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REVIEW

SEPARATION OF OESTROGEN CONJUGATES IN URINE AND SYNTHETIC MIXTURES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHODS

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CONTENTS

1. INTRODUCTION

The assay of oestrogens in biological fluids has a number of clinical applications, the chief one being the determination of oestrogen levels in maternal urine (or blood) in order to aid the assessment of the intrauterine viability of the foetus [1]. As a result of the importance of these applications, there now exists such a considerable literature on the subject of the methodologies available for this assay that it is being reviewed annually by one of us [2]. The most commonly determined analyte is the 24-h urinary total oestrogen excretion level and this has been statistically correlated with week of gestation for "normal" pregnancies [3] leading to the production of reference ranges

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suitable for clinical use. In our latest paper [4] on the subject of this assay we listed a number of the analytical difficulties experienced with it. Now, it should be noted that whilst the oestrogens are excreted almost totally as conjugates, they are normally assayed as free oestrogens (produced by a suitable hydrolysis procedure) which assists the analyst by reducing the molecular complexity of the oestrogens in the biological matrix.

There have been a number of detailed studies of the contributions made by the various oestrogens to 24-h urinary total oestrogen excretion rates. As a result of these investigations it is generally agreed that, for the three classical oestrogens (oestrone, oestradiol and oestriol), oestriol accounts for more than 90% of their total excretion rate in late human pregnancy [5]. Moreover, a recent Belgian study [6] has shown that this total oestriol excretion level is made up of contributions from the following four conjugates whose approximate percentages (in oestriol equivalents) are given in parentheses: oestriol-16-glucuronide (67%); oestriol-3-glucuronide (21%); oestriol-3-sulphate-16-glucuronide (8%) and oestriol-3-sulphate (3%)[*]. It should perhaps be noted that less than 1% of the total oestriol excretion rate was ascribed to the free steroid. It is logical to predict that analysis of the individual intact oestrogen conjugates instead of total oestrogens would give results having a higher clinical efficiency (i.e. fewer false positive and false negative results) for present applications of oestrogen assays. Further, such multicomponent analysis (*complete* oestrogen conjugate profiling) might also prove of value in the diagnosis of additional abnormal conditions. At present the only published approach to this problem of multicomponent analysis of complex biological fluids has involved the use of the sophisticated, expensive and time-consuming technique of combined gas chromatography—mass spectrometry. Before this analytical technique can be employed, the conjugates have first to be chromatographically separated, then hydrolysed to form free oestrogens which are then silylated in order to make suitable volatile derivatives [7]. This type of method is clearly unsuitable for general routine clinical use.

In contrast, the modern technique of high-performance liquid chromatography (HPLC) has the ability to separate and quantitate complex aqueous mixtures in short periods of time (min), with little or no pre-treatment of sample, and is, therefore, easily automated for routine use. Theoretically, it should be possible to devise an HPLC system which can separate the four major oestriol conjugates of pregnancy urine listed above. If this *partial* oestrogen conjugate profiling and individual assay could be achieved, then the routine assay of the total oestriol content would follow and the problems of poor precision encountered with the present common methods (for total oestrogens) [8] would be obviated. For these reasons, we have closely examined the published literature [2] on the subject of HPLC analysis of the oestrogen conjugates in general and of the above four oestriol conjugates in particular in order to determine if a system suitable for routine use has yet been published. Further, we have abstracted the key experimental details in a unified manner (Tables 1—3) to aid assessment of the present state of the

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[*] These four oestriol conjugates are abbreviated to E_3-16-G, E_3-3-G, E_3-3-S-16-G and E_3-3-S, respectively, for the remainder of the paper.

art and hence to identify possible areas for improvement. The results of these combined bibliographic—abstracting studies will now be presented and discussed.

2. SURVEY OF REPORTED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATIONS OF OESTROGEN CONJUGATES

Tables 1—3 contain the key experimental details for a total of eighteen HPLC separations of one or more of the four oestriol conjugates discussed above (and of certain other oestrogen conjugates) abstracted from a total of fourteen papers. The elution order data shown in the tables is taken from chromatograms presented by the various authors. The separations are divided into three tables so as to group them according to the apparent mechanism of separation. The papers are arranged in chronological order within each table and each of these will now be discussed in turn.

