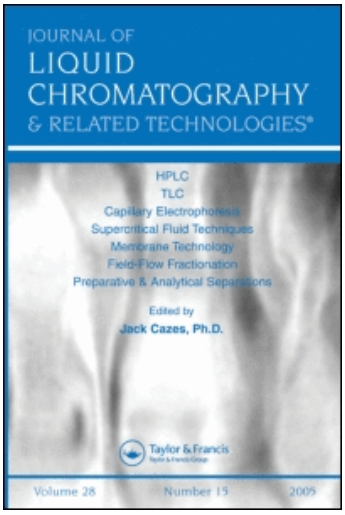

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Analysis of Hygrine in Coca Leaves Using a Novel High-Performance Liquid Chromatographic Method

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ANALYSIS OF HYGRINE IN COCA LEAVES USING A NOVEL HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD

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ABSTRACT

A novel high-performance liquid chromatographic (HPLC) method is described for the determination of hygrine in leaves of Erythroxylum coca and Erythroxylum novo. The analysis was performed on a strong cation exchange HPLC column with a mobile phase consisting of MeOH: 0.1 M KH_2HPO_4, pH 7 (75%:25%, v/v) and a UV detector set at 220 nm. Recoveries of hygrine averaged about 64% from extracts fortified with 2.5 to 10.0 mg of hygrine per 10 ml of extract. Hygrine contents in leaves of E. coca and E. novo were determined as 0.09 and 0.10% of the dry weight, respectively.

INTRODUCTION

Hygrine (N-methyl-2-acetonyl pyrrolidine) is an alkaloid found in leaves of a variety of plants such as Datura, Hyoscyamus, Nicotiana and Erythroxylum. Recently, this pyrrolidine alkaloid was found to be the precursor of the tropane alkaloids hyoscyamine and scopolamine, both of which are widely used in the preparation of many pharmaceutical products (1,2).

Hygrine in plant tissue has been traditionally analyzed by conventional chromatographic techniques, such as thin-layer chromatography (TLC)(2) and capillary gas chromatography (CGC)(3). In a recent study (4), hygrine from coca extracts was derivatized with heptafluorobutyric anhydride and then analyzed by CGC. Although CGC has been routinely used for the analyses of many tropane alkaloids (3), this method has not been validated for hygrine, in respect to linearity, precision, and recovery of hygrine from coca leaf extracts.

In the present paper, a modified procedure for extracting hygrine from coca leaves and a high-performance liquid chromatographic (HPLC) method are described. The HPLC method is simple and reliable for routine analysis of hygrine. This analytical HPLC method was developed as part of a project aimed at isolating, via preparative HPLC, some of the unknown tropane alkaloids that are found in coca extracts (6). More than 45 trace-level tropane alkaloids were recently detected in coca extracts using CGC-MS (mass spectrometry) (4).

EXPERIMENTAL SECTION

Chemicals: Methanol, ethanol and chloroform of HPLC grade were purchased 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 subsequently run through a HP Model 661A water purifier (Hewlett-Packard Co., Avondale, PA). This treatment produced extraordinary pure water with a resistivity > 18 Ωm-cm.

Hygrine Synthesis: Hygrine was synthesized by the procedure initially reported by Galinovsky et al. (7) and later modified by Leete et al. (8). N-methyl pyrrolidone (1.0 g) (Aldrich Chem. Co., Milwaukee, WI) dissolved in ethyl ether was partially reduced to the amino-aldehyde by refluxing with lithium aluminum hydride. After the ether in the mixture was removed by rotary evaporation, acetone dicarboxylic acid (1.0 g) (Aldrich Chem. Co.) dissolved in 0.1 M sodium dihydrogen phosphate was added to the mixture. Hygrine was separated and purified by vacuum distillation (10 mm) between 75 and 85°C which gave a colorless oil (0.13 g, 13% yield); density 0.934. The chemical purity of the synthesized hygrine was higher than 90 % as determined by CGC analysis in our laboratory and CGC/MS analysis at the Drug Enforcement Administration Special Testing and Research Laboratory, Mclean, VA.

