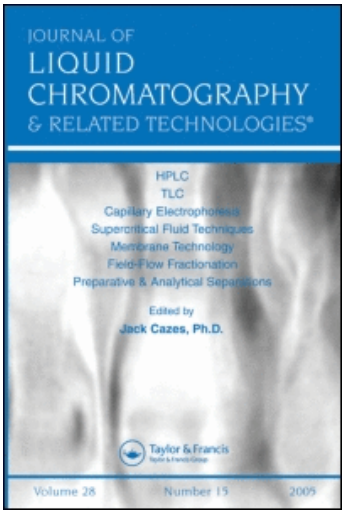


Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Determination of Free Estriol in Amniotic Fluid by High Performance Liquid Chromatography

S. U. Sheikh[a]; J. C. Touchstone[a]
[a] University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania

To cite this Article Sheikh, S. U. and Touchstone, J. C.(1994) 'Determination of Free Estriol in Amniotic Fluid by High Performance Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 17: 17, 3813 — 3820
To link to this Article: DOI: 10.1080/10826079408013996
URL: http://dx.doi.org/10.1080/10826079408013996

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(17), 3813-3820 (1994)

DETERMINATION OF FREE ESTRIOL IN AMNIOTIC FLUID BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

S. U. SHEIKH AND J. C. TOUCHSTONE
University of Pennsylvania
School of Medicine
Philadelphia, Pennsylvania 19104

ABSTRACT

The estrogen levels of amniotic fluid and maternal plasma may be altered in those cases with a fetus which presents Down syndrome. This paper describes the methodology for determination of free estriol in amniotic fluid. Fresh amniotic fluid was extracted using solid phase extraction techniques. Analtech C18 Spice cartridges were used to extract estriol (E3) prior to chromatography. After elution of the analyte E_3 from the cartridge, the solvent was evaporated under nitrogen in a warm water bath at 60°C. The extract was reconstituted in methanol and E3 separated by high-performance liquid chromatography. A reversed phase C18 Partisil ODS-3 (4.6 mm X 25 cm) column was developed with acetonitrile - methanol (65:35 v/v) as mobile phase. Subambient temperature (-20°C) improved the resolution and aided separation from extraneous material. Quantitation followed intrapolation against a standard curve prepared under identical conditions. Measurement of amniotic fluid E_3 may provide insight into the reported alteration of maternal plasma E_3 level in Down syndrome. Results so far indicate that in Down syndrome the amniotic fluid E_3 levels wee below the detection limits.

INTRODUCTION

Estriol (E_3) levels in blood serum in Down syndrome and normal patients is a controversial issue. No report indicates the level of estriol in blood and amniotic fluid in Down syndrome, and Trisomy - 18. Three components in serum - α-fetoprotein, free estriol (E_3) and human chorionic gonadotropin (β-hCG) have been analyzed in order to detect Down syndrome. A report by Carrick, et al, showed that maternal serum "free" estriol (E_3) was low in Down Syndrome (1). Greenberg et al also reported lower values for these constituents in maternal plasma (2). The

concentration of the "unconjugated" estriol increases approximately 20 percent per week in the second trimester. Maternal estriol E_3 is mostly conjugated, while the unconjugated estriol is of fetal origin.

Merkatz et al (2) showed that a low maternal serum a-fetoprotein is associated with Down fetuses. Several other studies (3,4) have confirmed this finding. The fetal liver is the source for this protein and the measurement of it is used on prenatal screening for neural tube defects. Approximately 20% of mothers with affected fetuses and 5% of mothers with unaffected fetuses have a level below 0.5 multiples of the median as compared to mothers of Down fetuses with 0.7 multiples of the median. There have been several studies on the relationship of free E_3 of the maternal plasma (3,4) to Down Syndrome. These have indicated that levels of (E_3) in the unconjugated form may correlate with the genetic status of the fetus.

The present report indicates that the free estriol (E3) in amniotic fluid may also be indicative of Down Syndrome. Undetectable levels of free estriol were found in these cases. In some cases of "normal" pregnancies the levels of free estriol were also below detection limits. Considering that the fetus is a major source of the estriol it might be more appropriate to assess estriol in the amniotic fluid rather than the maternal blood. A description of the methods used and the results obtained in normal and affected pregnancies are presented. Considering that amniotic fluid is routinely drawn for genetic studies and the small amounts required for the method as described the utility of amniotic fluid estriol merits further investigation.

MATERIAL AND METHODS

All specimens were from 15-18 weeks gestation. Fresh or frozen amniotic fluid samples were extracted using solid phase extraction (SPE) techniques; then aliquots of reconstituted samples were subjected to HPLC at subambient column temperature (-20°C) to resolve E_3.

All solvents were EM Omnisolv, purchased from E.M. Science. Steroids were from Sigma Chemical Company, USA. Water was demineralized, filtered and degassed before use. Sample solutions were made in methanol and kept at 4°C. All solvents were degassed by filteration.

A modular liquid chromatograph (LC) equipped with LDC pump and a variable wavelength UV Spectroflow 773 detector, was interfaced with a Hewlett Packard HP 3385A integrator. The column was a Whatman Partisil 10 ODS (250 x 4.6 mm). The mobile phase was acetonitrile-methanol (65:35 (v/v)). Detection was at 280 nm. Flow rate was 1 ml/min.

