra of (A) estriol and (B) d_3-estriol after conversion to the PFB ether heptafluorobutyryl ester derivatives.

SPE yield with Bond Elut C_{18} cartridges was 92.9±1.5%, as checked by addition of the internal standard to the spiked plasma samples after the extraction procedure. There was no estriol found in an additional elution with methanol. During the aqueous washing step, estriol was fully retained on the cartridge. When the adsorbed sample was washed with *n*-hexane, however, partially elution occurred and recovery was reduced to approx. 85%. Derivatization reactions with PFBBr and HFBA occurred quantitatively. No trimethylsilyl derivative was detected after PFBBr reaction, and no increase in signal intensity was observed after longer reaction with HFBA. No formation of byproducts has been observed. Typical single-ion monitoring (SIM) mass chromatograms obtained after analysis of estriol in human plasma are given in Fig. 2A (after SPE) and Fig. 2B (after extractive alkylation).

3.2. Analytical method validation

3.2.1. Low-level method

The calibration graphs established were linear within the tested range of 5 and 650 ng/l plasma, using 1 μg/l of internal standard. With concentrations above 1 μg/l, the signal becomes saturated. The calibration curve with the statistical data is shown in Fig. 3A.

The limit of detection of the mass spectrometer was found to be 50 fg (on column) at a signal-to-noise ratio of at least 4:1. The lower limit of detection for the complete procedure was 1 ng/l

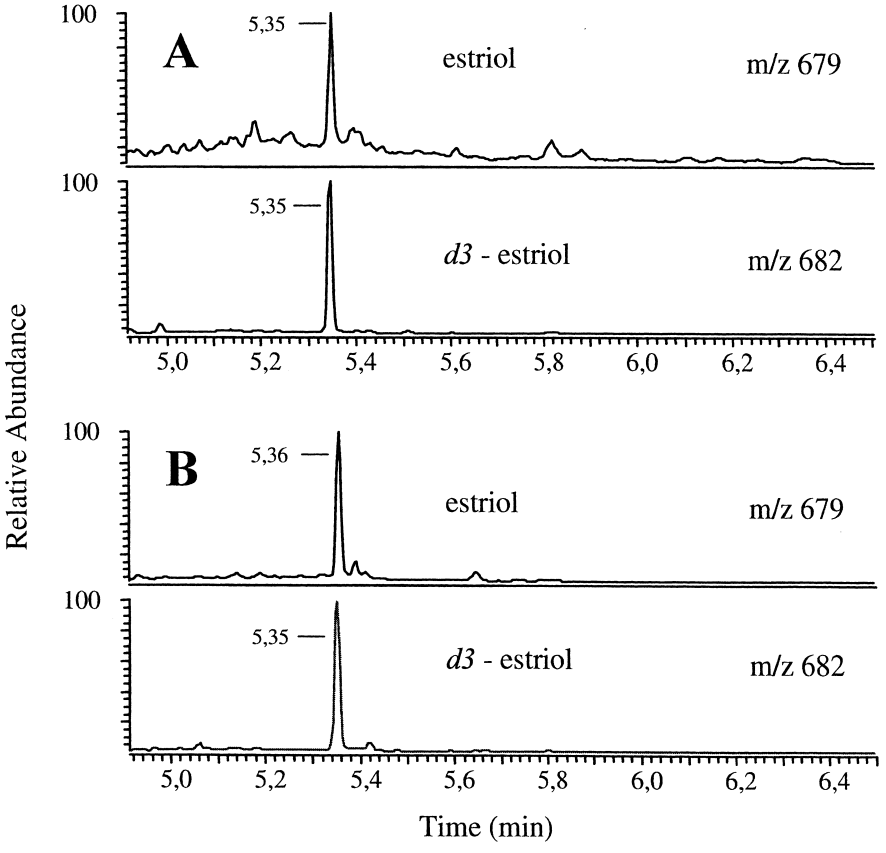

Fig. 2. SIM chromatograms obtained after analysis of a human plasma sample (A) after solid-phase extraction and (B) after direct extractive alkylation. The corresponding plasma levels are 42.0 ng/l and 4.2 μg/l, respectively.

