of 10% HCl to the cold mixture. Thus, 2.5 g. of thioketo acid, m.p. 185°, was obtained, after recrystallization. Then 2.5 g. of the thioketo acid was treated with a free NH_2OH solution, prepared from 3 g. of $NH_2OH \cdot HCl$, 0.7 g. of Na, and 20 cc. of EtOH. The reulting solution was heated for about 0.5 hr., the solvent was distilled off under a reduced pressure. The residual solid mass was dissolved in 5 cc. of 5% NaOH solution, and cooled with ice after filtration. When cool, the solution was acidified with 10% HCl and an oximino acid was obtained. The whole amount of the oximino acid, after having been completely dried, was converted to the nitrile by warming in Ac_2O under reflux for 0.5 hr. After the removal of Ac_2O from the reaction mixture by distillation, it was shaken with Et_2O and H_2O. The Et_2O extract of the product was washed with Na_2CO_3 solution, dried, and Et_2O was distilled off, giving 1.5 g. of (X), $b.p_{3\sim5}$ 145～150°.

b) From *p*-Nitrotoluene : *p*-Nitrotoluene was oxidized with CrO_3 into *p*-nitrobenzaldehyde. The aldehyde group of the product was converted to $CN\text{-}CH_2$ group according to the rhodanine synthesis. *p*-Nitrophenylacetonitrile thus obtained was reduced to *p*-aminophenylacetonitrile which was then coverted to (X) by the procedure as stated in (a).

4-(*p*-Bromobenzyl)quinoline (IX)—*p*-Bromophenylacetonitrile (X) (1 g.) and 4-chloroquinoline (I) (1 g.), both of which were freshly distilled, were combined in Et_2O in the presence of $NaNH_2$ (0.7 g.), the mixture was treated in the same manner as for (III) and (IV), and 4-(*p*-bromobenzyl)quinoline (IX) was obtained as a yellow oil, $b.p_2$ 167°. It is a very weak base, its hydrochloride readily hydrolyses in the air by the presence of moisture. Attempt to obtain pure crystals of the hydrochloride failed. In order to identify this bromo compound with that obtained earlier, its picrate m.p. 188°, was prepared. No m.p. depression was shown on admixture of these picrates, thus indicating that the two bromo compounds are identical. Further, perchlorate of (IX) was prepared as cubic crystals, m.p. 151.5～152.5°, after drying over P_2O_5. *Anal.* Calcd. for $C_{16}H_{12}NBr \cdot HClO_4$: C, 48.19; H, 3.29. Found : C, 48.12; H, 3.17.

Summary

4-Benzylquionline (V) was synthesized and from its nitration product, 4-(*p*-nitrobenzyl)quinoline (VI) was isolated. It was reduced to the amino compound and substituted with bromine atom via diazonium group.' 4-(*p*-Bromobenzyl)quinoline (IX) thus obtained was identified with an authentic sample derived from 4-chloroquinoline (I) by the action of *p*-bromophenylacetonitrile (X).

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10-3

53. Kōiti Kimura and Hiroshi Hikino : Studies on the Constituents of Ephedra. I. Determination of Alkaloids in Ephedra.*

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The routine methods generally used for the determination of ephedrine have been acid-base titration and direct Kjehldahl steam-distillation.[1] As a special method, a colorimetric method based on the Nagai reaction was described by Feng,[2] the formation of iodoform from ephedrine was proposed as a method of estimation by Sánchez,[3] and biological methods have been used by several workers.[4] However, none of them proved adequate as a method of microdetermination.

On the other hand, ninhydrin reaction, found by Ruhemann as a color test for α-

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** Kita-toneyama, Toyonaka, Osaka-fu (木村康一, ヒキノヒロシ).

1) W. W. Hilty : J. Am. Pharm. Assoc., **33**, 28(1944); L. H. Welsh : *Ibid.*, **33**, 96(1944).
2) C. T. Feng : Chin. J. Physiol., **1**, 337(1928).
3) J. A. Sánchez : J. Pharm. Chin., **22**, 489(1933).
4) P. S. Pittenger : J. Am. Pharm. Assoc., **17**, 634(1928); T. S. Githens : *Ibid.*, **22**, 391(1933).

amino acids, has been used for colorimetric determination of α-amino acids by numerous investigators. More recently, it was found that ninhydrin produced the same color with some amines, including ephedrine,[5] as with α-amino acids. Then separatory colorimetric estimation of ephedrine and ephedrone, applying this reaction, was reported,[6] and assumption of the mechanism of this color reaction with the amines was described.[7]

The purpose of the present work is to find a quick and sensitive analytical procedure on the application of ninhydrin reaction to the microdetermination of alkaloids in the crude drug, ephedra.

