Journal of Chromatography, 341 (1985) 271—278
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2568

DETERMINATION OF OESTRIOL IN PREGNANCY URINE BY NORMAL-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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(First received November 13th, 1984; revised manuscript received January 17th, 1985)

SUMMARY

A method for the determination of oestriol in pregnancy urine by normal-phase high-performance liquid chromatography with electrochemical detection is described. A large-volume wall-jet cell with an $Ag—Ag^+$ reference electrode was used as the detector system. The limit of detection obtained is comparable to that of electrochemical detection following reversed-phase liquid chromatography. One of the advantages of electrochemical detection with normal-phase systems is that adsorption problems are minimized.

INTRODUCTION

During the later months of pregnancy, especially after the 12th week of gestation, large amounts of oestrogens are produced in the body and excreted [1]. The oestrogens, which are formed in the foeto-placental unit, consist mainly of oestrone, oetradiol and oestriol. Of the three, oestriol is the major oestrogen secreted. The monitoring of the levels of oestriol in urine excreted by pregnant women is a widely accepted test for determining the health of the foetus during the later stages of pregnancy [2, 3]. A deficiency in the excretion levels of oestriol is indicative of a possible malfunction of the placenta.

At present, the main method employed by hospitals for the determination of oestrone in urine is based on a highly specific Kober colour reaction [4], which indicates the total amount of oestrogen present. This can lead to an erroneous conclusion about the condition of the patient as it is really the level of oestriol that is the crucial indicator [5]. Therefore, the Kober colour reaction is usually followed by a spectrofluorimetric determination of the oestriol concentration [6].

0378-4347/85/$03.30

An alternative method to determine selectively the various oestrogen steroids would be to employ high-performance liquid chromatography (HPLC) [7]. The separated oestrogen mixture may be monitored by UV [5, 7], fluorescence or, more recently, electrochemical detection [8—13]. Reports describing the application of HPLC with electrochemical detection to oestrogen steroids have mostly involved reversed-phase systems. Claims have been made that the electrochemical detection of oestrogen steroids is about twenty times more sensitive than UV detection [11]. In contrast to numerous papers published on the electrochemical detection of oestrogen steroids following reversed-phase HPLC [5, 8, 12], little work has been reported on their detection following normal-phase HPLC. One of the few studies dealing with the latter is that of Hiroshima et al. [14]. However, their approach is completely different from that presented in this paper.

The difficulty of employing electrochemical detection in normal-phase HPLC arises from the low dielectric constant of the eluents. The choice of the supporting electrolyte and a suitable reference electrode thereby present difficulties. Indeed, several workers have precluded the use of electrochemical detection with normal-phase HPLC on these grounds [12]. Hiroshima et al. [14] solved the problem by using post-column addition of an electrolyte in a solvent with a high dielectric constant. The ratio of the volume of this conducting solution to that of the low-dielectric-constant eluent was about 3:1. Hence the solvent mixture entering the detector actually has a high dielectric constant. One of the drawbacks of using this approach is that the post-column addition of solution results in dilution in addition to an increase in the volume flow-rate. This results in a decrease in the current response because of the lower solute concentration and its decreased residence time at the working electrode. There is, therefore, a reduction in the efficiency of electrolysis and sensitivity. In the work of Hiroshima et al. [14] the reference was an Ag—AgCl system. This reference is generally not recommended for non-aqueous work because of the junction potentials that arise.

In recent work, however, it was shown that electrochemical detection in normal-phase HPLC can, in fact, be employed, with the addition of tetraalkylammonium salts as the supporting electrolyte to the eluent itself, and with the use of an Ag—Ag^+ electrode as the reference system [15]. High limits of detection for the oestrogen steroids (100 pg levels), comparable to those obtained with reversed-phase systems, can be achieved. This contrasts with the results of Hiroshima et al. [14], where detection limits were ten times lower. In this paper, we describe a method for the electrochemical detection of oestriol in pregnancy urine using a large-volume wall-jet cell (WJC). The benefits of using a large-volume WJC in amperometric detection have been discussed in previous papers [15, 16]. We also compare the oestriol levels determined by this electrochemical detection technique with those obtained using a spectrofluorimetric method.

