Spectrophotometric Methods for the Analysis of Mixtures of Oestradiol-17α and Oestradiol-17β

TORLEIV LUNAAS

Department of Reproductive Physiology and Pathology, Veterinary College of Norway, Oslo, Norway

The conversion of oestradiol-17α into the pink, fluorescent Kober colour is shown to take place in good yield within 5 min in hot, 40 % (v/v) sulphuric acid containing quinol. Under these conditions the colour produced by oestradiol-17β is comparatively very weak. When heating for 40 min in 52 % (v/v) sulphuric acid containing quinol, the production of the Kober colour takes place from both of the epimeric oestradiols. A method, based on these findings, is proposed for the differential analysis of the oestradiols in mixtures. By including extraction of the Kober colour complex into methylene chloride for fluorimetry, the sensitivity in quantitative determinations is considerably increased in comparison with the sensitivity in colorimetric measurements.

Oestradiol-17α and oestradiol-17β, of which the latter exhibits by far the higher oestrogenic potency, have been demonstrated to occur in the bovine placenta and in equine urine. In cattle as well as in the rabbit and in the dog, oestradiol-17α seems to be a predominant metabolic product of oestradiol-17β. Practicable methods for the analysis of the two epimeric oestradiols in mixtures would thus be desirable in further studies on the occurrence and metabolism of oestrogens in domestic animals.

Differential analysis of oestradiol-17α and oestradiol-17β has previously been achieved by bioassay in combination with an oxidation procedure. In the stallion’s urine, oestradiol-17β may be determined by selecting conditions for hydrolysis under which interfering oestradiol-17α is effectively destroyed. The epimers may be separated by chromatography on paper and on thin layers of silica gel. In the author’s experience, methylation of the compounds facilitates their separation by the latter method (suitable system, 96 % ethanol:benzene, 5:95). In the methods based on column chromatography developed for the routine determination of oestrogens, the epimers are not separated. However, with such methods a mixture of the oestradiols are readily obtained, and the α-epimer may be detected and estimated by the

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modified Kägi-Miescher reaction and with a Kober reagent containing an iron salt and phenol. The present communication deals with the conditions for separate colour development of oestradiol-17α in the presence of oestradiol-17β. The conditions have been re-investigated with the aim of including the Ittrich procedure for the selective extraction of the Kober colour, thus allowing for improved specificity and sensitivity in quantitative determinations of oestradiol-17α.

MATERIAL AND METHODS

Solutions of oestradiol-17α* and of oestradiol-17β** were prepared in absolute ethanol and stored at 4°C.

Sulphuric acid (d 1.84, zur Anal., E. Merck) was diluted with distilled water to desired concentrations.

Quinol (techn.) was dissolved in 96% ethanol and stored at 4°C.

2% (w/v) solutions of p-nitrophenol (puriss., Fluka) in tetrachloroethane (puriss., Fluka) or in methylene chloride (techn.) were filtered prior to use and stored at room temperature.

For the development of colour, the steroids were treated with the aqueous sulphuric acid in Pyrex glass tubes (2.5 cm × 15 cm) which were fitted with glass cones.

Optical densities were read against appropriate blanks in a Beckman model DU spectrophotometer. The corrected optical densities were obtained from the reading at the wave length of maximal absorption and the readings 35 μν above and below this wave length by using the equation $E_c = 2E_{max} - (E_1 + E_2)$.

Fluorescence spectra were recorded with a Beckman model DU monochromator with photomultiplier combined with an attachment for fluorimetry. As incident light a beam of an unfiltered wolfram continuum was used. In fluorimetry of the Kober colour complex in methylene chloride a corrected fluorescence intensity was calculated as the difference between the intensities at the wave length of maximal emission (548–549 μν) and at 520 μν.

The procedure finally adopted for the separate colour development of oestradiol-17α in the presence of oestradiol-17β was as follows. After evaporation of solvents from the reaction tubes, quinol was added as 0.2 ml of a 2% (w/v) ethanolic solution, which was taken to dryness under reduced pressure with gentle heating. The residues were then treated with 3 or 4 ml of 40% (v/v) aqueous sulphuric acid by heating in boiling water for 5 min.

When colour development of both epimers was desired, the residues were treated with 52% (v/v) sulphuric acid by heating for 40 min.*

For the extraction of the Kober colour formed the reaction mixtures were diluted with 5 ml water and, after cooling, shaken with methylene chloride containing p-nitrophenol.**

In fluorimetry of the Kober colour complex in 5 ml methylene chloride, the intensity of the fluorescent light was linearly related to the amounts of steroids up to about 2 μg. Inner filter effects were marked in fluorimetric measurements of larger amounts. The range suitable for colorimetry was 1–10 μg.

