ELSEVIER

Journal of Chromatography B, 723 (1999) 39–45

JOURNAL OF CHROMATOGRAPHY B

Simultaneous determination of estriol and estriol 3-sulfate in serum by column-switching semi-micro high-performance liquid chromatography with ultraviolet and electrochemical detection

Noriko Tagawa[a], Hisao Tsuruta[b], Aya Fujinami[a], Yoshiharu Kobayashi[a,*]

[a]*Clinical Chemistry Laboratory, Kobe Pharmaceutical University, 4-19-1 Motoyamakita-machi, Higashinada-ku, Kobe 658-8558, Japan*
[b]*Analytical Chemistry Research Laboratory, Irika Instruments Inc., 8-47-1 Fukakusa Nishiura-cho, Fushimi-ku, Kyoto 612-0029, Japan*

Received 8 September 1998; received in revised form 11 November 1998; accepted 30 November 1998

Abstract

A column-switching HPLC with semi-microcolumn enabled us a direct and simultaneous analysis of estriol (E3) and estriol 3-sulfate (E3 S) in human serum in combination with ultraviolet (for E3 S) and electrochemical (for E3) detectors. The mobile phases (phosphate buffer pH 7.0) contained 5 m*M* tetra-*n*-butylammonium ion (TBA) as a counter ion for E3 S. Serum samples were diluted with 200 m*M* phosphate buffer (pH 7.0) containing 100 m*M* TBA, then injected to the pre-column. After serum proteins had flowed out from the pre-column, E3 and E3 S were transferred to the enrichment column. Subsequently the analytes were eluted to the analytical column. Detection limits of E3 and E3 S in human serum were 2.5 ng/ml and 295 ng/ml. Serum E3 and E3 S levels (mean±SD) of umbilical artery from 18 full-term healthy neonates were 33±23 ng/ml and 1.26±0.69 μg/ml, respectively.

Keywords: Steroids; Estriol; Estriol 3-sulfate

1. Introduction

Estriol (E3) and estriol 3-sulfate (E3 S) are the most abundant estrogen circulating in serum of fetus and pregnant women. These steroids are produced in placenta from two principal precursors, which are originated from fetus: 16α-hydroxydehydroepiandrosterone (16-OH-DHEA) and 16-OH-DHEA sulfate. The concentrations of E3 and E3 S are 4–8 times higher in the fetus than in the maternal circulation. Moreover, in fetal serum as in maternal serum, the highest level of these steroids was observed at delivery [1,2]. However, the physiological significance of these steroids in fetus is not fully understood. To investigate the feto-placental function during pregnancy, E3 and E3 S levels in urine, in serum and in amniotic fluid have been measured by HPLC using various detectors: UV [3], fluorescence [4–8] and electrochemical (EC) detector [9–15], GC–MS [16–19], micellar electrokinetic chromatography [20,21], EIA [22,23], and RIA [24–28]. None of these methods are capable of measuring E3 and E3 S simultaneously. Moreover, most of these methods require sample preparation steps such as extraction from the biological fluids, enzymatic hydrolysis or acidic solvolysis and derivatization. These procedures are tedious and time-consuming, and sometimes disturb the analytical results. For

*Corresponding author. Fax: +81-78-441-7559.

0378-4347/99/$ – see front matter
PII: S0378-4347(98)00555-6

immunoassay, it is necessary to obtain a specific antibody to each steroid of interest. Therefore, it is desirable to determine serum E3 and E3 S levels in fetus accurately, rapidly and conveniently. Here we report simultaneous determination of E3 and E3 S in serum using column-switching HPLC along with UV and EC detectors.