Experimental

Colorimetric Procedure—The following procedure was devised in the analysis of pure ephedra alkaloids, modifying the procedure of colorimetric determination of amino acids.[8]

A 1.0-cc. aliquot of the sample solution is placed in a glass-stoppered tubes, 1.0 cc. of 0.5% ninhydrin, 0.5 cc. of 0.05% ascorbic acid, and 1.0 cc. of pyridine are added to each tube, the tubes are stoppered tightly, and placed in a boiling water bath for 45 mins. The tubes are removed from the bath and cooled in cold water. The tubes are agitated vigorously until the red color due to the exsistence of indanone-enediol disappears through contact with air. This is diluted with water to the 10.0-cc. mark and mixed well. Within at least 0.5 hr. after the end of color development, the color is read by a spectrophotometer* set at 570 mμ against a blank of the reagents which have been put through the same treatment at the same time as the sample.

The procedure described above was arrived at after investigations of all factors which had a bearing upon the development of the final color as follows :

(1) Wave length of maximum absorption : Absorption curves of the color, developed by the procedure above-mentioned, using a solution of each sample of ephedra alkaloids, showed two maximum extinction peaks at the wave lengths of about 405 and 570 mμ, as illustrated in Fig. 1. Thus the color formed from ephedra alkaloids is the same as Ruhemann purple from common amino acids. Therefore, optical density was measured at 570 mμ.

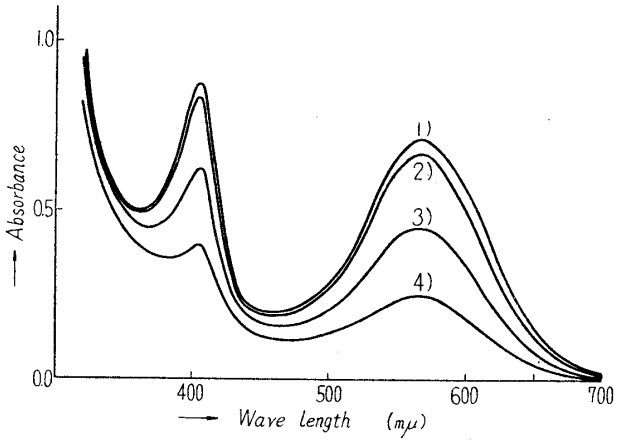

Fig. 1. Absorption Spectra of Coloring Matter from Ephedra Alkaloids

1) *l*-Ephedrine (50 γ)
2) *dl*-Norephedrine (50 γ)
3) *d*-ψ-Ephedrine (50 γ)
4) *l*-Methylephedrine (10 mg.)

(2) Rate of color development : When all the reagents were pipetted into the test tubes with 1.0-cc. aliquot of sample solution containing 50 γ or 100 γ of *l*-ephedrine per cc. and the tubes placed in a boiling water bath, the increase in absorbance was very rapid at first, then slowed down gradually. Rate curves for color formation are shown in Fig. 2. It was desirable that the heating time be prolonged to prevent the error due to deviation of the reaction time. On the other hand, however, the evaporation of liquid affected color formation if the heating time was too long. Evaporation due to incompleteness of tight stopper caused decrease in optical density of the final color. Thus to obtain the best reproducibility, the heating was carried out for 45

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6) S. Ose, R. Huzimoto : The 74th Annual Meeting of the Pharmaceutical Society of Japan, at Kyoto, April 5, 1954.
7) M. Yamagishi : J. Pharm. Soc. Japan, **74**, 1042(1954).
8) M. Yamagishi, T. Yoshida : *Ibid.*, **73**, 675(1953).
* All absorption measurements throughout this investigation were made with a Beckman Quartz Model DU spectrophotometer using glass cuvets with light path of 1.0 cm.

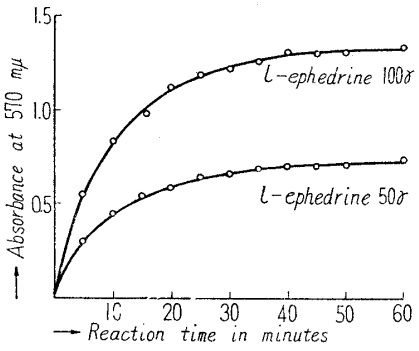

Fig. 2.
Change of Color Intensity with Reacting Time

mins. in which the full color development was nearly complete.

