narcotine by chemical and mechanical stimulation methods using guinea pigs. The N-oxides of both *l*- α - and *l*- β -narcotines had better action than their original salts and the antitussive action of *l*- β -narcotine N-oxide was more effective than that of dihydrocodeine when tested by the mechanical stimulation method.

Both *l*- α - and *l*- β -narcotines showed some activity in tests of anti-acetylcholine action on frog rectus abdominis muscle, respiration and blood pressure of rabbits, relaxation and histamine antagonizing action on guinea pig smooth muscle, and acute toxicity of mice. These compounds had no effect of increasing the analgesic action in combination with morphine, inhibition of intestinal propulsion in mice, or constipative action. N-oxides of both *l*- α - and *l*- β -narcotines had weak or entirely no effect in these tests, except for a marked antitussive action and relaxation of tracheal muscle in comparatively high dosage.

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81. Kazukichi Kato : A New Color Reaction of Steroid with
Anhydrous Aluminum Chloride and Anisaldehyde. I.
A Colorimetric Determination of Ethylestrenol.

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Ethylestrenol (17 α -ethyl-17 β -hydroxyestr-4-ene) is an anabolic hormone widely used. There is no colorimetric method for this type of steroid, except that with concentrated sulfuric acid.¹⁾ Sulfuric acid, however, is not a specific reagent for steroid as well known, and gives only a yellow color to ethylestrenol. In fact, in the assay of a preparation of this steroid it tends to give an error owing to simultaneous coloration of other inseparable components, for example, an antioxidant.

In Liebermann-Burchard's reaction^{2,3)} of this steroid, a violet color is immediately observed, but it fades so rapidly, that this reaction is unsuitable to the quantitative determination. Commonly used color reactions specific for functional groups of steroid are not successfully applied for ethylestrenol, because it has only one double bond, one ethyl and one hydroxyl group.

It gives no color in K $\ddot{a}$ gi-Miescher's reaction,⁴⁾ in which steroid is heated with concentrated sulfuric acid and bromine or aromatic aldehyde, for this reaction is specific for the steroid having a secondary α -hydroxyl group at its 17-position.

In research for a more rational method of the assay, the combination of anhydrous aluminum chloride and anisaldehyde was found to be most suitable for this steroid, and therewith a new colorimetric determination was achieved.

Experimental

Material—Ethylestrenol : Ethylestrenol (N. V. Organon, Holland) was dried at 30° to constant weight under reduced pressure (5 mm. Hg). m.p. 94.6°. Anal. Calcd. for C₂₀H₃₂O : C, 83.33; H, 11.11. Found : C, 83.43; H, 11.09.

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Sample Solution—A quantity of ethylestrenol was accurately weighed, and dissolved in CHCl_3 to make a certain volume.

Reagents and Test Solution—1) Anhyd. AlCl_3 , JIS 1st class. 2) Anisaldehyde : L. Light Co. (England). b.p. 248° . 3) 5% anhyd. AlCl_3 solution : 5 g. of anhyd. AlCl_3 was dissolved in nitrobenzene to make 100 ml. (prepared just before use) 4) 4.5% Anisaldehyde solution : 4.5 g. of anisaldehyde was dissolved in nitrobenzene-benzene (1:1 by volume) to make 100 ml. 5) Nitrobenzene : JIS special class. 6) Benzene : JIS special-class. 7) CHCl_3 : JIS special class.

Apparatus for Measurement—Hitachi EPU-2A spectrophotometer and Hitachi EPS-2 autorecording spectrophotometer.

General Procedure—To 1 ml. of the sample solution containing 100~200 μg . of ethylestrenol in a 10 ml. volumetric flask, 1 ml. of 5% anhyd. AlCl_3 solution and 1 ml. of 4.5% anisaldehyde solution were added and shaken thoroughly. After being warmed at $35\sim 40^\circ$ for 60 min., it was cooled rapidly to a room temperature and diluted with nitrobenzene-benzene (1:1) solution to 10 ml. Its absorbance at 580 $\text{m}\mu$ was measured within 30 min. As a blank, 1 ml. of CHCl_3 was operated in the same manner to be referred to the sample solution in the measurement.

Assay of Preparation—An accurately weighed quantity of the powdered sample equivalent to about 10 mg. of ethylestrenol was suspended in 10 ml. of H_2O , and shaken vigorously with 20 ml. of CHCl_3 for 5 min. The CHCl_3 layer was filtered into a 100 ml. volumetric flask, and the H_2O layer was extracted with four successive 10 ml. portions of CHCl_3 , and the extracts were filtered into the flask. The combined filtrates were diluted to 100 ml. with CHCl_3 , and dried with 5 g. of anhyd. Na_2SO_4 . Two ml. of this solution was accurately taken into a 10 ml. volumetric flask, and evaporated to dryness on a water bath. One ml. of CHCl_3 was added to the residue, and evaporated again in the same manner. The residue was dissolved in 1 ml. of CHCl_3 , and operated by the procedure of the colorimetric determination. Other components contained usually in the preparation did not disturb the assay.