2. Experimental

2.1. Chemicals and materials

The chemicals used in this study were obtained from the following sources: E3 and E3 S (Sigma Chemical, St. Louis, MO, USA); 0.5 *M* tetra-*n*-butyl-ammonium hydroxide solution for HPLC (Wako Pure Chemical Industries, Osaka, Japan); disodium hydrogen phosphate, potassium dihydrogenphosphate and acetonitrile for HPLC (Nacalai Tesque, Kyoto, Japan); arylsulfatase from *Helix pomatia* (EC.3.1.6.1), (Boehringer Mannheim, Mannheim, Germany) and other chemicals were of analytical grade. Water was purified with a Milli-Q system (Nihon Millipore Kogyo, Yonezawa, Japan). Steroid-free serum was prepared from pooled human serum according to the method of Heyns et al. [29].

2.2. Mobile phase

As the mobile phase for the pre-column, 200 m*M* phosphate buffer (pH 7.0) containing 5 m*M* tetra-*n*-butyl-ammonium ion (TBA) was used and 5 m*M* phosphate buffer (pH 7.0) containing 5 m*M* TBA was used as the mobile phase for the enrichment column and for the analytical column. The pH was adjusted with phosphoric acid.

2.3. Chromatographic system

The HPLC system, Nanospace SI-1 (Shiseido, Tokyo, Japan), consisted of 3 pumps (model 2001), a six-port switching valve (model 2011), a column oven (model 2004), an autosampler (model 2003), a UV–VIS detector (model 2005), an EC detector (model 2005) and a degassing unit (model 2010). The columns (Shiseido) used in this system were a pre-column: Capcell MF Ph-1 (20 mm×4 mm I.D., polymer-coated mixed function pre-column), an enrichment column: Capcell Pak C_{18} UG120 (35 mm× 2.0 mm I.D., 5 μm particle size) and an analytical column: Capcell Pak C_{18} UG120 (250 mm×1.5 mm I.D., 5 μm particle size). The data were integrated with an S-Micro Chrom 4.1 (Shiseido) using a personal computer running MS-Windows 95® (Microsoft, Redmond, WA, USA). The column switching HPLC system is shown schematically in Fig. 1.

2.4. Chromatographic conditions

The mobile phase for retention of steroid sulfate in the pre-column (Pump 1) was 200 m*M* phosphate buffer (pH 7.0) containing 5 m*M* TBA. The mobile phase (Pump 2) for elution of steroid sulfates from the pre-column and for concentration of those steroids in the enrichment column was 5 m*M* phosphate buffer (pH 7.0) containing 5 m*M* TBA–acetonitrile (95:5, v/v). The mobile phase for the analytical column (Pump 3) was 5 m*M* phosphate buffer (pH 7.0) containing 5 m*M* TBA–acetonitrile (76.5:23.5, v/v). The flow rates of Pump 1, 2 and 3 were 0.5,

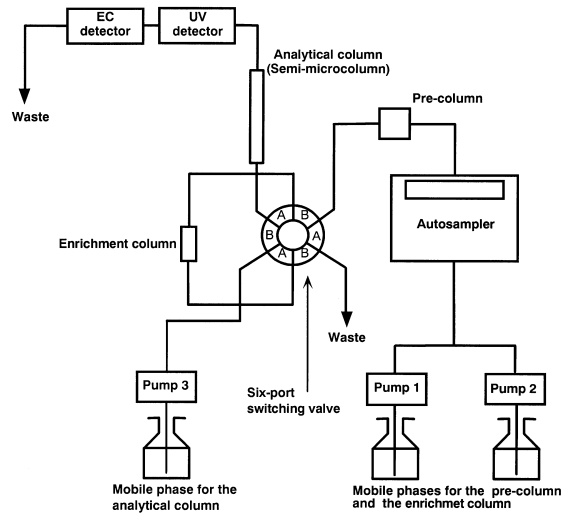

Fig. 1. Schematic diagram of the column-switching HPLC system During analysis, the position of the six-port high-pressure valve was switched to A, B and A, successively. A position (Pump 1): the steroids were retained in the pre-column and the serum proteins were discarded; B position (Pump 2): the steroids were concentrated in the enrichment column; A position (Pump 3): the steroids were transferred to the analytical column via the enrichment column.