(3) Stability of the color: A few series of measurements of the absorption, taken at various times from a few mins. up to 6 hrs. after the end of heating, are indicated in Table I. It was found that the solutions were ready for measurement immediately after dilution and the color intensity could be kept in nearly constant readings during the observation period of 0.5 hr. After a prolonged standing, however, the solutions were observed to undergo gradual decrease in color. Therefore, optical density of the color was measured within 0.5 hr. after the color formation.

Table I. Change of Color Intensity with Standing at Room Temperature

Duration	*l*-Ephedrine		
	20 γ	50 γ	100 γ
10 mins.	0.353	0.708	1.314
20 〃	0.351	0.707	1.314
30 〃	0.349	0.706	1.314
60 〃	0.345	0.701	1.303
2 hrs.	0.344	0.699	1.299
6 〃	0.343	0.696	1.296

Preparation of Calibration Curves—A standard solution containing 100 mg.(as a free base) of each sample of the ephedra alkaloids per 100 cc. was prepared and aliquots of this solution were diluted to give concentrations of 10~100 γ/cc. The procedure described above was used. A plot of the absorbancies against concentrations was found to be linear and passed through the origin. Fig. 3 is the graph of the standard calibration. This indicates the applicability of Beer's law over a suitable range of concentration, at least up to 100 γ.

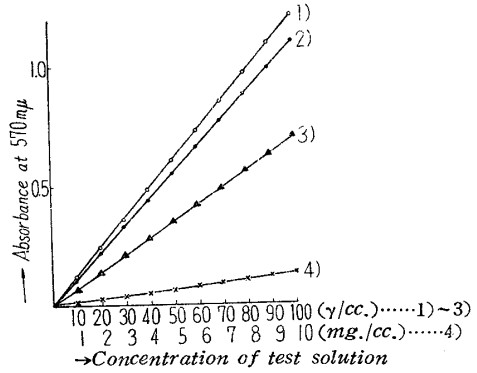

Fig. 3.
Standard Curves of Relation between Absorbance and Concentration of Ephedra Alkaloids
1) Ephedrine (*l*-, *dl*-)
2) Norephedrine (*dl*-)
3) ψ-Ephedrine (*d*-, *dl*-)
4) Methylephedrine (*l*-, *d*-, *dl*-)

Precision—A statistical study on the precision of the absorbancy measurements under proposed condition was made by repeated analysis of each sample with various concentrations of the alkaloid (*l*-ephedrine). The blank for the color reagents varied from one lot to another, but the readings for the alkaloid coloration remained almost constant. Table II shows details of the statistical treatment of these data. At 100 γ level, the analysis was very satisfactory with regard to the precision and even at 50 γ the precision remained good, judged from coefficients of variation. With alkaloid content below 20 γ, however, poor results were obtained.

Examination of Interference of Ninhydrin-coloring Substances—In order to test the effect of interference to this colorimetric method of amino acids and ammonium salts contained in ephedra, following experiments were made.

Table II.

l-Ephedrine content, y (γ/cc.)	10	20	30	50	100
Number of test, n	20	20	20	20	20
Average absorbancy, $\bar{x}$	0.1259	0.2440	0.3625	0.6049	1.2124
Standard deviation, σ*	0.0037871	0.0047573	0.0058289	0.0071591	0.0090161
Coefficient of variation, c.v.	3.03	1.95	1.61	1.18	0.744

* Standard deviation was calculated as $\sigma=\sqrt{\frac{\sum(x_i-\bar{x})^2}{n-1}}$

The regression line from all data is expressed by the following formula :
$y=82.6x$ (x : observed absorbancy)

(1) Detection of amino acids contained in ephedra : First, these amino acids were detected qualitatively by the following procedure.

One g. of a finely powdered sample of ephedra (*Ephedra distachya* Linné) was extracted consecutively with several 20-cc. portions of boiling 50% EtOH for 2 hrs. under reflux. All extracts were combined, evaporated, treated with 5 cc. of 1% HCl, and again evaporated. The residue was extracted successively with small portions of hot water. All extracts were combined and evaporated. The residue was dissolved in 0.3 cc. of 50% formic acid and 0.005-cc. aliquot of this test solution was applied to two-dimensional paper partition chromatography. The water-poor phase of a BuOH-80% formic acid-H_2O (9 : 1 : 3) mixture and 80% phenol served as the solvents. The chromatograms were run by ascending method for 16~20 hrs. After development, the solvent was allowed to evaporate at room temperature. The papers were then sprayed with 0.2% ninhydrin in BuOH saturated with water and color development was allowed to take place at approximately 70°. The other papers were treated with special reagents for detecting each amino acid.

