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Separation and Quantitative Analysis of Ephedra Alkaloids by Gas Chromatography and Its Application to Evaluation of Some *Ephedra* Species collected around Himalaya

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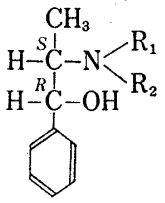
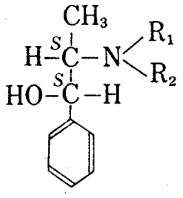
Gas chromatographic analysis of Ephedra alkaloids is described. A satisfactory separation of these alkaloids and quantitative determination for ephedrine(I) and pseudoephedrine(II) were achieved in use of oxazolidine formation with acetone. On application of this procedure, the evaluation of some *Ephedra* spp. collected around Himalaya is also reported.

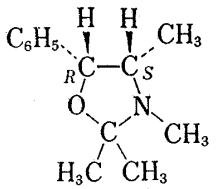
It has been well known that the oriental crude drug "Ma-Huang," the aireal part of *Ephedra* spp. contains the sympathomimetic amines, *l*-ephedrine (I), *d*-pseudoephedrine (II) and some other related alkaloids.[2] As to the evaluation of this crude drug, JP VIII describes the titration of the total alkaloids. But the quantitative estimation of each alkaloid must be necessary because of the some difference of the physiological actions between these constituents.

Several attempts have been reported for the separation and the analysis of these alkaloids; the copper complex method,[3] the salting out chromatographic method,[4] the method based

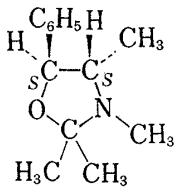




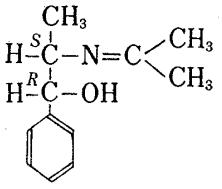
		R_1	R_2
l-ephedrine (I)	*d*-pseudoephedrine (II)	H	CH_3
l-norephedrine (III)	*d*-norpseudoephedrine	H	H
l-methylephedrine (IV)	*d*-methylpseudoephedrine	CH_3	CH_3



Ia



IIa



IIIa

Chart 1

1) Location: *1-2-3 Kasumi, Hiroshima-shi.*
2) L. Reti, "The Alkaloids", Vol. III, ed. by R.H.F. Manske and H.L. Holmes, Academic Press, New York, 1953, p. 339.
3) C.T. Feng, *Chinese J. Physiology*, **1**, 397 (1927).
4) S. Kori and M. Kono, *Yakugaku Zasshi*, **81**, 166 (1961); *idem, ibid.*, **81**, 170 (1961).

on the solubility difference between the salts of the alkaloids,[5] and the preparative thin-layer chromatography followed by the colorimetry.[6] The present authors have intended the development of the more satisfactory procedure by gas liquid chromatography (GLC) coupled with the preparation of the derivatives which are characteristic to these alkaloids.

On treatment with acetone, I and II afforded the corresponding oxazolidine derivatives (=acetonides), Ia and IIa[7] and norephedrine (III) yielded the Schiff base (IIIa), while methylephedrine (IV) was recovered unchanged.

On GLC of the Ephedra alkaloids even under the various conditions, the separation of I and II, a pair of *erythro* and *threo* isomers has been shown to be unsuccessful. Whereas, GLC of the mixture of acetonides, Ia and IIa,[8] was found to give sharp peaks with clear separation. The present paper deals with the separation and the quantitative determination of I and II by GLC in use of this evidence. On application of this procedure, the evaluation of some *Ephedra* spp. collected around Himalaya is also reported.

Experimental

Apparatus——A Shimadzu Model GC-4A Gas Chromatograph equipped with a hydrogen flame ionization detector and glass column (2 m in length and 4 mm in internal diameter) was used. A flow rate of carrier gas (N_2) was 33 ml/min. The column was packed with 15% polyethylene glycol 6000 on Celite 545 (mesh 60—80). The column temperature was 175° and the injection part and the detector were kept at 190°.