0.5 and 0.1 ml/min, respectively. The pre-column and the analytical column were kept at 40°C and the enrichment column was run at room temperature. When the column-switching valve was at the A position, the mobile phase for the pre-column was passed through Pump 1, and sample loading and primary separation of steroids from serum proteins were performed. Subsequently, the valve was switched to the B position and Pump 2 was used to flow the mobile phase solvent for elution of the steroids from the pre-column and for concentration of the steroids in the enrichment column. Position B was maintained for 3.0–15.0 min after the injection of serum sample. Then, the valve position was returned to A and the mobile phase for the analytical column was passed via Pump 3. The steroids concentrated in the enrichment column were transferred into the analytical column in the back-flash mode. E3 was detected electrochemically at 0.65 V vs. Ag/AgCl reference electrode and E3 S was monitored at 210 nm with the UV detector.

2.5. Preparation of standard serum

Standard serum for E3 and E3 S was prepared as follows: 0.1 ml of methanol solution of E3 (1 μg/ml) and of E3 S (94.4 μg/ml) was pipetted into a tube. The solvent was evaporated under vacuum, then 1.0 ml of steroid-free serum was added to the tube. The mixture was vortexed and stood at 4°C overnight. After filtration with a 0.22 μm pore size membrane, the standard serum was serially diluted with steroid-free serum (E3: 2.5–100 ng/ml; E3 S: 0.3–9.44 μg/ml).

2.6. Sample treatment for HPLC

Before HPLC analysis, standard sera and serum samples were treated as follows: 35 μl of standard sera and of serum sample was diluted with 231 μl of 200 m*M* phosphate buffer (pH 7.0) containing 100 m*M* TBA and 14 μl of methanol. After the diluted standard sera and serum samples had been filtered with a 0.22 μm pore size membrane, 200 μl aliquots of these solutions were subjected to HPLC. Standard sera and serum samples were stored at −30°C until use.

3. Results and discussion

3.1. Retention of E3 and E3 S in the pre-column

To investigate the effect of retention of E3 and E3 S in the pre-column, the end of the pre-column was connected directly to the UV detector. The peak area for E3 and E3 S was monitored at 210 nm. Before injection of serum sample containing E3 and E3 S to the pre-column, 35 μl of serum sample was diluted with 231 μl of 200 m*M* phosphate buffer (pH 7.0) containing 100 m*M* TBA and 14 μl of methanol. Then, 200 μl of the sample was applied to the pre-column. The resolution of the pre-column is generally low so high concentrations of E3 and E3 S in the diluted serum sample (each 2.5 μg/200 μl) were used to obtain peaks in this experiment. As shown in Fig. 2A, E3 S was retained in the pre-column during the exclusion of the serum proteins which was seen at the position of void volume. E3 was also retained, however, the peak position was delayed. To elute E3 faster, the pre-column was flowed with 5 m*M* phosphate buffer (pH 7.0) containing 5 m*M* TBA–acetonitrile (95:5, v/v) in 3 min after the injection of the sample by Pump 2. Then, the peak of E3 appeared at 12 min (Fig. 2B). According to these results, column-switching conditions for the present HPLC system were determined. The eluate of the pre-column from 3 to 15 min after injection of samples was transferred to the enrichment column with the switching valve (position B, Pump 2). Then, E3 and E3 S, which were trapped in the enrichment column, were introduced into the analytical column by Pump 3 with back-flash mode (switching valve, position A). As shown later the section on recovery, E3 and E3 S in serum were recovered at between 96.8 to 101% under the above conditions.

