Analysis of Hygrine and Cuscohygrine in Coca Leaves Using Gas Chromatography and High-Performance Liquid Chromatography

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The alkaloids hygrine and cuscohygrine in *Erythroxylum coca* var. Coca leaves were analyzed by a gas chromatographic (GC) method using a dimethylsilicone capillary column and a high-performance liquid chromatographic (HPLC) method using a weak cation exchange column. Hygrine content in *E. coca* leaves was determined as 0.12% by GC and 0.07% by HPLC, whereas cuscohygrine content was 0.25% by GC and 0.21% by HPLC.

Keywords: *Hygrine; cuscohygrine; alkaloids; Erythroxylum; coca extracts*

INTRODUCTION

Hygrine (*N*-methyl-2-acetonylpyrrolidine) and cuscohygrine [1,3-bis(1-methyl-2-pyrrolidinyl)-2-propanone] are two pyrrolidine alkaloids (Figure 1) found in a variety of plants such as *Datura*, *Hyoscymus*, and *Erythroxylum* (Ionkova et al., 1994). In the literature, considerable attention has been paid to the role of pyrrolidine alkaloids in the biosynthesis of more complex plant alkaloids such as nicotine, atropine, and cocaine (Leete, 1990). Hygrine is the established precursor of the tropane alkaloids hyoscyamine and scopolamine, both of which are used in the preparation of several pharmaceutical products.

Gas chromatography (GC) has been widely used for the analysis of pyrrolidine and tropane alkaloids in coca plants (Johnson and Emche, 1994; Moore et al., 1994). Recently, we reported a simple and reliable high-performance liquid chromatographic (HPLC) method for the analysis of cuscohygrine in coca leaf extracts, using a weak cation exchange column (Glass and Johnson, 1996). The HPLC analysis of hygrine using a strong cation exchange column was reported in an earlier paper (Glass, 1995).

In the present paper, the HPLC analysis of both hygrine and cuscohygrine on a single weak cation exchange column is described. A comparison between the new HPLC method and the GC method is presented for the analysis of these two alkaloids in *E. coca* leaf extracts.

MATERIALS AND METHODS

Chemicals. The purity of hygrine and cuscohygrine, which were synthesized according to a procedure already described (Glass, 1995), was >90%. Methanol, ethanol, and chloroform of HPLC grade were obtained from EM Science (Gibbstown, NJ). All other chemicals were of reagent grade or better. Water used to prepare solutions and mobile phases was initially deionized and was run through a HP Model 661A water purifier (Hewlett-Packard Co., Avondale, PA).

Plant Materials. *Erythroxylum coca* leaves were collected from plants that were grown from seeds under greenhouse conditions in Beltsville, MD, and in field plots in Hawaii. Air-dried *E. coca* leaves were ground into a powder using a Wiley mill and were stored in jars at room temperature in a desiccator. The powdered leaf samples were extracted within 2 h after powdering to minimize the loss of volatile alkaloids.

GC Analysis. GC analysis of the alkaloids was performed according to the method reported by Johnson and Emche (1994). A HP Model 5890 instrument (Avondale, PA) equipped with a flame ionization detector and a HP 2934A integrator were used in the alkaloid analysis. A dimethylsilicone capillary column [DB-5, 15 m × 0.25 mm (i.d.), 0.25 μm film thickness] was obtained from J&W Scientific (Rancho Cordova, CA). Helium (99.95% purity) at approximately 60 cm/s linear velocity was used as the carrier gas. The injection and detector temperatures were 250 and 285 °C, respectively. The temperature program consisted of an initial oven temperature of 70 °C followed by a ramp rate of 25 °C/min for 8 min and a hold at 280 °C for 1 min. The HP 7673A automatic sampler/injector was used to make 1-μL injections in a splitless mode. The HP 5890A GC Chemstation data system processed the chromatographic data such as peak areas, retention times of the various peaks, and the amount of alkaloids in each chromatogram. Hygrine and cuscohygrine external standards were used for the quantitation of these alkaloids. Calibration curves for hygrine and cuscohygrine were obtained by varying the concentrations (0.1-1.0 mg/mL) in methanol.

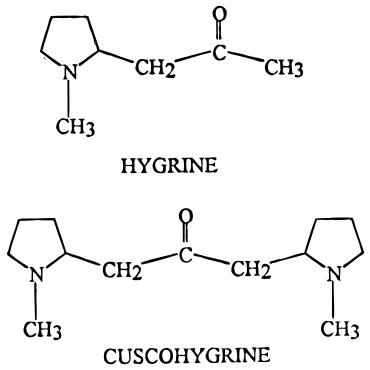

Figure 1. Chemical structures of hygrine and cuscohygrine.