Acetonide Formation of *l*-Ephedrine (I)——*l*-Ephedrine(I) (12.4 mg) was refluxed in anhydrous acetone (20 ml) with silica gel (80 mg)[9] for 6 hr. Before cooling, silica gel was filtered off and washed three times with hot acetone. The filtrate and the washings were combined and evaporated to dryness under reduced pressure to give colorless oil (Ia), GLC: A single peak at retention time 11.5 min. Mass Spectrum *m/e*: 204 (M^+-1) and 162 (M^+-43). IR^{Nujol}: no OH, no NH, and no C=O bands. NMR (in $CDCl_3$) δ ppm: 7.26 (5H, br.s. aromatic), 5.01 (1H, d. $J=8$ Hz, CH–O), 3.15 (1H, dq. $J=8$ and 6.5 Hz, C<u>H</u>–CH_3), 2.26 (3H, s. N–CH_3), 1.23 and 1.52 (3H each, s. C=$(CH_3)_2$, 0.65 (3H, d. $J=6.5$ Hz, C<u>H</u>$_3$–CH) and no signals due to the impurities.

Acetonide Formation of *d*-Pseudoephedrine(II)——*d*-Pseudoephedrine(II) was treated with anhydrous acetone in the same way as I, reacting more readily than I to give IIa, GLC: A single peak at retention time 10.1 min. Mass Spectrum *m/e*: 204 (M^+-1) and 162 (M^+-43). IR^{Nujol}: no OH, no NH, and no C=O bands. NMR (in $CDCl_3$), δ ppm: 7.32 (5H, br.s. aromatic), 4.46 (1H, d. $J=9$ Hz, CH–O–), 2.55 (1H, dq. $J=9$ and 6 Hz, C<u>H</u>–CH_3), 2.29 (3H, s. N–CH_3), 1.34 and 1.43 (3H each, s. C=$(CH_3)_2$), 1.10 (3H, d. $J=6$ Hz, C<u>H</u>$_3$–CH) and no signals due to the impurities.

Treatment of Norephedrine(III) with Acetone——*l*-Norephedrine(III) was treated as above to give the Schiff base (IIIa), GLC: A single peak at retention time 16.8 min. $IR_{max}^{CCl_4}$ cm^{-1}: 3630 (OH), 1655 (–C=N–), and 1600 (aromatic). NMR (in CCl_4) δ ppm: 7.24 (5H, br.s. aromatic), 5.03 (1H, d. $J=7.5$ Hz, <u>H</u>–C–O–), 3.85 (1H, m. <u>H</u>–C–CH_3), 1.92 (1H, br.s. OH), 1.55 and 1.43 (3H each, s. N=C=$(CH_3)_2$), 0.71 (1H, d. $J=6.5$ Hz, –C–CH_3). The Schiff base (IIIa) was reduced with $NaBH_4$ in MeOH to give *N*-isopropylnorephedrine, NMR (in CCl_4) δ ppm: 1.10 (6H, d. $J=7$ Hz, –CH=$(CH_3)_2$), 2.90 (2H, m. N–C<u>H</u>–CH_3 and N–C<u>H</u>=$(CH_3)_2$, the coupling was confirmed by the double resonance technique.

Plant Materials——The specimens of *Ephedra intermedia* collected at Ningsar in Afganistan and at Shingash in Baluchistan, and *E. nebrodensis* var. *procera* at Shingash were obtained by courtesy of Dr. K. Nanba, Dainippon Seiyaku Ltd. *Ephedra gerardiana* var. *sikkimensis* was collected by one of the authors, O. Tanaka at Laya (alt. 3600 m) in Bhutan as the member of The Third Botanical Expedition of University of Tokyo to Eastan Himalaya in 1967. *Ephedra* spp. (unidentified) were collected at Jomosom (alt. 3000 m, Nepal-1) and at Modi (alt. 2500 m, Nepal-2) by Dr. N. Ishibashi as the member of The Hiroshima University Scientific Expedition to Nepal Himalayas, 1973.

Extraction and Quantitative Determination of I and II in *Ephedra* spp. and Ma-Huang——Dried Ephedra plants or commercial crude drug Ma-Huang (5 g) were exhaustively extracted with aldehyde free MeOH (50 ml×4) for 8 hr. The solvent was distilled off and the residue was dissolved in 1% H_2SO_4. After removing the acidic and neutral substances by filteration and by extraction with ether, the acidic aqueous