EXPERIMENTAL AND RESULTS

1. Conditions for colour production of oestradiol-17α in aqueous sulphuric acid.

From previous work in this laboratory it is known that the colour production takes place very rapidly when oestradiol-17α is submitted to the two stage Kober reaction with the quinol-containing reagent designed for oestradiol-17β. According to a recent study on the two stage Kober reaction, maximal

* Courtesy of Dr. L. L. Engel, Massachusetts General Hospital, Boston.
** Courtesy of Ciba, Basel.

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colour production of oestradiol-17α is obtained by heating in 66 % sulphuric acid containing quinol for 4 min and subsequently for 6 min after dilution of the acid to 45.5 %. It was found that heating of oestradiol-17α in more diluted acid for a short time was sufficient to induce development of the pink Kober colour, and that the colour to some degree developed in absence of quinol. In search for conditions yielding a favourable ratio between the colour productions of oestradiol-17α and oestradiol-17β, several series of colour developments of the two compounds were carried out by varying the concentrations of acid and quinol and the time of heating. It was found that the presence of quinol increased the colour production of both epimers and that a concentration of about 1 mg quinol per ml dilute acid was sufficient for maximal effect. In a subsequent series, 0.2 ml of a 2 % (w/v) solution of quinol was added to the tubes containing the steroid solutions (0.2 ml) and the mixture taken to dryness prior to addition of 4 ml dilute acid used for colour development.

As may be seen from Fig. 1, the colour production of oestradiol-17α increased with the concentration of the acid and, after about 5 min, decreased with the time of heating. It may also be seen that the colour production of oestradiol-17β was negligible at acid concentrations below 45 % (v/v) when heated for less than 10 min. Since the colour production of oestradiol-17α heated for 5 min in 40 % acid was satisfactorily reproducible (see Table 1) these conditions were finally adopted, they yielded a ratio between the corrected optical densities of oestradiol-17α and oestradiol-17β of 100:1 — 3. In the modified Kägi-Miescher reagent (sulphuric and acetic acid, 1:20), the ratio between the colour productions was about the same as in 40 % sulphuric acid when heating for 5 min was applied. The corrected optical densities of oestradiol-17α in the sulphuric-acetic acid reagent were about one third of those in 40 % sulphuric acid in water.

2. Stability of oestradiol-17α during evaporation of solvents. During the course of the investigation it was observed that aerobic evaporation of 5—15 ml
Table 1. Colour production of oestradiol-17α and of oestradiol-17β under two different reaction conditions. Corrected optical densities ($E_c + s.d., n = 10$) of 10 µg of the steroids developed in 3 ml aqueous sulphuric acid containing 4 mg quinol by heating in boiling water.

<table>
<thead>
<tr>
<th>Concentration, % (v/v) of sulphuric acid and time of heating</th>
<th>$E_c$ *</th>
</tr>
</thead>
<tbody>
<tr>
<td>40 %, 5 min</td>
<td>0.854 ± 0.030</td>
</tr>
<tr>
<td>52 %, 40 min</td>
<td>0.710 ± 0.016</td>
</tr>
</tbody>
</table>

* As calculated from $E_c = 2E_{max} - (E_1 + E_2)$ where $E_1$ and $E_2$ represent readings 35 µu above and below the wave length of maximal absorption.

Table 2. Wave lengths in µu of maximal absorption and fluorescence (in brackets) of oestradiol-17α and oestradiol-17β and of their 3-methyl ethers. Colour development in the modified Kågi-Miescher reagent (sulphuric and acetic acid) and in aqueous sulphuric acid containing quinol. Subsequent extraction of the Kober colour complex into methylene chloride containing p-nitrophenol.

<table>
<thead>
<tr>
<th>Reagent and time of heating for colour development</th>
<th>Wave length, µu</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oestradiol-17α</td>
</tr>
<tr>
<td>Sulphuric and acetic acid, 1:20, 5 min</td>
<td>523 (546)</td>
</tr>
<tr>
<td>Aqueous sulphuric acid, 40 %, 5 min</td>
<td>517 (539)</td>
</tr>
<tr>
<td>Methylene chloride extract</td>
<td>532 (548)</td>
</tr>
<tr>
<td>Aqueous sulphuric acid, 52 %, 40 min</td>
<td>519 (540)</td>
</tr>
<tr>
<td>Methylene chloride extract</td>
<td>532 (548)</td>
</tr>
</tbody>
</table>

benzene or hexane from the tubes containing oestradiol-17α and quinol, rendered the chromogenicity of the steroid seriously erratic, especially at the sub-microgram level. This was found to be due to the presence of the quinol. The ethanolic solution of quinol was subsequently added to the tubes after removal of other solvents and rapidly taken to dryness under reduced pressure with gentle heating on a water bath. This procedure gave a standard deviation.