Standard Solutions: A 55-ul volume of hygrine was dissolved in 50 mL of methanol to give a standard solution of concentration 1.0 mg/mL. Standards ranging in concentrations from 0.5 to 0.05 mg/mL were prepared by serial dilutions using methanol. These standards were stored in amber vials at 0°C.

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. Hygrine was separated on an Adsorbosphere SCX (strong cation exchange) column (25.0 cm x 4.6 mm i.d., 5 μm; Alltech Associates Inc., Deerfield, IL). The SCX column was used without a guard column. The mobile phase consisted of methanol: 0.1 M KH_2PO_4, pH 7.0 (75:25, v/v) delivered isocratically at 1.2 mL/min resulting in a column head pressure of about 1250 psi. Detection was made with a Model UV2000 dual wavelength detector (Thermo Separation Products, Fremont, CA) operated at 220 nm (0.05 AUFS). Chromatographic data were obtained on a Model 427 Integrator (Beckman Instruments, Inc, Fullerton, CA) with a chart speed of 0.5 cm/min and an attenuation of 32 mv full scale.

Calibration Curve: The area counts of the individual peaks and the corresponding concentrations were used to construct the standard

curve for hygrine. The curve followed Beer's law in the range 0.1 to 1.0 mg/mL.

Extraction Procedure: The original procedure for extracting alkaloids from coca leaves (9) was modified for the extraction of hygrine in the present investigation. E. coca and E. novo leaves were collected from plants grown from seeds under greenhouse conditions as described in an earlier alkaloid study in this laboratory (5). Air-dried leaves (0.1-10.0 g) were crushed by hand and were refluxed with 95% ethanol (50-300 ml) for 30 min. The extract was passed through Whatman No. 41 filter paper to remove all particulate matter. The solvent was removed by rotary evaporation at 60°C under vacuum (2-10 mm). The residue was re-dissolved in 50 ml of chloroform and then transferred to a separatory funnel. The chloroform extract was shaken separately with two 25-ml volumes of 1.5% citric acid in water (w/v) which were then combined in a beaker. The aqueous layer was adjusted to pH 5.5 with 1.2 M sodium bicarbonate and was subsequently shaken with two 25-mL aliquots of chloroform for the purpose of removing cocaine and other interfering alkaloids from the aqueous phase. The aqueous layer was then adjusted to pH 7.5 with 1.2 M sodium bicarbonate and to the final pH of 8.8 with 1.0 M sodium hydroxide which was done to keep the final volume near 150 ml. Hygrine and the remaining alkaloids were partitioned into chloroform by mixing the aqueous layer with 50 mL of chloroform in a glass bottle which was shaken for 1.5 hr on a rotary mixer. The mixture was sub- sequently collected in a separatory funnel and was allowed to stand for 15 min. The chloroform layer was reduced to a volume of 1-2 mL on the rotary evaporator. The extract was diluted to a final 10-mL volume with methanol for HPLC analysis.

Fortified Samples: Sub-samples (10 mL) of a crude E. novo leaf extract were fortified with 0.5, 2.5, 5.0 and 10.0 mg of hygrine from the working standard solutions of hygrine (1.0 mg/mL). A minimum of two replicates were made of each fortification level. The crude leaf extract was prepared by refluxing 2.0 g of dry E. novo leaves with 100 mL of 95% ethanolic solution for 30 min as described earlier in the extraction procedure. Recoveries of hygrine from the fortified samples and other quantitative data were analyzed by the Axum statistical program (TriMetrix, Seattle, WA) to determine the 95% confidence intervals.