The chromatograms prepared in this manner indicated that a large quantity of aspartic acid, glutamic acid, alanine, glutamine, methionine, valine, leucine (isoleucine), and proline was present and a small quantity of cysteine (cystine), serine, glycine, and lysine in this sample of ephedra. It was doubtful whether phenylalanine was present or not, because Rf value of this amino acid corresponded nearly with that of ephedra alkaloid.

(2) Estimation of ninhydrin-coloring substances contained in ephedra : Secondly, the following method was employed for the quantitative determination of all ninhydrin-coloring substances contained in ephedra. One g. of the finely powdered sample of ephedra, used in the preceding experiment, dried and accurately weighed, was exhaustively extracted with 7 successive 20-cc. portions of boiling 50% EtOH for 2 hrs. each under reflux. All extracts were filtered through the same filter and evaporated. The residue was treated with 5 cc. of 1% HCl and again evaporated. The residue was completely extracted successively with 10-cc. portions of hot water. The extracts were collected and diluted accurately to 100.0 cc. A 10.0-cc. aliquot of this solution was again diluted to exactly 25.0 cc. and 1.0-cc. aliquot of this test solution was used for the procedure described earlier.

The result indicated that all ninhydrin-coloring substances contained in 4.0 mg. of this sample of ephedra developed the color, 1.060 of optical density. On the other hand, estimating the alkaloid content in the same sample of ephedra by the method to be described next, 0.371 of absorbance was given for the extinction per 4.0 mg. of this sample. Therefore, the balance of 0.689, was calculated as 33.9 γ of leucine. Thus 1.00 g. of this sample contained 8.48 mg. of ninhydrin-coloring substances as leucine, besides the alkoloids.

(3) Control test with amino acids and ammonium salt : Experiments were carried out finally, by the procedure to be described next, taking 50 mg. each of the amino acids detected in ephedra, and ammonium sulfate, in order to test whether or not these affected this colorimetric method. As a result it indicated that those scarcely colored, compared to the blank prepared from the reagents. Thus it was observed that this quantitative ninhydrin method, applied to the crude drug, was hardly interfered by amino acids and ammonium salt present in ephedra.

Application of Samples—The following procedure was taken for the analyses of samples.

One g. of ephedra, finely powdered and dried, was weighed accurately, placed in a suitable flask, 20 cc. of ether and 1 cc. of 10% NH_4OH were added, stoppered tightly, and the mixture shaken intermittently for 1 hr. The ether extract was filtered rapidly, 10 cc. of ether added to the residue, and the mixture again shaken frequently during 0.5 hr. The extract was filtered through the original filter, the flask and residue were washed with 10 cc. of ether, and the combined ether extracts was evaporated on a steam bath until the odor of ether was no longer perceptible. The residue was dissolved in 20 cc. of ether, transferred to a separatory funnel, and the vessel was washed with three 2-cc. portions of ether. The combined ether solution was extracted, first with 2 cc. of 0.1N HCl by shaking for 3 mins., and then with three 2-cc. portions of water. The combined acid and aqueous extract was warmed on a steam bath to evaporate

the ether, transferred to a 100-cc. volumetric flask, and diluted to the mark with water. This was mixed thoroughly and 1.0-cc. aliquot of this test solution was submitted to the procedure described earlier. A blank test was carried out with all the reagents used in the same manner.

Accuracy and Reproducibility—A check of the comparative accuracy of this colorimetric method was afforded by a comparison of analytical results obtained by the method given in the Japanese Pharmacopoeia VI on the same sample. Comparative results for several samples assayed by this colorimetric method and the titration method are listed in Table III. The results are in good agreement.

Table III. Check of Comparative Results obtained by Two Methods

Samples		Proposed colorimetric method	J. P. VI Titration method
Species	Remarks		
E. distachya	Japanese drug market (I)	0.802**	0.868
E. distachya	Japanese drug market (II)	0.946	0.919
E. distachya	Japanese drug market (from north China)	0.233	0.242
E. distachya	Cult. in Japan	0.701	0.707
E. gerardiana	Cult. in Japan	0.579	0.624
E. sp.	Wild in Pakistan	1.165	1.171

** All results are expressed in alkaloid (as ephedrine) content (%).

The reproducibility of this method was evaluated by statistical approach. The results of replicate analyses of a certain sample at various levels, shown in Table IV, gave an indication of the reproducibility of the method. At 5-g. level of a sample, the procedure of the colorimetric method was carrried out after the scale of the titration method of J. P. VI.