2.1. Anion-exchange chromatography

Table 1 summarizes the experimental details, abstracted from four publications [9—12], of six separations of mixtures of oestrogen conjugates obtained using columns of appropriate support materials. The latter may be divided into two types: the first being formed of chemically modified celluloses (Table 1, refs. 9, 10 and 11); the second is Partisil SAX (Table 1, ref. 12), which is a silica gel bonded with a quaternary ammonium functional group.

A study of the elution order data given in Table 1 shows that all of these anion-exchange columns can indeed separate mixtures of oestrogen conjugates. Each of the synthetic mixtures studied contained at least one of the three oestriol mono-conjugates of interest, but unfortunately none of them contained the mixed di-conjugate E_3-3-S-16-G, probably because it is not commercially available. However, theoretically there should be no difficulty in separating this di-conjugate from the three mono-conjugates by anion-exchange chromatography because it should elute much later than any of the latter. Experimental evidence indicating the validity of this statement is afforded by the numerical anion-exchange chromatographic data for the di-conjugate, oestradiol-3-sulphate-17-glucuronide (see Table II of ref. 11).

We will now consider the separations achieved with these two types of anion-exchange material starting with the most extensively studied, the modified celluloses. Of the five separations of oestrogen conjugates shown in Table 1 using columns of these support materials [Table 1, refs. 9, 10 and 11], all were operated at relatively low eluent pressures because of the non-rigid nature of the support and this led to the generally long elution times listed in the table. The highest elution pressure employed with these columns (36 bar) was achieved by the expedient of mixing a (rigid) diatomite powder with the cellulose. A study of the separations reported in ref. 11 leads to the conclusion that all four oestriol conjugates under discussion should be separable from aqueous synthetic mixtures using anion-exchange cellulose columns operated under HPLC conditions. Indeed, a chromatogram presented in this paper, and abstracted in Table 1, probably does show separation of these four oestriol

TABLE 1

SEPARATION OF OESTROGEN CONJUGATES BY ANION-EXCHANGE CHROMATOGRAPHY

	Ref. 9	Ref. 10
Sample	Synthetic	Synthetic
Column		
Support	ECTEOLA-cellulose (Baker 300)	ECTEOLA-cellulose (Baker 300)—diatomite (5:1, v/v)
Particle diameter (μm)	13	7 for Baker 300
Length (cm)	25	10
I.D. (mm)	3	4
Temp. (°C)	25	70
Elution		
Pressure (bar)	NG*	36
Flow-rate (ml/min)	NG	NG
Mobile phase	0.125 *M* sodium chloride + 0.05 *M* sodium acetate, pH 5.0	0.025 *M* perchlorate + 0.01 *M* phosphate, pH 6.8
Detection	UV, 220 nm	UV, 220 nm
Elution time** (min)	14	28
Elution order***	E_3-3-G, E_1-3-G, E_2-3-G, E_3-16-G, E_2-17-G	E_1-3-G, E_3-3-S, E_1-3-S, 17α-E_2-3-S, 17α-Eq-3-S, Eqe-3-S, 17α-Eqe-3-S

*NG = not given.

**Elution time is the time taken for the last oestrogen to elute off the column.

***E_1 = oestrone, E_2 = oestradiol, E_3 = oestriol, Eq = equilin, Eqe = equilenin, G = glucuronide, S = sulphate.

conjugates since the sample applied was an Amberlite XAD-2 methanol extract of pregnancy urine. Six Kober-positive (i.e. oestrogenic steroids) peaks were obtained after an elution time of 75 min, four of which may be ascribed to the four oestriol conjugates. The two additional peaks could possibly be due to oestrone-3-glucuronide and 16-hydroxyoestrone-3(?16α)-glucuronide as these are excreted at comparable rates to the oestriol sulphate-glucuronide [13]. The finding of fourteen low ultraviolet- (UV)-absorbing peaks (220 nm) from the same urine sample in the same time period indicates that the modified cellulose columns are capable of considerable separation of multicomponent mixtures such as urine.