3.2. Detection limit and calibration curves for E3 and E3 S

Since serum levels of E3 and E3 S are low in fetus, we employed a semi-microcolumn as an analytical column and an EC detector (for E3) in order to obtain high resolution and sensitivity. The detection limits for E3 and E3 S were 2.5 ng/ml (62.5 pg/injection) at a S/N ratio of 3 and 295

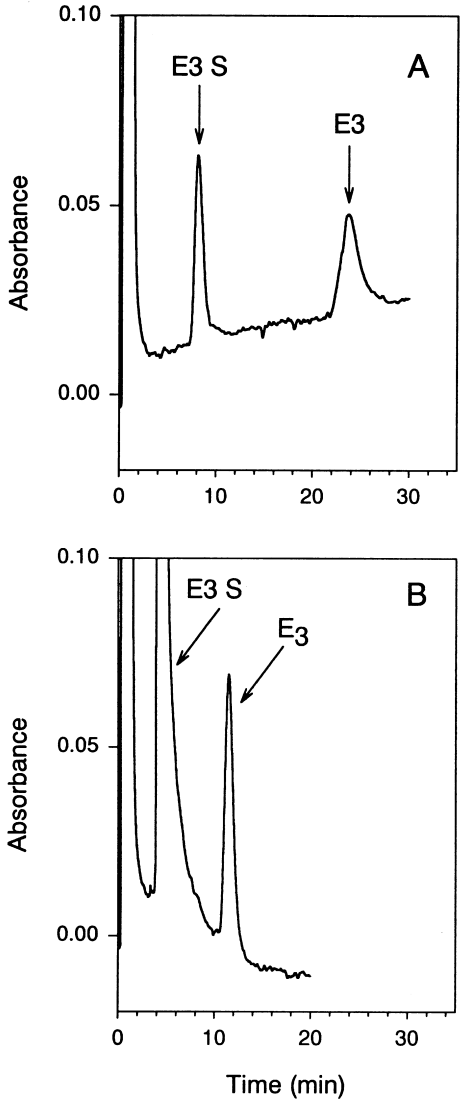

Fig. 2. Retention of E3 and E3 S in the pre-column. The end of the pre-column (Capcell MF Ph-1 20 mm×4 mm I.D.) was connected directly to the UV detector. The peak area for E3 and E3 S was recorded at 210 nm. Before injection of serum sample containing E3 and E3 S to the pre-column, 35 μl of serum sample was diluted with 231 μl of 200 m*M* phosphate buffer (pH 7.0) containing 100 m*M* TBA and 14 μl of methanol. Then, 200 μl of the sample (E3 and E3 S: 2.5 μg each) was applied to the pre-column. (A) The pre-column was flowed with 200 m*M* phosphate buffer (pH 7.0) containing 5 m*M* TBA. (B) The pre-column was flowed with 5 m*M* phosphate buffer (pH 7.0) containing 5 m*M* TBA–acetonitrile (95:5, v/v) in 3 min after the injection of the sample by Pump 2.

ng/ml (14.7 ng/injection) at a S/N ratio of 4, respectively. The calibration curves obtained with the standard serum of E3 and E3 S were linear from 2.5 to 100 ng/ml for E3 and from 0.3 to 9.44 μg/ml for E3 S. The detection limit for E3 was superior than in previous reports [9,10,12–15]. Recently, direct measurement of steroid sulfates with reversed-phased HPLC–MS has been reported [30]. To our knowledge, there has been no report on the direct measurement of E3 S in serum by HPLC.

3.3. Dilution study

Human serum was diluted with steroid-free serum to 1/2, 1/4 and 1/8, then the levels of E3 and E3 S were measured by the present method. The E3 and E3 S values of 1/1 were 47.3 ng/ml and 5.98 μg/ml, respectively. Linear relationships that went through the origin were obtained between the levels of E3 or E3 S.

3.4. Precision

Precision of the present method was evaluated by 6 runs of the neonatal serum samples. The intra-assay coefficients of variation (C.V.) for E3 and E3 S were 0.9% (11.3±0.1 ng/ml, mean±SD) and 1.1% (1.88±0.02 μg/ml), respectively. The inter-assay C.V. for E3 and E3 S were 7.5% (10.6±0.8 ng/ml) and 4.9% (1.02±0.05 μg/ml), respectively.