HPLC Analysis. A Model 8800 ternary gradient HPLC pump (Spectra-Physics, San Jose, CA) was used with a Model 7125 Rheodyne valve (Cotati, CA) fitted with a 5-μL loop. Detection was made with a Model UV2000 dual-wavelength detector (Thermo Separation Products, Fremont, CA) operated at 220 nm (0.05 AUFS). Hygrine and cuscohygrine were separated on Synchropak CM100 weak cation exchange (WCX) column [10.0 cm × 4.6 mm i.d., 5 μm); SynChrom, Inc. (Lafayette, IN)]; the mobile phase consisted of methanol/0.05 M KH_2PO_4, pH 7.0 (75:25, v/v). The analytical column was used without a guard column. The calibration curves were constructed by plotting the concentrations of external standards (0.1−1.0 mg/mL) versus area counts of the corresponding peaks.

Extraction Procedure. Powdered leaf tissue (~5 g) was combined with 100 mL of 95% ethanol in a flask that was allowed to stand for 30 min. The extract was stirred for 30

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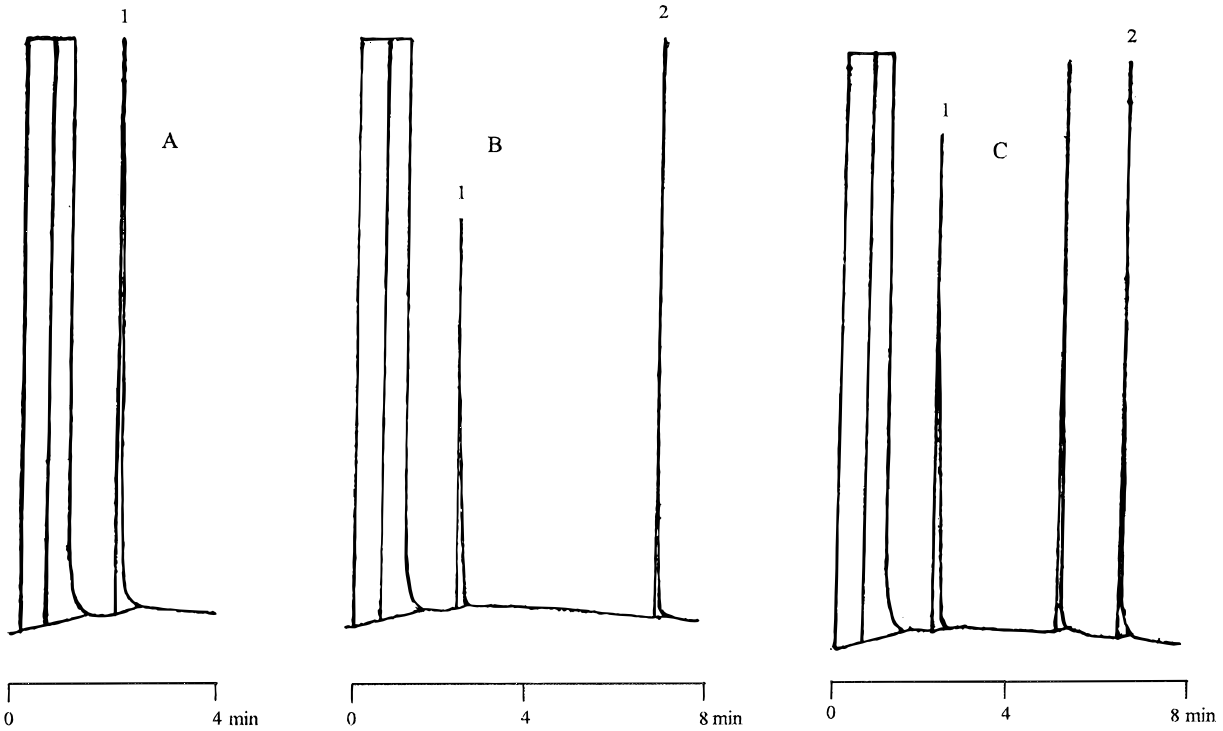

Figure 2. GC chromatograms obtained from (A) standard solution of hygrine (1.0 mg/mL), (B) standard solution of cuscohygrine (0.25 mg/mL), and (C) an *E. coca* leaf extract. Peak 1 is hygrine with a retention time of 5.4 min. In chromatogram C, *E. coca* extract (final volume of 8 mL) originated from a leaf mass of 5 g.