Interestingly, from a combined study of the abstracted elution order data in Table 1 and retention data for similar systems reported in the corresponding references, it is found that the elution order of oestrogen mono-glucuronides from cellulose anion-exchangers is primarily dependent on the site of conjugation since the steroid A-ring conjugated oestrogens tend to elute before those conjugated at the steroid D-ring. (It should perhaps be noted that the same

Ref. 11 (i)	Ref. 11 (ii)	Ref. 11 (iii)		Ref. 12
Synthetic	Synthetic	Pregnancy urine XAD-2 extract		Synthetic
ECTEOLA-cellulose (Baker 300)	ECTEOLA-cellulose (Whatman ET 41)	ECTEOLA-cellulose (Baker 300)		μPartisil 10 SAX
13	11	11		10
25	25	25		25 × 2
3	3	3		4.6
25	70	70		NG
16	30	20		83
NG	NG	NG		0.8
0.025 *M* perchlorate + 0.01 *M* phosphate, pH 7.0	0.25 *M* perchlorate + 0.01 *M* phosphate, pH 8.5	0.025 *M* perchlorate + 0.01 *M* phosphate, pH 6.8		0.1 *M* sodium chloride pH 4.8
UV, 220 nm	UV, 220 nm	UV, 220 nm	Kober, fluorimetric	UV, 254 nm
25	31	75	75	23
E_3-3-G, E_1-3-G, E_2-3-G, E_3-16-G, E_2-17-G	E_3-3-S, E_1-3-S, Eq-3-S, 17α-Eq-3-S, Eqe-3-S, 17α-Eqe-3-S	14 peaks None of the peaks identified	6 peaks	(E_3-16-G, E_3-17-G), E_3-3-G, E_2-17-G, E_2-3-G, E_1-3-G, E_2-3-S, E_1-3-S

order of elution for these conjugates has been reported for a non-HPLC separation using a modified cross-linked dextran as the anion exchanger [7].) Further, within the group of oestrogen ring-A glucuronides the elution order is dependent upon the exact nature of the oestrogen moiety and is as follows: oestriol, oestrone, oestradiol. In addition, both of these types of relationship between structure and elution order are also found to hold for the series of classical oestrogen mono-sulphates [11]. Oestrogens conjugated with sulphate groups are invariably eluted after the corresponding glucuronide conjugates. To summarize, the expected elution order of the four oestriol conjugates under discussion from the modified cellulose anion-exchangers given in Table 1 is E_3-3-G, E_3-16-G, E_3-3-S, E_3-3-S-16-G.

The second type of anion-exchange chromatographic system abstracted in Table 1 consists of a single separation of a synthetic mixture of oestrogen conjugates obtained by the use of two columns of Partisil SAX arranged in series [12]. The mixture separated only included two of the four oestriol conjugates of interest (both glucuronides). Interestingly, a study of the

TABLE 2

SEPARATION OF OESTROGEN CONJUGATES BY ION-PAIR CHROMATOGRAPHY

	Ref. 14	Ref. 15 (i)	Ref. 15 (ii)	Ref. 16	Ref. 17
Sample	Synthetic	Synthetic	Synthetic	Synthetic	Pregnancy urine XAD-2 extract
Column					
Support	LiChrospher Si 100 (coated with 0.1 *M* TEABr*, pH 7.4)	LiChrosorb RP-18	LiChrosorb RP-18	LiChrosorb RP-18 (coated with *n*-pentanol)	Hypersil ODS
Particle diameter (μm)	10	5	5	10	5
Length (cm)	NG**	15	15	15	12.5
I.D. (mm)	NG	3	3	3.2	4.5
Temp. (°C)	25	70	70	25	NG
Elution					
Pressure (bar)	30	250	250	23	NG
Flow-rate (ml/min)	NG	NG	NG	0.27	1
Mobile phase	Dichloromethane—*n*-pentanol (9:1, v/v) saturated with TEABr	0.05 *M* phosphate pH 8.0, + 0.1% (w/v) CTMABr***—acetonitrile (6:4, v/v)	0.02 *M* phosphate pH 5.0, + 0.1% (w/v) CTMABr—methanol (3:7, v/v)	Sodium phosphate buffer, ionic strength 0.1, pH 6.4, saturated with *n*-pentanol + 0.041 *M* TPABr§	0.05 *M* phosphate pH 8.0, + 0.1% (w/v) CTMABr—methanol (5:5, v/v)
Detection	UV, 254 nm	UV, 223 nm	UV, 223 nm	UV, 254 nm	UV, 220 nm
Elution time§§ (min)	6	2	4	20	13 (for E_3-16-G)
Elution order§§§	E_2-3-S, E_2-17-S, E_3-17-S, E_3-3-S	E_3-3-G, E_3-17-G, E_2-17-G, E_1-3-G, E_3-3-S	E_3-3-S, E_2-17-S, E_2-3-S, 17α-Eqe-3-S, E_1-3-S	E_3-17-G, E_1-3-G, E_2-3-G, E_2-17-G	E_3-3-G, E_3-16-G, resolved from 7 other (unidentified) peaks

*TEABr = tetraethylammonium bromide.