Discussion

In the following discussion, unless otherwise described, data were obtained on the sample solution containing 200 μg . of ethylestrenol per 1 ml.

Absorption Spectrum of Colored Solution

The colored solution obtained from 1 ml. of the sample solution by the above procedure gave an absorption spectrum as shown in Fig. 1, and its absorption maximum exists at 580 $\text{m}\mu$. Absorbance of the blank is about 0.06 at this wave length.

Fig. 1. Absorption Spectra

(1) : Colored solution obtained from 200 μg . of ethylestrenol
(2) : Blank solution

Fig. 2. Calibration Curve

Calibration Curve

Sample solutions, which contained 35~420 $\mu\text{g.}$ of ethylestrenol per 1 ml. respectively, were operated by the procedure mentioned above, and a calibration curve was obtained, as shown in Fig. 2.

Effect of the Reaction Time and Temperature on Absorbance

Results were obtained as shown in Table I, when the reaction time and temperature were varied, while other conditions were left as mentioned in the procedure of the colorimetric determination. Absorbance does not change in a range of the temperature 35~40° and of the reaction time 55~70 minutes. Though absorbance increases, as the reaction temperature becomes higher, absorbance of the blank also increases, as shown in Table II. Accordingly, 35~40° was adopted as the reaction temperature, and 60 minutes as the reaction time for the determination procedure.

TABLE I. Effect of the Reaction Time and Temperature on Absorbance

Reac. time (min.) Reac. temp. (°C)	40	50	55	60	65	70
30	0.416	0.441	0.440	0.441	0.445	0.450
35	0.440	0.450	0.454	0.453	0.456	0.455
40	0.434	0.449	0.451	0.459	0.454	0.455
45	0.442	0.487	0.491	0.496	0.500	0.501

TABLE II. Effect of the Reaction Time and Temperature on Absorbance of the Blank

Reac. time (min.) Reac. temp. (°C)	40	50	55	60	65	70
30	0.045	0.048	0.046	0.050	0.048	0.052
35	0.048	0.050	0.055	0.055	0.060	0.062
40	0.057	0.057	0.058	0.060	0.072	0.076
45	0.069	0.076	0.082	0.092	0.099	0.107

Effect of the Concentration of the Reagents

Results were obtained as shown in Table III, when the concentration of anhydrous aluminum chloride and anisaldehyde were varied, while other conditions were left as mentioned in the determination procedure.

TABLE III. Effect of the Concentration of the Reagents on Absorbance

AlCl ₃ (%) Anisaldehyde(%)	4.50	4.75	5.00	5.25	5.50	5.75	6.00
3.50	0.416	0.383	0.359	0.311	0.257	0.223	0.173
4.00	0.450	0.456	0.456	0.462	0.373	0.228	0.223
4.25	0.452	0.453	0.454	0.454	0.394	0.237	0.231
4.50	0.463	0.460	0.463	0.452	0.406	0.282	0.229
4.75	0.452	0.463	0.453	0.460	0.415	0.321	0.266
5.00	0.455	0.455	0.462	0.456	0.405	0.358	0.307
5.50	0.396	0.397	0.413	0.432	0.453	0.474	0.467

Absorbance does not change in a range of the concentration of anhydrous aluminum chloride 4.50~5.25% and of anisaldehyde 4.0~5.0%, and absorbance of the blank does not change in these ranges. Therefore, 5.0% solution of anhydrous aluminum chloride and 4.5% solution of anisaldehyde were adopted for the determination procedure.

Stability of the Colored Solution

Results were obtained as shown in Table IV, when the colored solution was allowed to stand for 60 minutes after being diluted with nitrobenzene-benzene (1:1) solution. Absorbance does not change within 50 minutes.

TABLE IV. Stability of the Colored Solution

Elapsed time (min.)	5	10	15	20	25	30	40	50	60
Absorbance	0.453	0.453	0.453	0.452	0.453	0.453	0.453	0.449	0.445

Error of the Assay

In order to ascertain the precision of the proposed method, an equally powdered sample of a preparation containing 1 mg. of ethylestrenol per 100 mg. was repeatedly assayed by the procedure described in Experimental (Table V). The error of the assay ($\hat{\sigma}$) is 1.33% ($n=6$), calculated on these data.

TABLE V. Results on an Equally Powdered Sample of a Preparation

	1	2	3	4	5	6	$\bar{x}$
Percentage to expected value	99.4	99.8	101.2	99.6	98.4	102.5	100.15

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Summary

A new colorimetric method has been established to determine ethylestrenol (17 α -ethyl-17 β -hydroxyestr-4-ene), which lacks a functional group available to known color reactions, and was assayed hitherto by the unspecific method with concentrated sulfuric acid. It gives a stable blue color with the reagents, anhydrous aluminum chloride dissolved in nitrobenzene and anisaldehyde in nitrobenzene-benzene (1:1). The assay is carried out in a range of 100~200 μ g. of the steroid per 1 ml., and successfully applied also to the determination of its preparation.

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