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of 3.2 % of the mean of the corrected optical densities in ten analyses of 10 μg oestradiol-17α in 10 ml benzene. In the absence of quinol the tubes with the dry residues of oestradiol-17α could be left on the boiling water bath for at least 15 min without any demonstrable influence on the subsequent colour production.

3. Colour development of oestradiol-17α and oestradiol-17β. No conditions could be found for the separate colour development of oestradiol-17β in the presence of oestradiol-17α. For the colour development of both epimers the one stage Kober reaction of Ittrich¹⁰ was adopted with the modification that the steroids (in 0.2 ml ethanol) were taken to dryness together with 0.2 ml 2 % quinol in ethanol and developed for 40 min with 52 % (v/v) sulphuric acid to which no quinol had previously been added. Under this condition the chromogenicity of oestradiol-17α was somewhat lower than when heated in 40 per cent acid for 5 min, but still higher than that of oestradiol-17β (Table 1).

Similar results were obtained by using a freshly prepared mixture of quinol in 52 % sulphuric acid¹⁰ as recommended for oestradiol-17β. Fresh mixtures of quinol (2 %, w/v) in 40 % acid could be used for the separate colour development of oestradiol-17α.

4. Extraction of the Kober colour according to Ittrich.¹¹ For the extraction of the colour formed from the oestriadiols, the reaction mixtures (3 or 4 ml) were diluted with water (5 ml) under cooling in tap water and then shaken vigorously with methylene chloride (usually 5 ml) containing 2 % (w/v) p-nitrophenol.¹¹ Methylene chloride was preferred for this purpose rather than tetrachloroethane since the latter appeared more prone to give opaque extracts less suited for fluorimetry. The tetrachloroethane extracts could, however, be perfectly cleared by shaking with a little dry sodium sulphate. This procedure did not seem to interfere with the stability of the colour.

At the wave lengths of maximal emission, the fluorescence intensity of oestradiol-17α was about twice as high in the methylene chloride extracts as in the sulphuric acid used for colour development. In Fig. 2 are recorded the absorption spectra of oestradiol-17α developed in 40 % sulphuric acid and of the Kober colour complex in methylene chloride. Also recorded is the fluores-

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Fig. 2. Absorption and fluorescence spectra of the colour produced from oestradiol-17α. Absorption spectrum of 20 μg in 4 ml 40 % (v/v) aqueous sulphuric acid with 4 mg quinol heated for 5 min (×) and of the Kober colour complex extracted into 4 ml methylene chloride containing 2 % (w/v) p-nitrophenol (○). Fluorescence spectrum of 1 μg similarly developed and extracted into 5 ml methylene chloride (○). An unfiltered wolfram continuum was used as activating light. E, optical density. F, fluorescent light intensity units.

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cence spectrum in the latter medium. The extraction procedure caused a shift in all absorption spectra and in their corresponding fluorescence spectra (see Table 2). Attempts to extract the colour formed from oestradiol-17a in the Kägi-Miescher reagent were unsuccessful.

5. Determination of mixtures of oestradiol-17a and oestradiol-17β. When estimating oestradiol-17a only, development in 40 % sulphuric acid for 5 min would frequently suffice since the colour production of oestradiol-17β under this condition is very small in comparison with that of oestradiol-17a. When the β-epimer exists in large proportions and when both epimers are to be determined, the mixture is developed also in 52 % acid.

For the calculation of the optical densities or fluorescence intensities due to either of the epimers, the following formulas may be applied:

\[
P_{52\beta} = \frac{P_{52} - mP_{40}}{1 - mn} \quad P_{40\alpha} = \frac{P_{40} - nP_{52}}{1 - mn}
\]

They are derived from the following equations:

\[
P_{52} = P_{52\alpha} + P_{52\beta} \quad P_{40} = P_{40\alpha} + P_{40\beta}
\]

\[
= mP_{40} + P_{52\beta} \quad = P_{40\alpha} + nP_{52\beta}
\]

where the values of \( P \) are the values of the corrected optical densities or of the corrected fluorescent light intensities:

\[
P_{52}, \quad \text{mixture in 52 % H}_2\text{SO}_4
\]

\[
P_{40}, \quad \text{in 40 %}
\]

\[
P_{52\alpha}, \text{oestradiol-17a} \quad \text{in 52 %}
\]

\[
P_{40\alpha}, \quad \text{in 40 %}
\]

\[
P_{52\beta}, \text{oestradiol-17β} \quad \text{in 52 %}
\]

\[
P_{40\beta}, \quad \text{in 40 %}
\]

Table 3. Determination of mixtures of oestradiol-17a and oestradiol-17β by colorimetry and fluorimetry.