min at room temperature and was then allowed to stand for 40 min before passing through filter paper. The solvent was reduced to 5−10 mL by rotary evaporation at 60 °C under vacuum (2−10 mm) to minimize the loss of hygrine and cuscohygrine. The extract was redissolved in 75 mL of chloroform. The flask was rinsed with 25 mL of an ethanol/water (3:1) mixture that was combined with the extract in a separatory funnel. The extract was shaken with a 75-mL volume of 1.5% citric acid in water (w/v) and was then transferred to a second separatory funnel. The extract was shaken again with a 25-mL volume of citric acid. The larger citric acid volume was combined with the smaller volume that was allowed to stand for about 15 min for phase separation. After the chloroform layer was discarded, another 50 mL of chloroform was combined with the citric acid layer. After the mixture was shaken and then the chloroform discarded, the aqueous fraction was poured into a beaker and was adjusted to pH 5.5 using $NaHCO_3$ powder. The aqueous fraction was shaken with two 40-mL volumes of chloroform for the purpose of removing cocaine and other interfering alkaloids. The aqueous layer was then adjusted to pH 8.8 with 10% NH_4OH in water. Hygrine and cuscohygrine were partitioned into chloroform by shaking the aqueous layer with two 40-mL volumes of chloroform in a separatory funnel. The chloroform extract was collected in a flask over anhydrous Na_2SO_4. The extract was then transferred to a round-bottom flask and was subsequently reduced to a volume of 0.5−1.0 mL on the rotary evaporator. The extract was diluted to 8 mL with methanol for GC and HPLC analyses.

Fortified Samples. Aliquots of a working standard solution (10.0 mg/mL) of hygrine and cuscohygrine were added to 2.5−5.0 g of powdered *E. coca* leaf tissue to yield 10 and 20 mg of hygrine and cuscohygrine per sample. The alkaloids were extracted from fortified samples according to the same procedure as described above. A minimum of two replicates was made of each fortification level.

RESULTS AND DISCUSSION

GC and HPLC Analysis. GC chromatograms of a hygrine standard (A), a cuscohygrine standard (B), and an *E. coca* leaf extract (C) are shown in Figure 2. Chromatogram A shows a hygrine standard (peak 1, retention time = 2.3 min), and chromatogram B shows a cuscohygrine standard (peak 2, retention time = 5.4 min). The hygrine observed in chromatogram B is attributed to the decomposition of cuscohygrine in the GC injection port or on the capillary column. The conversion of cuscohygrine to hygrine in the GC injection port was reported in a recent coca alkaloid study (Moore et al., 1995). Chromatogram C shows the separation of hygrine and cuscohygrine in an *E. coca* extract. The unlabeled peak between hygrine (peak 1) and cuscohygrine (peak 2) has been identified by GC/MS analysis as methylecgonine (unpublished data). Figure 3 illustrates the HPLC chromatograms of (A) a hygrine standard (retention time = 4.2 min) and (B) a cuscohygrine standard (retention time = 6.8 min). Hygrine also is observed in chromatogram B, and its presence is attributed to the decomposition of cuscohygrine on the HPLC column, as was reported in our earlier study (Glass and Johnson, 1996). Cuscohygrine was retained longer on the cation exchange column than hygrine because of the greater positive charge distribution on the cuscohygrine molecule. The two nitrogen atoms on cuscohygrine (Figure 1) create the greater positive charge distribution, which is more strongly held by the negatively charged sites of the cation exchange phase.

Table 1 shows the slopes, intercepts, and correlation coefficients obtained from the linear regression analysis of the chromatographic data. The limits of detection (LOD) for hygrine were estimated at about 0.05 mg/mL on the HPLC column and 0.1 mg/mL on the capillary GC column. LOD for cuscohygrine was estimated as 0.1 mg/mL on both the GC and HPLC columns.

Recovery of Alkaloids. Table 2 summarizes the recoveries of hygrine and cuscohygrine from fortified leaf samples (2.5−5.0 g). Recoveries of the two alkaloids were considerably higher using the GC analysis. A