**NG = not given.

***CTMABr = cetyltrimethylammonium bromide.

§TPABr = tetrapropylammonium bromide.

§§Elution time is the time taken for the last oestrogen to elute off the column.

§§§E_1 = oestrone, E_2 = oestradiol, E_3 = oestriol, G = glucuronide, S = sulphate.

tabulated chromatographic data for oestrogen mono-glucuronides shows that the specific nature of the oestrogen moiety is the major determinant of the elution order since these conjugates are eluted in the general order oestriol, oestradiol, oestrone. This is in contrast to the aforementioned finding for separations performed using columns of modified celluloses as for these the site of conjugation is of primary importance. Further, even the elution order of the two oestriol glucuronides of interest from Partisil SAX differs from that obtained using the modified cellulose anion exchangers; i.e. E_3-16-G is eluted before E_3-3-G. But in all of the anion-exchange systems it is found that glucuronides are eluted before the corresponding sulphates.

2.2. Ion-pair chromatography

Table 2 summarizes the experimental details, abstracted from four publications [14—17], of five separations of mixtures of oestrogen conjugates obtained using columns operated in ion-pair chromatographic modes. We shall first consider the three separations abstracted in Table 2 involving the use of columns of octadecyl-silica support materials and cetyltrimethylammonium bromide (CTMABr) as the ion-pair forming surfactant. The table of abstracted data shows that these systems separate synthetic and natural mixtures of oestrogen conjugates in much shorter periods of time (2—13 min) than that required for the previous group of separations carried out using modified celluloses. This finding is a direct consequence of the use of modern rigid support materials which can be operated at elution pressures about an order of magnitude greater than those compatible with the modified cellulose columns.

Considering now the application of these chromatographic systems to the problem of separating the four oestriol conjugates under discussion, a study of Table 2 shows that only synthetic mixtures containing a maximum of two of these conjugates have been studied. The first of these separations abstracted in the table shows that it is possible to separate E_3-3-G, oestriol-17-glucuronide (E_3-17-G) and E_3-3-S from a synthetic mixture within 2 min. The paper from which this is abstracted is ref. 15, and a detailed study of this paper showed that it contains numerical chromatographic data (Table III of ref. 15) which indicate that E_3-17-G co-elutes with E_3-16-G. Thus, it is predicted that an aqueous synthetic mixture of E_3-3-G, E_3-16-G and E_3-3-S would be separated, in this order, within 2 min by this chromatographic system. Interestingly, in the application of this type of high-speed separation to the analysis of pregnancy urine [17], two of the nine UV-absorbing chromatographic peaks eluted within 13 min were assigned to E_3-3-G and E_3-16-G (see Fig. 1). A comparative study of the elution order data for the chromatographic systems abstracted in Table 2 that employ CTMABr leads to the following three correlations. First, that the oestriol glucuronides are eluted before the oestriol sulphates and, secondly, that oestriol ring-A conjugates are eluted before the ring-D conjugated isomers. Additional support for these elution orders is afforded by the tabulated numerical data given in ref. 15. Thirdly, this same tabulated data also indicate that, within the two groups of classical oestrogen mono-sulphates and mono-glucuronides, the elution order is primarily

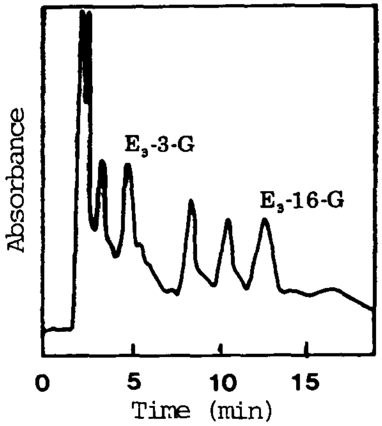

Fig. 1. HPLC chromatogram of a pregnancy urine extract. Re-drawn from Fig. 2. of ref. 17.