EXPERIMENTAL

Electrochemical system

The large-volume WJC is, in principle, similar to the glass—PTFE cell used in

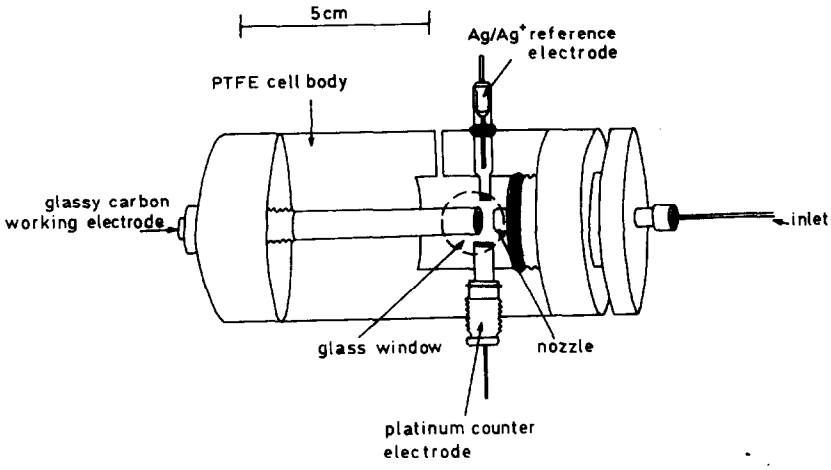

Fig. 1. Cross-section of the wall-jet cell.

previous studies [15, 16]. The main differences as as follows: (1) the cell used in this study is constructed alsmost entirely of PTFE, with the exception of a glass window press-fitted on to the cylindrical cell body; (2) the WJC in this instance operates independently of the PAR Model 303 electrode system because it has its own working, reference and counter electrodes; and (3) the cell volume is about 20 ml, compared with 35 ml for the glass-PTFE cell. Fig. 1 shows a cross-section of the PTFE WJC.

The working electrode was a 5 mm diameter glassy carbon disc (Tokai, Tokyo, Japan), which was press-fitted on to a PTFE casing. A copper lead afforded electrical contact between the glassy carbon disc and the polarographic analyser. Similarly, a 3 mm diameter platinum disc was fitted on to a PTFE casing to serve as the counter electrode. The reference electrode used was an $Ag—Ag^+$ electrode [15]. The silver wire of the reference electrode was dipped into a saturated solution of silver nitrate and tetrabutylammonium fluoroborate $[(C_4H_9)_4BF_4]$ in ethanol. The reference solution was contained in a glass tube with a ceramic frit at the end, which was in contact with the external cell solution. All potentials quoted are with respect to this reference electrode. The nozzle of the WJC was positioned 4 mm away from the working electrode to ensure the back wall of the cell did not interfere with the flow of the hydrodynamic boundary layer [16, 17]. The WJC was controlled by a PAR Model 174A polarographic analyser (Princeton Applied Research, Princeton, NJ, U.S.A.). The current output of the Model 174A was recorded on a Perkin-Elmer Model R-100 recorder (Perkin-Elmer, Norwalk, CT, U.S.A.).

HPLC system

All chromatographic separations were performed on a Perkin-Elmer Series 4 microprocessor-controlled solvent delivery system. The eluents were deaerated with helium and kept under pressure in the solvent chamber throughout the HPLC analysis. The chromatographic column used was a Lichrosorb Si 60 (10 μm) 250 × 4.6 mm I.D. normal-phase column (Merck, Darmstadt, F.R.G.) and the pressures applied were typically 6.6—6.8 MPa. The flow-rate of the eluents was set at 1 ml/min. The samples were injected through

a Rheodyne Model 7105S 175-μl loop injector valve (Rheodyne, Berkeley, CA, U.S.A.) using a 10-μl syringe. The connection between the chromatographic column and the WJC was 10 cm × 0.16 cm I.D. PTFE tubing.

Chemicals

All reagents and chemicals were of analytical-reagent grade and used without further purification. The HPLC eluents used were *n*-hexane and ethanol (Merck). The $(C_4H_9)_4BF_4$ supporting electrolyte was prepared at a concentration of 0.05 *M* in the ethanol eluent, which also contained 0.5% ammonia solution (Ajax Chemicals, Sydney, Australia). The oestrogen standards (oestrone, oestradiol and oestriol), obtained from Sigma (St. Louis, MO, U.S.A.), were prepared in methanol.

Sample collection

The pregnancy (38—40 weeks) urine samples were supplied by Kandang Kerbau Hospital, Singapore.

Sample preparation

The hot acid hydrolysis of pregnancy urine (20 ml) was carried out according to the procedure described by Gelbke et al. [18]. After the hydrolysis and extraction steps, the extracts were evaporated to dryness under vacuum and the residue was dissolved in 1 ml of methanol.

RESULTS AND DISCUSSION

Calibration for oestrone, oestradiol and oestriol

Based on a previous study carried out using normal-phase HPLC, the three oestrogen standards were satisfactorily resolved with *n*-hexane—ethanol (80:20) as the eluent. The optimum potential applied to the working electrode was determined to be +0.4 V versus $Ag—Ag^+$. Under these operating conditions, a detection limit of the order of picomoles can be achieved, which is comparable to those quoted for reversed-phase electrochemical detection.