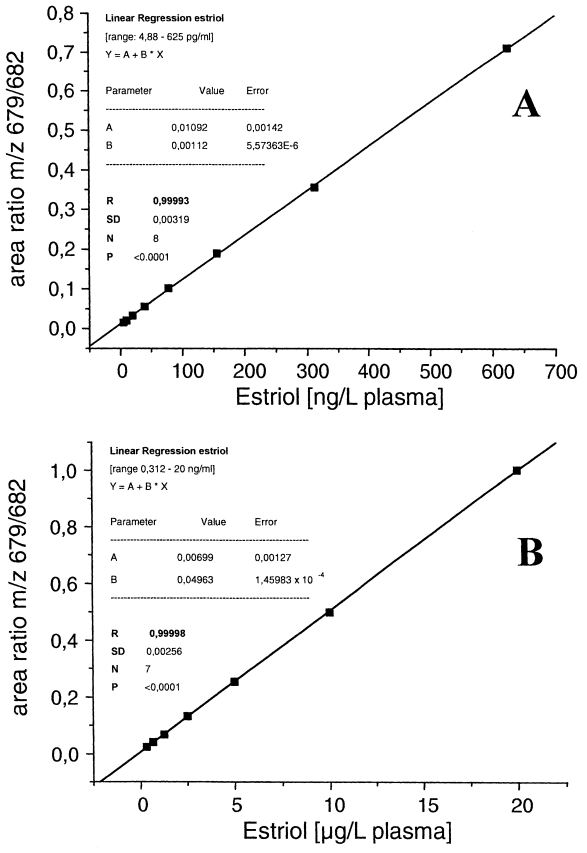

Fig. 3. Calibration graphs and statistics for the determination of estriol in human serum by (A) the solid-phase extraction method and (B) the direct extractive alkylation method. A 1-ng amount of internal standard was used.

plasma (corresponding to 80 fg on column), estimated by analyzing spiked plasma samples. For routine measurements, the limit of quantitation was set to 5 ng/l plasma.

The inter- and intra-assay relative standard deviations (RSDs) are presented in Table 1, as well as the analyte recovery in the spiked samples.

3.2.2. High-level method

The calibration graphs established were linear within the tested range of 0.313 and 20 μg/l plasma, using 1 ng of internal standard. The calibration curve with the statistical data is shown in Fig. 3B. For routine measurements, the limit of quantitation was set to 0.313 μg/l plasma.

The inter- and intra-assay RSDs are presented in Table 2, as well as the analyte recovery in the spiked samples.

There was no interference measureable from estrone, estradiol, ethinylestradiol, levonorgestrel, desogestrel, dehydroepiandrosterone and testosterone at concentrations of 1 μg/l each.

Table 1
Inter- and intra-assay precision and accuracy of estriol determination in human plasma after solid-phase extraction (n=6)

	Spiked amount (ng/l)		
	39	156	650
Inter-assay			
Mean (ng/l)	41.3	158	617
SD	0.53	3.63	17.9
RSD (%)	1.29	2.30	2.89
Recovery (%)	105.8	100.9	95.0
Intra-assay			
Mean	41.5	154	624
SD	0.64	2.23	9.21
RSD (%)	1.53	1.44	1.47

4. Discussion

4.1. Gas chromatography–mass spectrometry

Several derivatives of estriol have been used for determination by GC–MS. As described earlier [31–33], PFB derivatives have proved to exhibit excellent electron-capture response due to the formation of

Table 2
Inter- and intra-assay precision and accuracy of estriol determination in human plasma after direct extractive alkylation (n=6)

	Spiked amount (μg/l)			
	0.313	0.625	2.5	10
Inter-assay				
Mean	0.279	0.623	2.56	10.2
SD	0.014	0.012	0.066	0.278
RSD (%)	5.17	1.92	2.57	2.74
Recovery (%)	89.3	99.7	102.4	102.0
Intra-assay				
Mean	0.620	2.42	21.2	
SD	0.010	0.071	0.536	
RSD (%)	1.58	2.95	2.52	

resonance-stabilized fragments under chemical ionization conditions. Thus, our first attempts were directed towards potential use of this derivative for estriol analysis. As expected, there is excellent fragmentation behaviour for quantitative analytical applications. As known from other phenolic compounds, the PFB ether derivative is stable to hydrolysis and thus circumvents stability problems encountered with the use of other derivatives of the phenolic hydroxyls [28,34]. Extractive alkylation with the phase transfer catalyst TBAHS is a mild and effective way to yield the desired derivative. Alternatively, PFB-Br in acetone with potassium carbonate (1 h at 75 °C) can be employed. HFB derivatization was carried out in order to produce a diagnostic ion at relatively high mass. The corresponding trifluoroacetates or pentafluoropropionates can also be prepared by using the appropriate anhydride reagent. PFB-trimethylsilyl (TMS) derivatives have been previously used for measurement of estrogens, but the mixed PFB-HFB derivative, which was not previously used for estriol analysis, was found to be advantageous due to its high m/z value used for detection. Although electron ionization has been frequently used for the determination of steroid derivatives by GC–MS, NICI was chosen in our case for several reasons. First, the soft ionization process yields fragment ions at high m/z values with high abundance, thus reducing instrumental and matrix background. Second, the derivative investigated did not yield an electron ionization spectrum of similar usability. Thus, the most prominent fragmentation process under electron ionization conditions is the formation of a pentafluorobenzyl tropylium radical after benzylic cleavage, a fragment ion which is not specific for the target molecule but occurs in most pentafluorobenzyl ether and ester derivatives. To take advantage of the extractive alkylation procedure described herein, NICI detection is highly recommended.