Table IV. Statistical Examination of Results obtained by Two Methods

Sample scale (g.)	Colorimetric method			Titration method		
	$\bar{x}$	σ	c.v.	$\bar{x}$	σ	c.v.
5	0.9110	0.01294	1.42	0.9525	0.01789	1.88
1	0.9211	0.02503	2.72	not applicable		

Number of test: 5
$\bar{x}$=average content (%) of alkaloid (as ephedrine).

It was observed that at 5 or 1 g. of the sample level, results of determination made according to the above procedure agree well with those obtained by the method of J. P. VI, and their precision was satisfactory.

Discussion

In the determination by the titration method of the assay of ephedra given in J. P. VI, the minimum limit is 5 g. of sampling scale and high expertness is required on account of the obscure end point for its judgement, and yet considerable deviations are not avoidable. The acid-base titration method is not always commendable even on such a large sample scale. By the present proposed method, the results must be lower than the true values, as the total alkaloid content is calculated as ephedrine which develops the color most intensely per unit mass among all ephedra alkaloids, especially when methylephedrine develops practically no color. However, this accuracy is considered satisfactory, as the main purpose of this method is to attain sensitivity.

Applying this procedure, a sample of ephedra with alkaloid content of 1% can theoretically be estimated even at 10-mg. level. Actually, however, by the present proposed method, low results and poor precision are given when the amount of the sample is smaller than 1 g. Among the most possible source of these errors, consideration must be given to incompleteness of extraction of alkaloids from the sample as a source of lowering the value of the results based on cutting down the sampling.

The authors are indebted to various persons for assistance in this research, especially to Dr. Masaharu Yamagishi, Research Laboratories of Takeda Pharmaceutical Industries, Ltd., for

his much helpful advice regarding the analytical techniques in the first stage of this work and to Mr. Takashima of this faculty for his help with the spectrophotometric measurements. Appreciation is expressed to the Research Laboratories of Dainihon Seiyaku Co. Ltd. and of Takeda Pharmaceutical Industries, Ltd. for providing some of the samples of ephedra alkaloids tested. Some of the analyzed samles of ephedra supplied by Amatsu Experimental Station of Forestry, Agricaltural Faculty, University of Tokyo, and the Botanical Research Laboratory of the Nippon Shinyaku Co. Ltd., are also gratefully acknowledged.

Summary

Alkaloids were extracted from a sample of ephedra with ammoniacal ether, transferred into acid solution, and diluted to a certain volume. Aliquots of this test solution were reacted under heating with ninhydrin in the presence of ascorbic acid and pyridine. The intensity of the color developed was measured at the maximum absorbancy peak at 570 mμ. Alkaloid content was calculated from the regression line, subtracting the blank from the observed value. It was found that this method was hardly interfered by amino acids and ammonium salt present in ephedra, through a control test with the quantity of these acids and salt about several times as much as those contained in ephedra.

For further check of the comparative accuracy and precision of this method of analysis, several sets of analytical data were evaluated. The results of estimation made according to this proposed procedure agreed well with those obtained by the method of Japanese Pharmacopoeia VI and their reproducibility was satisfactory.

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54. Masayuki Onda and Mituo Sasamoto : Analogs of Rauwolfia Alkaloids. IV. Synthesis of 2-Substituted Tetrahydro-β-carbolines : Ring Closure of 2-Carboxy-3-indoleacetobenzylamide.

(*Tokyo Research Laboratory, Gohei Tanabe & Co., Ltd.**)

In the preceding paper of this series,[1] we reported on the synthesis of over 10 analogs of Rauwolfia alkaloids, several of which were found to possess reserpine-like activity. Therefore we attempted to synthesize 2-substituted tetrahydro-β-carbolines because of pharmacological interest.

The most common of the synthesis of tetrahydro-β-carboline will be the Pictet-Spengler reaction of tryptamine with aldehydes or α-ketocarboxylic acids and the Bischler-Napieralski reaction of acylated tryptamines, but these methods are not so easy as with isoqunolines and necessitate more steps via tryptamine synthesis.

This paper is concerned with the synthesis of 2-benzyl-1,2,3,4-tetrahydro-β-carboline from 2-carboxy-3-indoleacetic acid[2] (I) which was prepared easily in a good yield from α-ketoglutaric acid and phenylhydrazine according to Fischer's procedure.

Attempt was first made to synthesize tetrahydro-β-carboline by the route shown in Chart 1 〔(I)→(II)→(III)→(IV)〕 but it failed because of the sensitivity of 2-hydroxymethyltryptophol (II) to acids. Thionyl chloride and phosphorus bromide converted (II) into a black, high-melting substance, which did not dissolve in organic solvents, and the

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2) R. Robinson, *et al.* : J. Chem. Soc., **1921**, 1602.