TABLE 3

SEPARATION OF OESTROGEN CONJUGATES BY REVERSED-PHASE CHROMATOGRAPHY

	Ref. 18	Ref. 15 (iii)	Ref. 19
Sample	Synthetic	Synthetic	Pregnancy urine
Column			
Support	μBondapak C_{18}	LiChrosorb RP-18	LiChrosorb RP-8 (coated with *n*-pentanol)
Particle diameter (μm)	10	5	5
Length (cm)	30	15	15
I.D. (mm)	6.4	3	4.5
Temp. (°C)	30.5	70	25
Elution			
Pressure (bar)	NG*	250	NG
Flow-rate (ml/min)	0.89	NG	NG
Mobile phase	H_2O—methanol (9.34:0.66, w/w)	0.05 *M* phosphate, pH 8.0—acetonitrile (8:2, v/v)	Phosphate buffer, ionic strength 0.1, pH 6.5—*n*-pentanol (9.81:0.19, v/v)
Detection	Refractive index	UV, 220 nm	UV, 280 nm
Elution time*** (min)	(34 or 54)?	3	13 (for E_3-16-G)
Elution order§	E_3-3-G, E_3-3-S, E_3-16-G	E_3-3-G, E_3-3-S, E_3-17-G, E_2-3-G, E_1-3-G	E_3-16-G resolved from 12 other (unidentified) peaks

*NG = not given.

**THF = tetrahydrofuran.

***Elution time is the time taken for the last oestrogen to elute off the column.

§E_1 = oestrone, E_2 = oestradiol, E_3 = oestriol, G = glucuronide, S = sulphate.

determined by the exact nature of the oestrogen moiety and is oestriol, oestradiol, oestrone.

The remaining two separations given in Table 2 [14, 16] are of synthetic mixtures of oestrogens reported in two separate papers and obtained using columns of 10-µm support materials and tetraethylammonium bromide and tetrapropylammonium bromide as the ion-pair reagents. The first paper reports a separation involving an oestriol conjugate of direct interest, namely E$_3$-3-S, and the second paper reports a separation of E$_3$-17-G. In view of the previous discussion of separations involving this type of chromatography, it would seem likely that this second separation would also have separated an oestriol conjugate of interest (E$_3$-16-G) had it been present in the test mixture.

2.3. *Reversed-phase chromatography*

Table 3 summarizes the experimental details, abstracted from seven publications [15, 18—23], of separations of mixtures of oestrogen conjugates obtained

Ref. 20	Ref. 21	Ref. 22	Ref. 23
Pregnancy urine (diluted)	Synthetic	Pregnancy urine (+ internal std., 16-epiE$_3$-17-G) extract	Synthetic
Spherisorb ODS	LiChrosorb RP-18	TSK gel LS -410 ODS-SIL	TSK gel LS-410 ODS-SIL
5	5	5	5
1.5	25	30	30
6.4?	9	4	4
21	NG	Ambient	Ambient
NG	172	NG	NG
0.4	2	1	1.5
Water—acetonitrile—acetic acid (26.5:6:2, w/w)	Convex gradient: 10% methanol in 0.01 *M* ammonium acetate, pH 6.9, to 100% methanol	0.05 *M* Na$_2$HPO$_4$, pH 3.0—THF** (6:1, v/v)	0.05 *M* Na$_2$HPO$_4$, pH 3.0—THF (6:1, v/v)
Kober, fluorimetric	UV, 280 nm	Electrochemical	UV, 280 nm
22	38	56 (16-epiE$_3$-17-G)	61
E$_3$-3-S?, E$_3$-3-G?, E$_3$-16-G	E$_3$-3-G, E$_3$-3-S, E$_3$-16-G, resolved from 12 other oestrogen peaks	E$_3$-16-G 16-epiE$_3$-17-G, resolved 6 other (unidentified) peaks	E$_3$-3-G, 16-epiE$_3$-3-G, E$_3$-17-G, E$_3$-16-G, 16-epiE$_3$-17-G, 16-epiE$_3$-16-G