5) M.I. Goryaev, S.A. Moshkevich, and R.N. Sazonova, *Zh. Priklad Khim.*, **32**, 2313 (1959).
6) K. Kimura, H. Shimada, S. Nomura, Y. Hisada, and T. Tanaka, *Yakugaku Zasshi*, **93**, 364 (1973).
7) E.D. Bergmann, *Chem. Rev.*, **53**, 309 (1953); J.B. Hyne, *J. Am. Chem. Soc.*, **81**, 6058 (1959).
8) E. Brochmann-Hanssen and A.B. Svendson, *J. Pharm. Sci.*, **51**, 938 (1962).
9) Kieselgel 60 for column chromatography 70—230 mesh, Merck.

solution was made alkaline with K_2CO_3 (pH 11) and then extracted with aldehyde-free ether. The resulting ethereal solution was washed with dil. alkali solution, dried over anhydrous Na_2SO_4, and evaporated to dryness affording the oily or sometimes crystalline basic substances, which were analyzed by GLC for the preliminary qualitative identification. This basic fraction was refluxed in anhydrous acetone with a suitable amount of silica gel[9] (five to ten times as much as this fraction) for 6 hr. The hot reaction mixture was filtered and the silica gel was washed with hot acetone repeatedly. The filtrate and the washings were combined and evaporated to dryness *in vacuo*. To the residue were added acetone (10 ml) and the internal standard, *l*-menthol (30 mg) and the solution was subjected to GLC analysis.

Results and Discussion

When refluxing in anhydrous acetone without dehydrating agents, the formation of the acetonide required 20 hr for II, and for I more than 48 hr. In order to accelerate the reaction, various reagents were examined. Anhydrous $CuSO_4$, a common dehydrating agent for the preparation of acetonides can not be used in this case owing to the formation of the complex salts with I and II. Among the reagents tested, the active silica gel for column chromatography proved most effective. Its catalytic action was most facilitated when 4 to 20 fold excess *vs.* I or II was used; normally the reaction completes within 4.5 hr.

Other reagents were found to increase the reaction rate in the following order; $Na_2SO_4 < K_2CO_3 < Al_2(SO_4)_3 < CaSO_4 <$ molecular sieve $<$ active alumina.

Norephedrine (III) yielded the Schiff base (IIIa) with acetone, and its structure was confirmed by infrared (IR) and nuclear magnetic resonance (NMR) spectra as well as the characterization of the hydrogenated product, *N*-isopropylnorephedrine.

Table I. Relative Retention Times of Ephedra Alkaloids and Their Derivatives with Internal Standard

Compounds	Relative retention time[a]
l-Menthol (internal standard)	0.38
Pseudoephedrine acetonide (IIa)	0.88
Ephedrine acetonide (Ia)	1.00
Norephedrine Schiff base (IIIa)	1.46
Methylephedrine (IV)	1.73 (broad)
Ephedrine (I)	1.73 (broad)
Pseudoephedrine (II)	2.24 (broad)
Norephedrine (III)	2.68 (broad)

a) retention time of Ia (11.5 min)=1.00
condition: 15% PEG 6000 on Celite 545, 2 m, 175°, N_2 flow rate: 33 ml/min

Relative retention times of the alkaloids and their derivatives are shown in Table I and a chromatogram of the typical separation is illustrated in Fig. 1. In contrast to the original alkaloids, the acetonides Ia and IIa exhibited the sharp and well separated peaks. The difference of the gas chromatographic behaviors of the acetonides results from the decrease in their polarity as well as the structural rigidity of the acetonides compared with the starting alkaloids. The calibration curves of Ia and IIa as illustrated in Fig. 2, indicate good linealities. Further it was found that no special calibration was needed even in the case of partial overlapping of the two peaks. The best GLC stationary phase was PEG 6000. Others such as SE-30, OV-1 and OV-17 were found to be less effective for the separation of Ia and IIa.

In GLC of the alkaloids fraction of *Ephedra*, small peaks sometimes appeared at the similar retention times to those of Ia and IIa. Their interference on the quantitative determination of Ia and IIa could be minimized by the rapid operation for the separation process of the basic fraction from the plant extract. The structure of these unidentified compounds are now under investigation.