3.5. Recovery study

To investigate the recovery of the present method, to human serum samples were added authentic E3 (5 ng/ml and 25 ng/ml) and E3 S (0.5 μg/ml and 2.5 μg/ml). The determinations were done 6 times. The recoveries for E3 were 98.2±5.8% at 5 ng/ml and 96.8±1.3% at 25 ng/ml. Those for E3 S were 101.0±4.6% at 0.5 μg/ml and 97.3±4.4% at 2.5 μg/ml.

3.6. Typical chromatograms of the serum samples

Typical chromatograms of steroid-free serum (blank) and neonatal serum sample (E3 8.2 ng/ml and E3 S 0.93 μg/ml) is shown in Fig. 3A and B. The serum samples were diluted before injection as

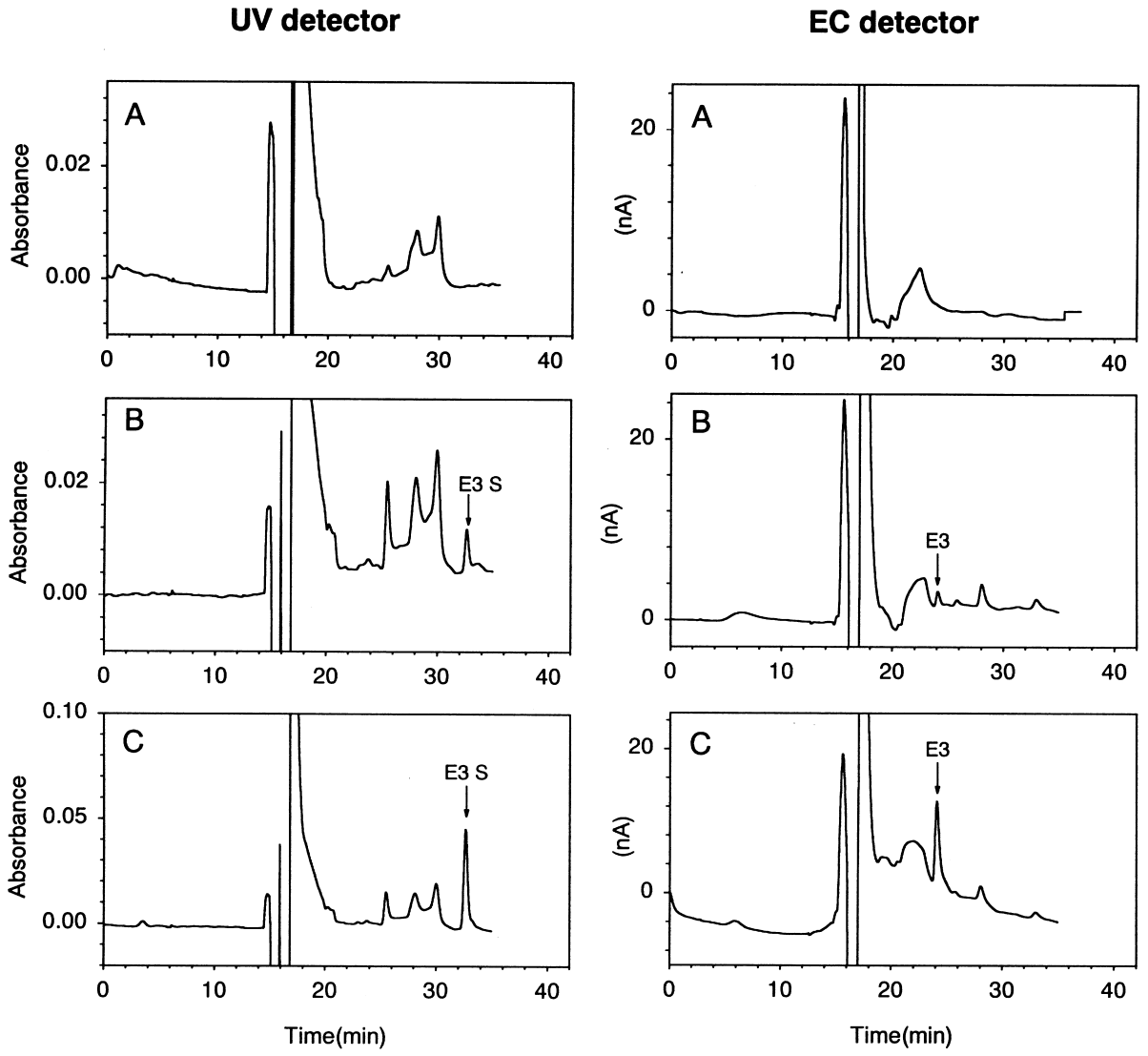

Fig. 3. Typical chromatograms of steroid-free serum (A), neonatal serum (B) and neonatal serum spiked with authentic E3 (65 ng/ml) and E3 S (6.5 μg/ml) (C). HPLC conditions are described in the Experimental section. Chromatograms for E3 S were recorded with a UV detector at 210 nm and those of E3 with an EC detector at 0.65 V vs. Ag/AgCl.

described in the section of sample treatment. These chromatograms were obtained simultaneously with an UV detector for E3 S and with an EC detector for E3. The retention times of E3 and E3 S were 24.1 and 32.6 min, respectively. The order of the elution of E3 and E3 S from the analytical column was reversed relative to that from the pre-column. This seems to be the elution of the mobile phase solution with back-flash mode. Chromatogram of the peaks of E3 and E3 S were identified by spiking with authentic E3 and E3 S (Fig. 3C). Moreover, the peak of E3 S was confirmed by enzymatic hydrolysis of the serum sample. This was done as follows: the serum sample was dissolved with 2 ml of 0.25 *M* acetate buffer solution (pH 6.0) containing 10 μl arylsulfatase and incubated at 40°C overnight. The sample was extracted with dichloromethane. Then the residue of the extract was dissolved with 200 m*M*