4.2. Preparation of [2,4,17β]-d_3-estriol

It is an ultimate criterium for the use as an internal standard in quantitative measurements, that the acquired isotope label remains stable throughout all procedures associated with sample preparation, clean-up and derivatization [35]. In the case of d_3-estriol, addition of the standard to human plasma did not evoke any changes of the isotopic pattern after 24 h at room temperature. Additionally, longer derivatization times did not alter the isotope label. No backexchange or instability of deuterium label has been found throughout the analytical procedure. The high quantitation mass, which is represented by the carboxylate anion thus carries the total amount of label in the molecule at m/z 682.

4.3. Plasma sample preparation

The analytical procedure allows the use of polypropylene tubes throughout the sample preparation sequence, thus reducing the risk of cross contamination and adsorption on polar glass walls. Although SPE in this case provides a very simple and straightforward sample preparation, the extraordinary high sensitivity of the analytical method allows the measurement of estriol from small sample quantities of 50 μl. In this case, no separate sample cleanup or extraction has to be performed, the first derivatization step performing both, sample derivatization and extraction in one. No further cleanup is needed for the GC–MS analysis, as show in a the sample chromatogram in Fig. 2B. The preparation of PFB ether derivatives by extractive alkylation thus provides a major advantage in sample preparation and enhances speed of analysis. As the derivative is exclusively used for NICI, the method requires this mode of mass detection, and electron ionization (EI) would not be applicable for the reasons stated above.

The low variability, even at low concentrations, is due to the inherent sensitivity of the NICI method and to the use of the stable isotope labeled internal standard. As pointed out earlier, mass spectrometry in combination with stable isotope dilution is a very powerful tool in external quality assessment schemes, and can be regarded as reference procedures to validate other analytical methods in steroid analysis [36].

The low-level method was applied to the analysis of estriol in real plasma obtained from male and female volunteers of various age. As expected, the plasma levels of postmenopausal and male volunteers could not be detected. Also, the levels of some premenopausal female volunteers were at or below the limit of detection. This is in accordance with the

literature, where normal values of estriol in young women are described at 7 to 10 ng/l plasma in the follicular and luteal phase, respectively [18–20].

In samples from pregnant women, however, both methods could be demonstrated to be highly useful in monitoring the estriol levels even at early gestational age. Table 3 shows the results of estriol determination in human plasma from obtained from pregnant women at weeks 7, 11, and 37 of gestation, respectively. Expectedly, the direct derivatization method could not detect estriol in samples of week 7 and 11, but readily determined estriol in late gestation at weeks 26–37. On the other hand, SPE made it possible to detect traces of the hormone at week 11. The levels measured at week 7 were below the limit of quantitation (<5 ng/l), but not below the limit of detection. Hence, a value of 3 ng/l plasma was readable from an extrapolated calibration curve, but with a signal-to-noise ratio less than 4:1. We have also followed the rise in plasma estriol levels in samples obtained from one single pregnant woman at different weeks of gestation by the SPE method, as shown in Table 4.

In conclusion, the PFB ether heptafluorobutyryl esters of estriol delivers a highly stable and sensitive derivative with excellent resonance electron capture properties demanded for NICI-MS analysis. The combination with stable isotope dilution internal standardization enhances specificity, and flexibility in sample pretreatment assures rapidity and reproducibility needed for the routine clinical monitoring of feto–placental function and maternal state.

Table 3
Estriol levels in human plasma samples obtained from women at different weeks of gestation

E3 (solid-phase extraction)	E3 (direct extractive alkylation)	Week of gestation	Subject
n.d.*	n.d.*	7	S.L.
42 ng/l	n.d.*	11	R.G.
n.m.**	4.2 μg/l	37	Z.A.
n.m.**	7.5 μg/l	37	R.J.
n.m.**	3.6 μg/l	26	A.K.
n.m.**	2.3 μg/l	34	L.L.

n.d.*: Below the limit of quantification.
n.m.**: Not measured, because concentration out of calibration range.

Table 4
Estriol levels in human plasma obtained from a single woman at different weeks of gestation

E3 (ng/l)	Week of gestation
5	7
13	8
54	12
230	13
250	14
699	16

Estriol was measured by the solid-phase extraction method.

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