using reversed-phase column chromatographic methods. As was found to be the case with all the previously discussed separations, none of the synthetic mixtures chromatographed included all four oestriol conjugates of interest. However, the first and the fifth of the separations listed in this section included all but the mixed oestriol di-conjugate. The two separations performed using columns of TSK gel at a pH of 3.0 [22, 23] are superior to those discussed previously in that they are able to separate E_3-16-G from E_3-17-G when both are present in a synthetic mixture or biological fluid, albeit after a long elution time (about 30—45 min). Indeed, the authors of one of these two abstracted papers [22] used this HPLC system to confirm that human pregnancy urine does not contain E_3-17-G, whilst bile from rats fed large amounts of oestriol was shown to contain significant quantities of both mono-conjugates. The resolution of the TSK gel column with respect to the separation of these isomeric oestriol glucuronides progressively decreased as the pH was raised [23]. Another separation of human urine performed on a reversed-phase column and abstracted in Table 3 (ref. 19) gave a complex UV chromatogram with a total of thirteen peaks resolved in 13 min, one of which was identified by the author as E_3-16-G (see Fig. 2). Again, the production of such a complex chromatogram indicates the high resolving power of these columns.

The second of the reversed-phase separations listed in Table 3 is a high-speed separation of three oestriol conjugates from a synthetic mixture of five oestrogen conjugates, achieved in approximately 1 min within a total elution time of only 3 min. This separation may be compared in terms of order of elution with the two rapid separations abstracted in Table 2 from the same paper and with the numerical chromatographic data which were also reported. When this was done it shows that the elution order of the conjugates off the reversed-phase column in the absence of CTMABr is E_3-3-G, E_3-3-S, E_3-17-G.

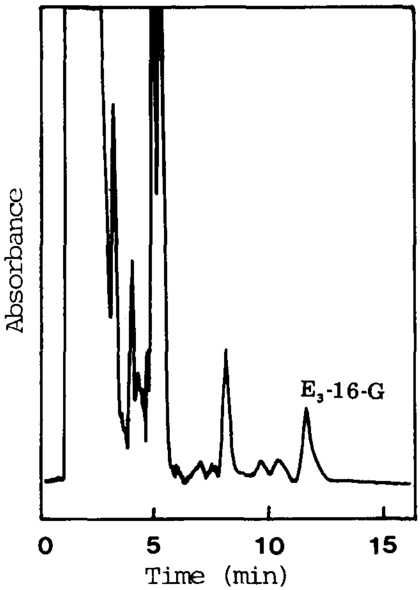

Fig. 2. HPLC chromatogram of pregnancy urine. Re-drawn from Fig. 4 of ref. 19.

This elution order differs from that found in the presence of the surfactant, which is E_3-3-G, E_3-17-G, E_3-3-S. (In both chromatographic systems the isomeric 16- and 17-glucuronides co-elute.) This difference in elution order of the oestriol conjugates means that the first of the correlations between elution order and molecular structure found for the previous type of chromatography does not apply to the present reversed-phase system. However, a study of the numerical elution order data for the reversed-phase system shows that the other two correlations are still applicable.

3. DISCUSSION ON THE APPLICATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY TO THE ROUTINE ASSAY OF OESTRIOL CONJUGATES

Although we are aware that there have been a number of reviews published recently which contain sections on the HPLC of oestrogen conjugates [24—26], the present account is considered distinctive because of the methodological details given in Tables 1—3 and for its delineation of the problem of routine analysis of the four major oestrogen conjugates present in human pregnancy urine (*partial* oestrogen conjugate profiling). To proceed, we shall first discuss the recent work of an American group which has devised a complex, multicolumn HPLC separation system for an extensive oestrogen conjugate profile (together with free oestrogens) of pregnant monkey urine [21]. The initial chromatographic stage in this system involves the use of a LiChrosorb RP-18 column operated in a convex gradient elution mode (Table 3, ref. 21). The resultant eluate was collected in six fractions which were evaporated to near dryness, reconstituted in the appropriate mobile phase and aliquots then subjected to further chromatography involving selective use of one of four HPLC systems. Even though synthetic mixtures of E_3-3-G, E_3-3-S and E_3-16-G can be separated by this method, we believe that such a complex analytical system is not suitable for routine clinical use and it will not therefore be considered further.