The column was cooled to -20°C using a cooling system provided by U.S. Coolers (Quincy, Ill). Quantitation followed intrapolation against a standard curve prepared from serial amounts of estriol under identical conditions as the samples.

Extraction procedure

Amniotic fluids are difficult to analyze by chromatography without specimen cleanup. The samples were prepared by extraction using solid phase extraction cartridges obtained from Analtech (Newark, DE) The C18 Spice cartridges were processed as outlined in Table 1. The procedure was carried out under vacuum of a water aspirator with the cartridges placed in a vacuum manifold. (Analtech, Newark, DE).

After the E3 fraction was eluted, the methanol was evaporated at 60°C using a stream of nitrogen. The residue so obtained was reconstituted in 10 µl of methanol for injection into the high performance liquid chromatograph. Recovery of 3H-estriol carried thru this procedure was routinely 91-93%.

TABLE 1

Solid Phase Extraction of Estriol from Amniotic Fluid

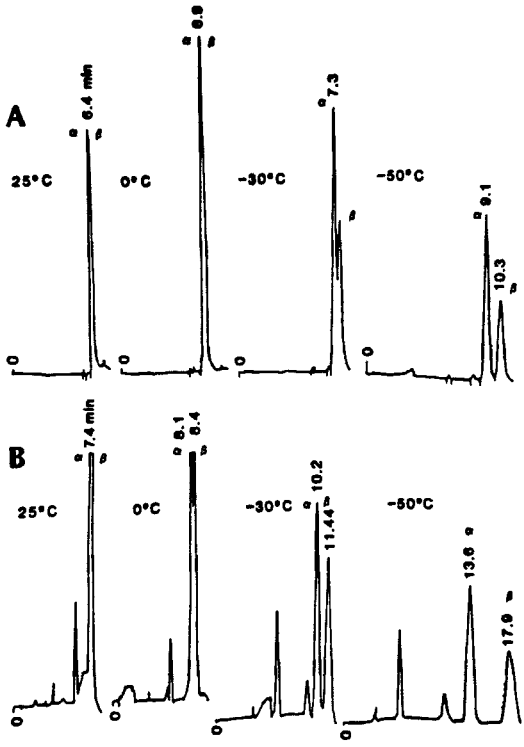
1. Conditioning - wash in sequence as follows:
   a. 2 ml of acetone - water (20:80)
   b. 2 ml of methanol
   c. methanol
2. Add 1 ml of amniotic fluid directly to the column.
3. Washing - add in sequence as follows:
   a. 2 ml of acetone-water (20:80)
   b. 2 ml of water
4. Drying - under vacuum aspirate air through the cartridge.
5. Elution of estriol follows:
   a. 1 ml of methanol
6. Evaporate in warm water bath (60°C) under nitrogen flow
7. Subject to HPLC.

Chromatography

The chromatography was carried out by injection of 5 µl of extract using a loop injector (Reodyne). For reproducible results the loop injector was necessary. The column cooled to -20°C gave improved separation of the estriol with a reasonable retention time (6).

RESULTS AND DISCUSSION

The use of subambient temperatures for separation of steroids has been described in previous work from this laboratory (6). It was deemed desirable to follow these procedures to assure confidence in the identity of the separated materials. Figure 1 shows how subambient temperatures facilitated separations of isomers of estradiol and of androstenediols (7). Figure 2 gives the results of HPLC of

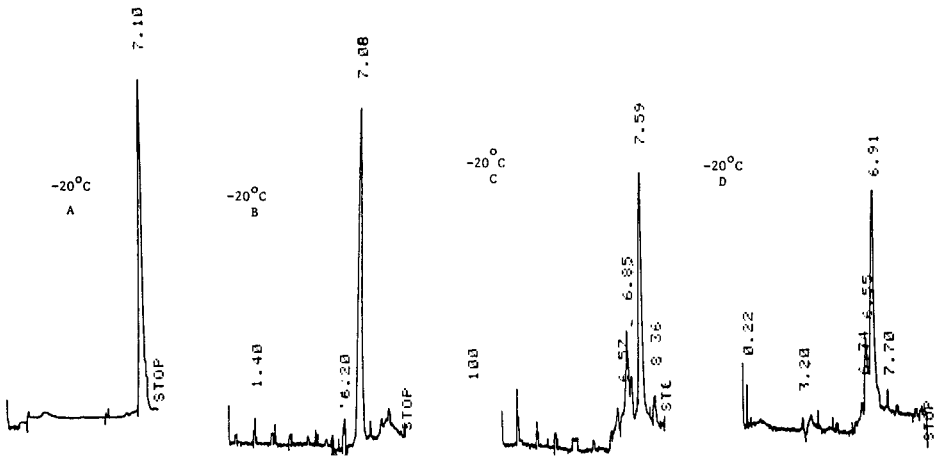


(A) Estradiol-17α and estradiol -17 β
(B) Androst-5ene-3β,17α-diol and androst-5ene-3β,17β-diol
Detection: Estrogen (280 nm) and androgen (200nm)