<table>
<thead>
<tr>
<th></th>
<th>( \mu g )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Present</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Oestradiol-17α</td>
<td>5</td>
</tr>
<tr>
<td>Oestradiol-17β</td>
<td>5</td>
</tr>
<tr>
<td>Oestradiol-17α</td>
<td>0.5</td>
</tr>
<tr>
<td>Oestradiol-17β</td>
<td>0.5</td>
</tr>
<tr>
<td>Oestradiol-17α</td>
<td>0.05</td>
</tr>
<tr>
<td>Oestradiol-17β</td>
<td>0.05</td>
</tr>
</tbody>
</table>

* The mean in 4 separate series of analyses, each in triplicates.

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The factors $m$ and $n$ varied slightly and were determined within each series of estimations of mixtures by developing known amounts of the steroids under both conditions.

In Table 3 are recorded some results in determinations of known mixtures of the epimers at different levels. It appears that the method is adequate for the estimation of the steroids when they are present in amounts of $0.05 \mu g$. The smallest amounts that could be distinguished from zero were $0.001 - 0.002 \mu g$ oestradiol-17$a$ or oestradiol-17$b$.

The ratios between the colour productions of the 3-methyl ethers of the epimers in 40 and in 52 % sulphuric acid, respectively, were about the same as of the non-methylated compounds. The method of Brown,\(^9\) which includes chromatography of oestrogens as the 3-methyl ethers, may therefore be used for the isolation of the oestradiols prior to quantitative determinations.

**DISCUSSION**

The method arrived at seems as practicable as that previously described by Haenni\(^9\) for the determination of mixtures of the epimeric oestradiols. The work of Haenni came to the attention of the author during the preparation of this paper. In both methods the separate colour development of oestradiol-17$a$ is achieved by heating for a short period of time in dilute sulphuric acid.

In the one stage Kober reaction\(^10\) as in the two stage reaction,\(^6\) the final pink colour of the oestrogens is formed via a yellow colour. The rates at which these conversions take place differ greatly for the epimeric oestradiols. Especially after methylation, oestradiol-17$a$ turns yellow-green and then pink almost instantaneously when heated in 52 % sulphuric acid containing quinol. Similar conversions of oestradiol-17$a$ apparently take place in the 40 % sulphuric acid reagent described, but virtually without concomitant colour production of oestradiol-17$b$. In the absorption spectrum of the colour produced from oestradiol-17$a$ in dilute acid, no shoulder could be recorded. This indicates a complete conversion of the yellow into the pink colour which may be selectively extracted using the procedure of Ittrich.\(^10,11\) The reagent proposed by Haenni\(^9\) contains iron (Mohr's salt) and phenol. In the present investigation, addition of small amounts of quinol was found satisfactory for good colour production. It has been pointed out that the sulphuric acid used (E. Merck) contains relatively much nitrate.\(^14\) This may be of importance for the conversion of the yellow into the pink colour which is thought to involve an oxidation step in colour development of oestrogens by the two stage Kober reaction.\(^15\)

The conversion of oestradiol-17$a$ into the pink, fluorescent substance in dilute sulphuric acid may be related to that occurring in the Kägi-Miescher reaction. Among 25 oestrogens examined,\(^16\) a distinctly positive Kägi-Miescher reaction has been found to be given only by oestradiol-17$a$ and 16-oxo-oestradiol-17$b$. It remains to be shown if the procedure described for the quantitative determination of oestradiol-17$a$ possesses a comparable specificity.

By introduction of the Ittrich extraction procedure\(^11\) for the quantitative determination of the oestradiols by fluorimetry,\(^13\) the sensitivity is increased by a factor of at least 10 as compared to the sensitivity in colorimetric measurements. It would thus appear that the method described might be applicable

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to distinguish between the epimeric oestradiols when they are present in amounts low enough to render recoveries subsequent to chromatographic separations more or less unprecise. In treating the oestradiols prior to the final colour development, it seems important to consider that their stability may differ appreciably, for example in hot acid hydrolysis of urine. In determinations of oestradiol-17β including column chromatography, quinol has been added to the eluates prior to the critical step of solvent evaporation. In this investigation, indications were obtained that the presence of quinol during solvent evaporation interferes with the chromogenicity of oestradiol-17a. It is possible, however, that addition of quinol is preferable when the eluates contain impurities originating from urine or other biological materials.

REFERENCES

4. Velle, W. Personal communication.

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