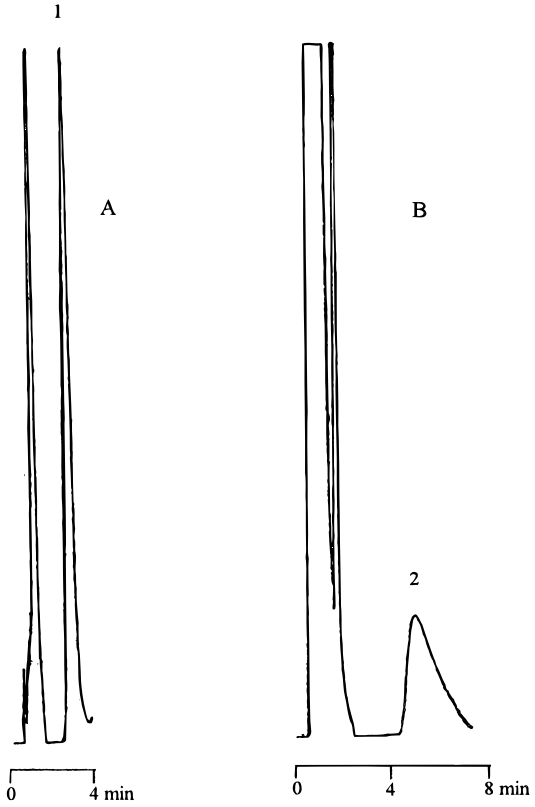

Figure 3. HPLC chromatograms obtained isocratically from (A) standard solution of hygrine (0.25 mg/mL) and (B) standard solution of cuscohygrine (0.25 mg/mL). The retention times were 4.2 min for hygrine (peak 1) with a flow rate of 0.5 mL/min as compared to 6.8 min for cuscohygrine (peak 2) with a flow rate of 1.5 mL/min.

Table 1. Values Obtained from Calibration Curves of Hygrine (H) and Cuscohygrine (C) Using GC and HPLC Methods

method	alkaloid	slope (*m*)	intercept (*b*)	correl coeff (*r*)
GC	H	4.87	−0.43	0.999
	C	5.44	−0.33	0.999
HPLC	H	10.1	−0.16	0.999
	C	2.87	−0.22	0.999

Table 2. Recovery of Hygrine (H) and Cuscohygrine (C) from Fortified *E. coca* Leaf Tissue

		recovery[a] (%)		
method	alkaloid added	mean	SD	CV (%)
GC	H, 10 mg	84.0	5.0	6.0
	H, 20 mg	83.0	7.0	8.4
	C, 10 mg	98.0	4.0	4.1
	C, 20 mg	95.0	6.5	6.8
HPLC	H, 10 mg	56.1	6.1	10.9
	H, 20 mg	71.1	9.6	13.5
	C, 10 mg	78.5	12.5	15.9
	C, 20 mg	72.0	2.0	2.8

[a] Results are the mean of two to four replicates, standard deviation, and coefficient of variation.

mean recovery of 83.5% (7.2% CV) was obtained for hygrine by GC, in comparison to a mean recovery of 63.6% (12.2% CV) by HPLC. This 63.6% hygrine recovery by HPLC using the weak cation exchange column agrees with the 63.7% hygrine recovery found earlier using the strong cation exchange column (Glass, 1995). For cuscohygrine, a mean recovery of 96.5% (5.5% CV) was obtained using GC as compared to a mean recovery of 75.3% (9.4% CV) using HPLC. In an earlier HPLC study of cuscohygrine (Glass and Johnson, 1996), a mean recovery of 64.4% (5.6% CV) was obtained on the weak cation exchange column.

Table 3. Comparison of Hygrine (H) and Cuscohygrine (C) Content in Air-Dried *E. coca* Leaf Tissue

			content[a] (%)		
site	method	alkaloid	mean	SD	CV (%)
Beltsville	GC	H	0.12	0.02	16.66
		C	0.25	0.03	12.00
	HPLC	H	0.07	0.01	14.29
		C	0.21	0.03	14.29
Hawaii	GC	H	0.08	0.01	12.50
		C	0.19	0.02	10.53
	HPLC	H	0.06	0.02	33.33
		C	0.17	0.03	17.65

[a] Results are the mean of two to four replicates, standard deviation, and coefficient of variation.

Alkaloid Content. Table 3 shows the results of a comparison between the GC and HPLC analysis of hygrine and cuscohygrine in greenhouse-cultivated *E. coca* leaves grown in Beltsville, MD, and field-cultivated *E. coca* leaves from Hawaii. The mean hygrine contents were 0.12% by GC and 0.07% by HPLC for the Beltsville-grown leaves. Cuscohygrine contents were found to be 0.25% by GC and 0.21% by HPLC. The precision was satisfactory for the GC and HPLC analysis with CVs of 12.0% and 14.3%, respectively. The hygrine and cuscohygrine contents found in the Hawaii-grown *E. coca* leaves (Table 3) are in good agreement with the hygrine and cuscohygrine contents in *E. coca* leaves from Beltsville; however, the precision was lower for the alkaloid content in *E. coca* leaves from Hawaii.

In conclusion, the present study reveals that the GC and HPLC methods are fairly comparable in accuracy and reproducibility for the analysis of hygrine and cuscohygrine. Both methods are quite suitable for routine analysis of these two alkaloids. The real potential for the HPLC method lies in its ability to be scaled-up to a preparative method for the isolation and purification of the many known and unknown alkaloids in coca leaf extracts.

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