Figure 1: A: estradiol - 17α and estradiol - 17β
B: androst-5ene-3β, 17α-diol and androst-5ene-3β, 17β-diol

Detection: estrogen (280 nm) and androgen (200 nm)

extracts of normal amniotic fluid and those of Down syndrome as well as Trisomy - 18. The chromatograms in each case indicate clear separation of estriol. The Down syndrome and Trisomy - 18 samples did not show the presence of free estriol. The identity of the peaks separated in the chromatograms which presumably are not estriol is not as yet known. Since these peaks are not present in the normal samples,



Estriol resolved at subambient temperature (-20°C) by HPLC. Chromatograms shown are A: Standard Estriol (E_3) peak with retenion time t_RE_3= 7.10; B: Normal Amniotic fluid, C: Down's Synodrome; D: Trisomy-18.
Detection of E_3 = 280 nm.

Figure 2: Estriol resolved at subambient temperature (-20°C) by HPLC. Chromatograms shown are
A: Standard estriol (E_3) peak with retention time t_RE_3=7.10
B: Normal Amniotic fluid
C: Down syndrome
D: Trisomy-18

Detection of E3=280 nm

it might be appropriate to attempt identification and determine whether there is a relationship if any to the genetic defect in question.

As seen in Table 2 the levels of free estriol in normal amniotic fluid were variable. In some cases only trace amounts were seen. However, no free estriol was seen in the amniotic fluid from the 20 cases diagnosed as Down syndrome or the 5 Trisomy - 18 cases.

The studies carried out in this work involved only the "free" estriol. Whether an investigation of a possible role of the "conjugated" estriol would provide further

Table II

Estriol In Amniotic Fluid[1]

Normal*					Down's Syndrome			
Sex	Age weeks	No of Specimens	E_3 Conc. ng/mL	$t_R E_3$	No of Specimens	E_3 Conc. ng/mL	$t_R X$
F	15	5	245, T, T, T,	7.08	(Trisomy-21)[2] 20	Not detected	7.59
M	15	5	T, 146, 48, T, T	"			
F	16	5	77, 99, 266.2, 306, 80.1	"			
M	16	5	61.3, 110.2, 28, 150.5, T	"			
F	17	5	T, T, 485.8, 264.9, T	"			
					(Trisomy-18) 5	-	-

T = trace, tRE_3 = retention time, E_3 = estriol, - = not detected, X = unknown compound, F = female, M = male

[1]Method involving Solid phase extraction and high-performance liquid chromatography.

[2]All sexes.

*Normal reported E3 level in plasma = 93 - 560 ng/mL

Gurpide, E., Giebenhain, M.E., Tseng, L and Kelly, W.G. Am. J. Obstet. Gyne. 109:897 (1971)

insight to the genetic defect of Down syndrome is not known. A comparison of the ratios of the "free" to the "conjugate" levels might provide useful information. In conjunction with concurrent estimation of α-fetoprotein and gonadotropin the amniotic estriol could provide the clinician with additional information of the status of the fetus.

CONCLUSION

The present method is comparable with other micro analytical methods. Solid phase extraction methodology and HPLC at subambient temperatures is applicable to other biologically important compounds.

REFERENCES

1. Canick, J.A., Knight, G.J., Palomaii, G.E., Haddow, J.E., Cuckle, H.D., Wald, N.J. (1988) Low second trimester maternal serum unconjugated oestriol in pregnancies with Down syndrome. Br. J. Obstet. Gynaecol., 95:330-333.

2. Merkatz, I.R., Nitowski, H.M., Macri, J.N., Johnson, W.E. (1984) An association between low maternal serum α-fetoprotein and fetal chromosomal abnormalities. Am. J. Obstet. Gynecol., 148:886-891.

3. Darnule, A., Schmidt, D., Weyland, B., Greenberg, F., Rose, E., Alpert, E. (1990) Serum HCG, AFP and unconjugated estriol levels in trisomy 19 pregnancies in mide-trimester. Am. J. Hum. Genet., 47 [Suppl]:A272.

4. Wald, N.J., Cuckle, H.S., Densem, J.W., Nanchahal, K., Canick, J.A., Haddow, J.E. (1988a) Maternal serum unconjugated oestriol as an antenatal screening test for Down syndrome. Br. J. Obstet. Gynaecol. 95:334-341.

5. Greenberg, F., Schmidt, D., Darnule, A.I., Wayland, B.R., Rose, E., Alpert, E., (1992). Maternal serum α-fetoprotein, β-human chorionic gonadotropin and unconjugated esteriol level in mid trimester trisomy 19 pregnancies. Am. J. Obstet. Gynecol., 166 (5):1388-92.

6. Sheikh, S.U., Touchstone, J.D. (1987) HPLC of Steroids in non-aqueous mobile phases at subambient temperatures. J. Liquid Chromatogr. 10, 2489.

7. ibid, (1987) Separation of steroid diasteriomers by HPLC at subambient temperature. Anal. Chem. 59, 1472.

Received: April 21, 1994
Accepted: June 20, 1994