In addition to the above complex, multicolumn separation, another American research group published earlier (1978) a simple isocratic HPLC system identical to that given in Table 1 (ref. 12), in which seven oestrogen conjugates (from a total of eight) were separated from a spiked urine extract. Indeed, in the list of oestrogens separated were two of the oestriol conjugates of interest, namely E_3-16-G and E_3-3-G. Unfortunately, the authors did not demonstrate any application of this method to the analysis of human pregnancy urine. Consequently, it is not immediately clear whether this method would be suitable for adoption by clinical laboratories. However, upon further study of the paper it became apparent that the method possesses a certain practical disadvantage which would seem to rule out its routine application, namely corrosion of the steel column and tubing by the sodium chloride in the eluent.

In the discussion now to follow, attention will be concentrated on those five papers in which one (E_3-16-G) or more of the oestriol conjugates have been separated from human pregnancy urine, in order to ascertain if any of the published methods could be recommended as a candidate for adoption as a routine assay procedure. Complete details of the HPLC methods employed have been abstracted and are given in Tables 1—3. These methods may be divided into two groups; namely, those in which the urine, either neat or diluted, is applied directly to the column (two methods), and those in which the urine is pre-treated before HPLC (three methods). If it is assumed that the various HPLC separations are equivalent, then, from the point of view of suitability as a routine assay procedure, the former methods are preferred and these will first be discussed in detail below.

The method to be initially considered is that published by Hermansson in 1980 [19]. In this procedure, 20 μl of untreated pregnancy urine were applied directly to a reversed-phase column (LiChrosorb RP-8) coated with *n*-pentanol by the expedient of using 1.9% (v/v) of this alcohol in phosphate buffer, pH 6.5, as the mobile phase. The resultant complex chromatogram (Fig. 2) obtained by using a UV detector operating at 280 nm included a well resolved peak, elution time about 13 min, which was assigned, on the basis of a co-chromatographic and mass spectroscopic study, to the most abundant oestrogen conjugate E_3-16-G. Apparently, therefore, the problem of measuring this conjugate routinely by HPLC would seem to be solved. However, when this method was further investigated in the authors' laboratory it became clear that the separation, in terms of elution time, is critically dependent upon the column temperature and is of limited use for studying pregnancy urines because of its low sensitivity [27]. Further, when an attempt was made to improve the latter by changing the monitoring wavelength from 280 to 215 nm, the previously clearly separated E_3-16-G peak became part of a new large non-resolved low UV-absorbing peak profile. For these reasons it is concluded that this method is not suitable for the routine assay of the E_3-16-G content of human pregnancy urine.

The second reported HPLC separation of oestriol conjugates present in human urine is that published in short note form by Keravis and Durand in 1980 [20]. In this method, a small aliquot of a diluted urine specimen is applied to a short column of Spherisorb ODS which is then eluted with a water—acetonitrile—acetic acid mixture. The eluate is then subjected to an on-line Kober fluorimetric procedure which leads to the production of a simple, completely resolved chromatogram containing a total of only three peaks which can be assigned to the following oestriol conjugates: E_3-16-G, E_3-3-S, E_3-3-G. Unfortunately, because of the brevity of the paper, this assignment of the peaks in the chromatogram and the exact chromatographic conditions are somewhat ambiguous (as indicated in Table 3). Further, the authors appear only to have measured E_3-16-G in the urine specimens studied even though they should have been able to measure all of the three conjugates listed above. Primarily, therefore, for these reasons this method cannot at present be recommended for routine HPLC assay of these oestriol conjugates in pregnancy urine.

The first of the HPLC studies of pre-treated pregnancy urines to be considered is that reported very recently by Shimada et al. [22]. In this paper the Japanese workers adopted the following complex pre-treatment procedure: first the conjugates present in samples of late-pregnancy urine are adsorbed onto a column of Amberlite XAD-2 resin which is then washed with water, eluted with methanol and the resultant eluate is evaporated to dryness and the residue reconstituted in a small (?) volume of methanol, which is then subjected to ion-exchange chromatography using a column of modified Sephadex (PHP-LH-20). The oestrogen conjugates were removed from this column by eluting with 2.5% ammonium carbonate in 70% methanol and this fraction was evaporated to dryness before reconstitution in methanol. An aliquot of the resultant solution was then subjected to HPLC under the conditions tabulated in Table 3. The predominant oestriol conjugate, E_3-16-G, was separated after an elution period of some 45 min. When samples of the pure mono-glucuronide were added to non-pregnancy urines, which were then analysed by the above method, recovery figures of about 80% were found. It should be noted that this method involved the use of an electrochemical detector; it does not, therefore, detect any of the other oestriol conjugates of interest since these molecules do not contain the requisite free phenolic group. It is not possible to state if this method separates more of the oestriol conjugates from urine until the experiments have been repeated using a suitable UV detector. The present authors have not performed these repeat experiments primarily because we are of the opinion that the complex sample pre-treatment procedure, together with its long HPLC elution time, rules out the adoption of this method for routine use.