As the oestriol excretion level in urine samples is expected to be of the order of tens of micromoles per day, the calibration plots for the three oestrogen steroids were obtained at nmol/μl levels. Fig. 2a shows a typical electrochemical chromatogram of the three-oestrogen mixture obtained by normal-phase HPLC, and Fig. 2b shows the calibration graphs for the three oestrogen standards at the nanomole level. The reproducibility for successive injections of the oestrogen mixture was determined to be within a relative standard deviation of 3%.

Recovery studies

In order to characterize the efficiency of the sample preparation technique used, a recovery test was carried out by adding known amounts of oestrogen standards to 20-ml volumes of water and male urine samples. The oestrogen standards were then recovered by the hot acid hydrolysis and extraction steps described earlier. The recoveries of the three oestrogens were then measured using the large-volume WJC following normal-phase HPLC. Fig. 3a and b shows

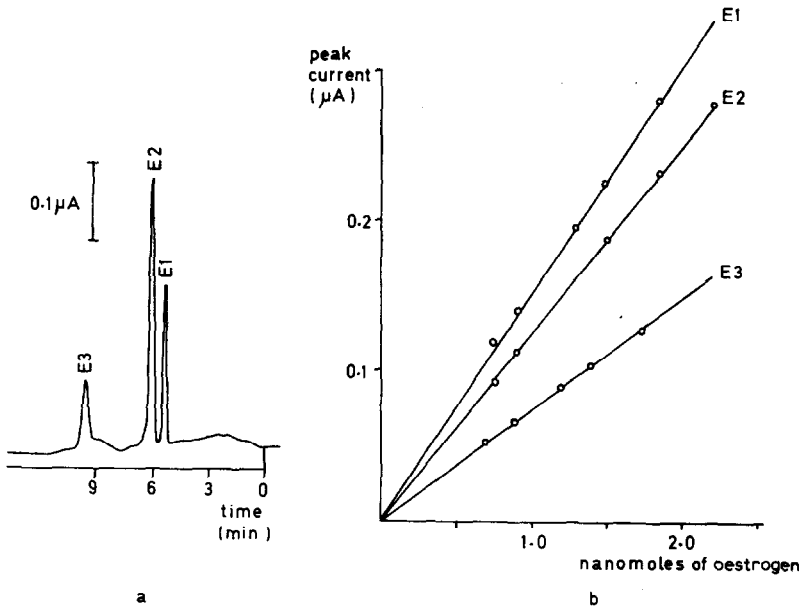

Fig. 2. (a) Chromatogram of the standard oestrogen mixture with electrochemical detection. Oestrone (E1) = 1.85 nmol; oestradiol (E2) = 3.7 nmol; oestriol (E3) = 1.74 nmol. Eluent, *n*-hexane—ethanol (80:20); flow-rate, 1.0 ml/min; working potential, +0.4 V. (b) Calibration graph for oestrogen standards.

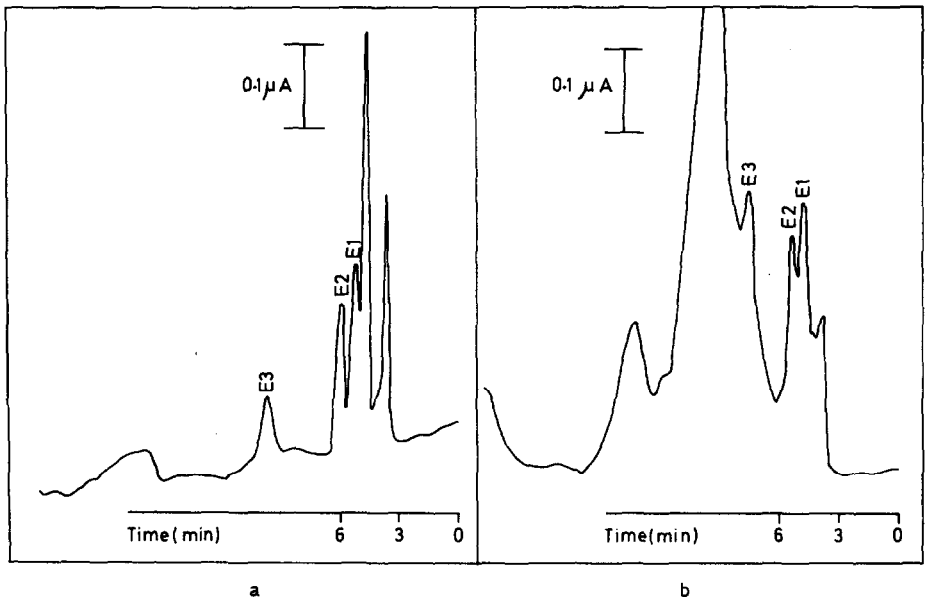

Fig. 3. Chromatogram of the recovered oestrogens in (a) a water sample and (b) a male urine sample with electrochemical detection. Conditions as in Fig. 2. E1 = oestrone; E2 = oestradiol; E3 = oestriol.