phosphate buffer (pH 7.0) containing 100 m*M* TBA and subjected to the present HPLC. As a result, the peak of E3 S disappeared, while that of E3 appeared (data not shown). The durability of the pre-column was studied with successive injections of the serum steroid standard (E3 S, serum 25 μl). Little change in the peak area was observed between the 1st and the 50th run. The lifetime of the pre-column used were over 50 runs (total serum volume, 1.25 ml or more).

3.7. Measurement of E3 and E3 S in serum of umbilical artery

The concentrations of E3 and E3 S in arterial cord serum from 18 healthy full-term infants were determined by the present method. The serum level of E3 was 33±23 ng/ml and that of E3 S was 1.26±0.69 μg/ml, respectively and these values were in agreement with the reported data [12,17,31–33]. The present column-switching method permits the direct and simultaneous determination of E3 and E3 S in serum without any clean-up procedures such as extraction, hydrolysis or derivatization.

4. Conclusions

A convenient column-switching HPLC was developed for the simultaneous determination of E3 and E3 S in serum samples. With this system, direct analysis has been accomplished by use of pre-column and higher sensitivity has been obtained with EC detector using a semi-microcolumn. The detection limits, precisions and recoveries of the present method were sufficient for determinations of E3 and E3 S in serum.

Acknowledgements

This work was supported in part by Kobe Pharmaceutical University Collaboration Fund and The Science Research Promotion Fund of The Japan Private School Promotion Foundation.