The next paper to be considered in which oestriol conjugates have been separated from pregnancy urine is that reported by Van der Wal and Huber [11]. This was published in 1977 and details of the separation are given in Table 1. A similar XAD-column—methanol extraction procedure was applied to the urine as in the previously discussed study. Now none of the peaks in the complex chromatograms, obtained using either a UV monitor or a Kober fluorimetric monitor, were identified. However, the authors indicated that they were continuing to work on the separation in order to develop a routine procedure for oestrogen conjugate urinary profiles and they were therefore contacted with a request for information on the progress of their studies. As a result of this request we were kindly sent a copy of Van der Wal's thesis [28] which contains a final 18-page chapter entitled "Rapid determination of estrogen conjugate profiles in human pregnancy urine. Preliminary report". A careful study of this section of the thesis unfortunately leads essentially to the same conclusion as before, namely, that none of the peaks in the complex chromatogram, obtained using an on-line Kober fluorimetric monitor, had been identified. It was also found that, in order to obtain these chromatograms reproducibly, the urine samples had first to be subjected to an XAD-column—methanol extraction pre-separation process. However, the chromatograms shown provide evidence that differing oestrogen profiles occur during a pregnancy and also that "various disorders" appear to affect these urinary steroid profiles. This finding supports the statement made in the Introduction that oestrogen conjugate profiling might prove of value in the diagnosis of abnormal clinical conditions. To conclude this discussion of these Dutch studies, it should be noted that it is now not possible to repeat these HPLC separations since the ECTEOLA-cellulose column support materials employed (Whatman ET-41 and Baker 300) are no longer commercially available [25].

The final paper to be considered is that published by Dixon et al. [17], details of which are given in Fig. 1 and the last column of Table 2. A study of this paper showed that the urine specimens were subjected to an XAD-2 column—modified methanolic extraction procedure, before injection onto a Hypersil ODS column and subsequent ion-pair chromatography. The major modification of the extraction of the adsorbed conjugates is a final elution of the XAD-2 column with a 60% methanol aqueous mixture to produce a solution which is said to give a less complicated chromatogram than that obtained if pure methanol is employed as eluent. There are a number of favourable features of this published study which, incidentally, was stated by the authors to be a preliminary one. First, the two major oestriol conjugates, E_3-16-G and E_3-3-G, have been rapidly separated and identified by co-chromatography and disappearance following enzymatic hydrolysis. The concentration levels of the former conjugate were quantified using a UV monitor (at both 220 and 278 nm) and also using an electrochemical monitor. Further, unlike the previously discussed rapid HPLC method of Hermansson [19], this type of method is not markedly affected by temperature changes [15]. Because of these advantages, we are of the considered opinion that this method is indeed a promising candidate method for the rapid, routine assay of at least the oestriol mono-glucuronides. Clearly, further detailed studies have yet to be performed in order to validate this recommendation involving the assay of large numbers of samples and leading to statistical estimates of both the precision of the method and correlation with a currently widely accepted method. Such studies are currently underway in our laboratories and the results will, we hope, form the subject of a subsequent publication on the routine HPLC analysis of oestriol conjugates in human pregnancy urine.

4. ACKNOWLEDGEMENTS

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5. SUMMARY

The analytical and clinical advantages that would be expected to follow the adoption by clinical laboratories of a routine HPLC method for the partial oestriol conjugate profiling of human pregnancy urine are outlined in the Introduction. In order to ascertain if a candidate method for this assay has yet been devised, a complete survey of the published HPLC separations of oestrogen conjugate mixtures is presented, in tabular form, and discussed. From this survey it is concluded that a number of good separations of these steroids from synthetic mixtures have already been published.

The third and final section of the paper contains the results of a detailed examination of those papers in which separation of oestriol conjugates present in pregnancy urine specimens have been reported. The paper is concluded with the recommendation that the method of Dixon, Lukha and Scott should be further investigated as a candidate method for adoption by clinical laboratories for the purpose of oestriol conjugate profiling.

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