the electrochemical chromatograms of the recovered oestrogens in water and male urine samples, respectively. The oestrogen peaks in the water sample were much better resolved than those in the male urine sample, because of the greater number of interfering peaks present in the latter. The recoveries of the oestrogens in both samples are given in Table I. The slightly higher recovery for the water sample is in agreement with the work of Gelbke et al. [18] on catechol oestrogens. The recovery of oestriol in both water and urine samples was the greatest of the three oestrogen steroids studied. Although the recoveries of oestrone and oestradiol were lower, the sample preparation technique used may be judged to be reasonably efficient for the extraction of oestrogen steroids from urine samples.

TABLE I

RECOVERY OF OESTRONE, OESTRADIOL AND OESTRIOL ADDED TO WATER AND MALE URINE SAMPLES (n = 3)

Sample	Recovery (%)		
	Oestrone	Oestradiol	Oestriol
Water	68	76	91
Male urine	64	70	86

Analysis of pregnancy urine

A typical electrochemical chromatogram of a pregnancy urine extract is shown in Fig. 4. The peaks obtained for oestrone and oestradiol are much smaller than the oestriol peak. Also, the oestrone and oestradiol peaks appear to be very poorly resolved on the shoulder of an earlier eluting peak. The poor resolution of both peaks makes quantification difficult. The oestriol levels in pregnancy urine samples are given in Table II, where the results obtained by

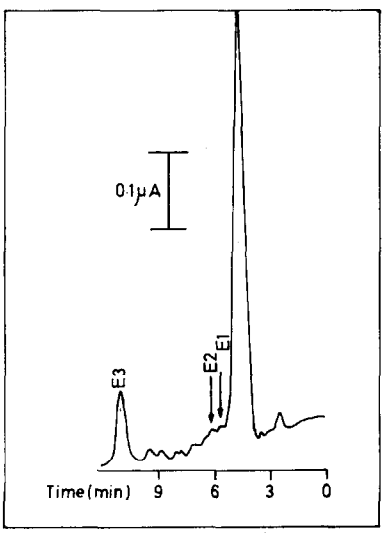

Fig. 4. Chromatogram of a pregnancy urine sample with electrochemical detection. Conditions as in Fig. 2. E1 = oestrone; E2 = oestradiol; E3 = oestriol.

TABLE II

LEVELS OF OESTRIOL IN NORMAL PREGNANCY

Subject	Weeks of gestation	Oestriol determined (μmol/day)	
		Electrochemical detection	Fluorescence detection
A	38	112.7	109.7
B	39	65.1	65.8
C	39	93.7	96.6
D	40	141.6	140.3
E	40	70.5	68.7
F	40	125.7	123.8
G	40	105.8	100.6

normal-phase electrochemical detection are compared with those obtained by spectrofluorimetry (excitation wavelength 520 nm, emission wavelength 550 nm) [7].

Potential selection

As the working potential is lowered, the peak heights of the three oestrogens decrease and the resolution is also reduced. This presumably occurs because the interfering peaks are due to compounds that have a less positive oxidation potential than the three oestrogens. Therefore, in this application, potential selection is not very useful. Although increasing the working potential generally enhances the peak height, it also results in a less stable background. In this work, we found that +0.4 V was an optimum potential that gave stable backgrounds and high sensitivity.

CONCLUSION

This work has shown that normal-phase HPLC with electrochemical detection can be used successfully to determine the oestriol excretion levels in pregnancy urine. The detection limits compare favourably with those reported for electrochemical detection following normal-phase separation and the adsorption problems are significantly less. The difficulty in quantifying oestrone and oestradiol levels in pregnancy urine is chromatographic in nature rather than a detector problem.

ACKNOWLEDGEMENTS

The award of a University Research Scholarship to B.T.T. and a Research Grant to K.P.A. and H.G. from the University are gratefully acknowledged. The authors are indebted to Prof. S.S. Ratnam, Dr. S. Arulkumaran and Mr. Stephen Koh of the Kandang Kerbau Hospital, Singapore, for the supply of urine samples. Thanks are due to Ms. Irene Teo for valuable technical assistance.

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