References

[1] K. Arai, Y. Kuwabara, K. Kihara, S. Okinaga, S. Sakamoto, Am. J. Obstet. Genecol. 114 (1972) 812–815.
[2] K. Arai, T. Yanaihara, Am. J. Obstet. Gynecol. 127 (1977) 879–883.
[3] S.U. Sheikh, J.C. Touchstone, J. Liq. Chromatgr. 17 (1994) 3813–3820.
[4] P.M. Kabra, F.H. Tsai, L.J. Marton, Clin. Chim. Acta 128 (1983) 9–17.
[5] F. Andreolini, C. Borra, A.D. Corcia, A. Lagana, R. Samperi, G. Raponi, Clin. Chem. 30 (1984) 742–744.
[6] T. Dohji, M. Fushimi, T. Kawabe, F. Kamiyama, M. Mori, N. Sugita, O. Tanizawa, J. Chromatogr. 311 (1984) 363–365.
[7] F. Andreolini, C. Borra, F. Caccamo, A.D. Corcia, I. Nicoletti, R. Samperi, F. Improta, Clin. Chem. 31 (1985) 1698–1702.
[8] Z. Kondo, T. Makino, R. Iizuka, J. Clin. Lab. Anal. 4 (1990) 410–413.
[9] K. Shimada, T. Tanaka, T. Nambara, J. Chromatogr. 178 (1979) 350–354.
[10] O. Hiroshima, S. Ikenoya, M. Ohmae, K. Kawabe, Chem. Pharm. Bull. 28 (1980) 2512–2514.
[11] Y. Sagara, Y. Okatani, Y. Takeda, A. Kambegawa, Folia Endocrinol. Jap. 57 (1981) 963–973.
[12] J. Noma, N. Hayashi, K. Sekiba, J. Chromatogr. B 568 (1991) 35–44.
[13] L.A. Kaplan, D.C. Hohnadel, Clin. Chem. 29 (1983) 1463–1466.
[14] N. Hayashi, K. Hayata, K. Sekiba, Acta Med. Okayama 39 (1985) 143–153.
[15] H. Gunasingham, B.T. Tay, K.P. Ang, J. Chromatogr. 341 (1985) 271–278.
[16] T. Laatikainen, J. Peltonen, P. Nylander, Steroids 21 (1973) 347–359.
[17] T. Laatikainen, J. Peltonen, Colloq. Inst. Natl. Sante Rech. Med.(Endocrinol. Sex. Periode Perinat., Int., Symp.) 32 (1974) 255–266.
[18] M. Iwai, H. Kanno, M. Hashino, J. Suzuki, T. Yanaihara, T. Nakayama, H. Mori, J. Chromatogr. 225 (1981) 275–282.
[19] K.Y. Tserng, R.K. Danish, J.S. Bendt, J. Chromatogr. 272 (1983) 233–241.
[20] K.C. Chan, G.M. Muschik, H.J. Issaq, P.K. Siiteri, J. Chromatogr. A 690 (1995) 149–154.
[21] A.J. Ji, M.F. Nunez, D. Machacek, J.E. Ferguson, M.F. Iossi, P.C. Kao, J.P. Landers, J. Chromatogr. B 669 (1995) 15–26.
[22] M. Ghosh, T.K. Dhar, E. Ali, B.K. Bachhawat, Clin. Chim. Acta 128 (1983) 223–231.
[23] M.S. Preti, S. Lodi, P. Busacchi, M. Filicori, C. Flamigni, Steroids 43 (1984) 469–479.
[24] D. Tulchinsky, G.E. Abraham, J. Clin. Endocrinol. Metab. 33 (1971) 775–782.
[25] H. Katagiri, F.Z. Stanczyk, U. Goebelsmann, Steroids 24 (1974) 225–238.
[26] J.E. Christner, M.C. Fetter, Steroids 24 (1974) 327–342.

[27] C.A. Miller, M.C. Fetter, R.C. Boguslaski, Clin. Chem. 21 (1975) 1805–1809.
[28] M. Levitz, H. Jirku, S. Kadner, B.K. Young, J. Steroid Biochem. 6 (1975) 663–667.
[29] W. Heyns, H.V. Baelen, P.D. Moor, Clin. Chim. Acta 18 (1967) 361–370.
[30] L.D. Bowers, Sanaullah, J. Chromatgr. B 687 (1996) 61–68.
[31] T. Laatikainen, J. Peltonen, Acta Endocrinol. 79 (1975) 577–588.
[32] T. Laatikainen, J. Pelkonen, D. Apter, T. Ranta, J. Clin. Endocrinol. Metab. 50 (1980) 489–494.
[33] D. Tulchinsky, J. Clin. Endocrinol. Metab. 36 (1973) 1079–1087.