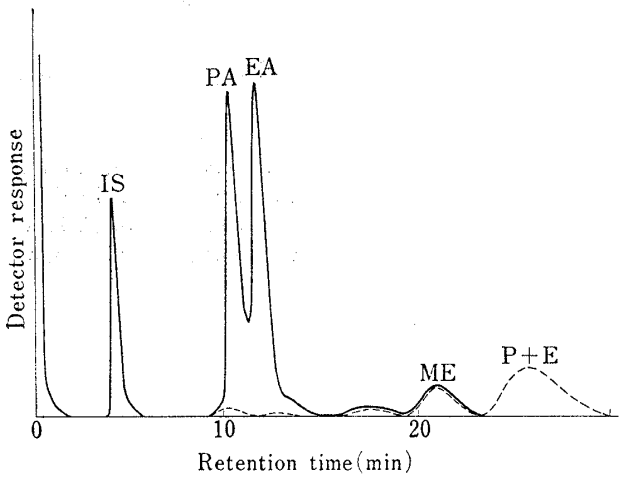


Fig. 1. Gas Chromatogram of Ephedra Alkaloids from Ephedra Plants

basic fraction: ------ before reaction with acetone
—— after reaction with acetone
IS: internal standard (*l*-menthol), PA: pseudoephedrine acetonide (IIa), EA: ephedrine acetonide (Ia), ME: methylephedrine (IV), P: pseudoephedrine (II), E: ephedrine (I)
condition: 15% PEG 6000 on Celite 545, 2 m column, 175°, N_2 flow rate: 33 ml/min

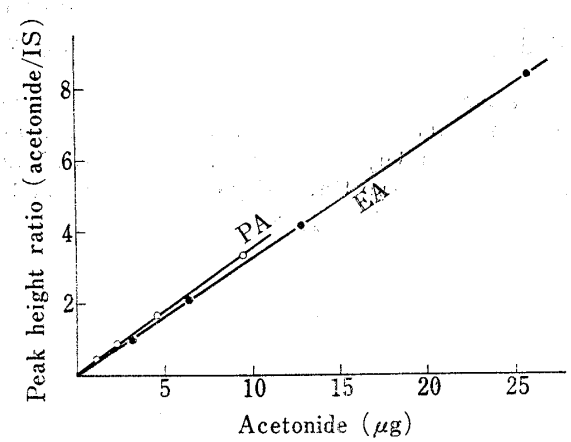


Fig. 2. Calibration Curves for Acetonides of Ephedrine (EA, Ia) and Pseudoephedrine (PA, IIa) using *l*-Menthol as an Internal Standard (IS)

Other ketones such as methyl ethyl ketone and diethyl ketone also afforded the corresponding oxazolidine derivatives from I and II but their separation on GLC proved unsatisfactory.

The analytical results of Ephedra plants collected around Himalaya and some of the commercial Ma-Huang in Japanese market are shown in Table II.

Table II. Contents of Ephedra Alkaloids

Species	(Origin)	I	II	IV	III
E. intermedia	(Afganistan)	0.006%	0.002%	−	−
	(Baluchistan)	0.35	0.18	+	−
	(cultivated)[b]	0.31	0.04	+	−
		0.29	0.05	+	−
E. gerardiana	(cultivated)[b]	0.04	0.97	−	−
		0.03	1.07	−	−
E. gerardiana var. *sikkimensis*	(Bhutan)	0.13	0.41	+	−
E. nebrodensis var. *procera*	(Baluchistan)	0.84	0.12	+	−
E. sp.	(Nepal-1)	0.73	0.41	+	−
	(Nepal-2)	0.63	0.15	++	−
Ma-Huang (commercial)	(Hiroshima)	0.26	0.21	++	−
	(Osaka)	0.58	0.17	+	−
	(Tokyo)	0.26	0.14	++	−

a) The developement of quantitative analysis of methylephedrine (IV) are under progress.
b) at Kasukabe Experiment Station of Medicinal Plants, attached to the National Institute of Hygienic Sciences

Our results indicates that *E. intermedia* and *E. nebrodensis* var. *procera* contained more ephedrine (I) than pseudoephedrine (II), though environmental variation was observed in the total alkaloids content. On the contray, *E. gerardiana* and *E. gerardiana* var. *sikkimensis* were found to be II-rich species. Two specimens collected in Nepal by the members of Scientific Expedition of Hiroshima University contained I in a high percentage that is attractive ephedrine source. The botanical identification of these species is now under progress. Commercial Ma-Huang showed wide variation of alkaloids contents as well as their components. Ac-

cordingly, it follows that the crude drug Ma-Huang should be evaluated by the quantitative determination of each alkaloid.

Further studies on the evaluation of the alkaloids contents of Ephedra plants cultivated in Japan and the commercial Ma-Huang will be reported elsewhere.

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