RESULTS AND DISCUSSION

HPLC Analysis: In the present investigation, the Adsorbosphere SCX (strong cation exchange) column has been found to be efficient column for analyzing hygrine in coca extracts. Both reversed phase chromatography (C_{18} column) and normal phase chromatography (NH_2 column) were efficient in resolving standards of hygrine but were incapable of resolving hygrine in coca extracts (6). The chromatograms in Figure 1 show the resolution of hygrine in (A) a standard solution of hygrine (0.25 mg/mL), (B) an extract fortified with 2.5 mg of hygrine per 10 mL of E. novo extract, and (C) an unfortified E. coca extract. The retention time for hygrine was about 13 min at a flow rate of 1.2 ml/min. The calculated number of theoretical plates (N) was 18,500 plates/m^2. The high pH of the potassium dihydrogen phosphate (pH 7) used in the mobile phase appeared to have adversely affected the column's stability by causing excessive baseline drift.

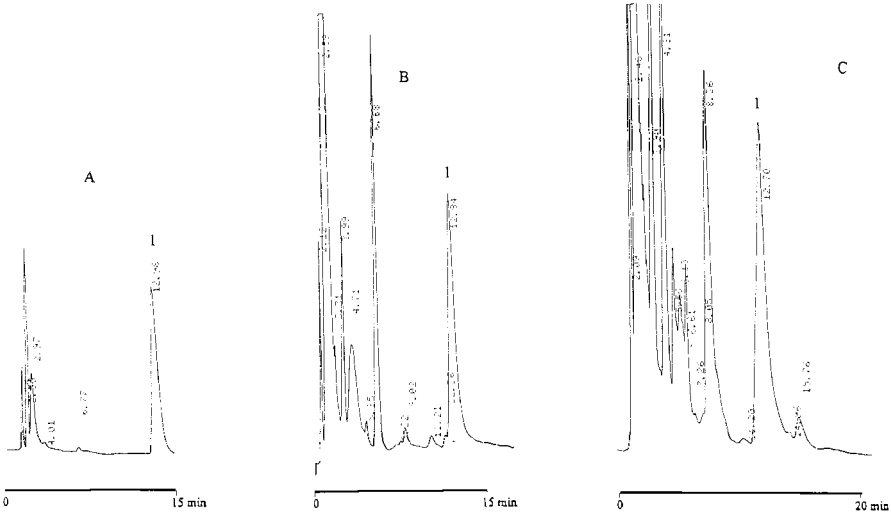

Figure 1. HPLC Chromatograms of hygrine in (A) a standard solution of hygrine (0.25 mg/mL), (B) an extract fortified with 2.5 mg of hygrine per 10 mL of E. coca extract, and (C) an unfortified E. coca extract. Peak 1 is hygrine.

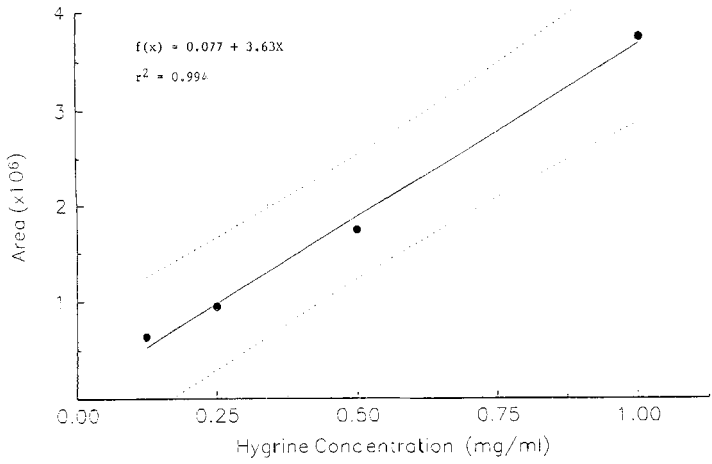

Figure 2. Hygrine standard curve. The dotted lines indicate a 95% confidence interval about the regression line.

TABLE I.

Recoveries (%) of Hygrine from Fortified Coca Extracts[a].

Hygrine Added (mg)		Hygrine Found (mg)	Recovery Content (%)	S.D.	C.V.[b] (%)
0.5	(n = 3)	N.D.[c]	----		
2.5	(n = 2)	1.62	64.80	0.80	1.20
5.0	(n = 3)	3.50	70.87	3.54	4.86
10.0	(n = 3)	5.53	55.27	3.60	6.51
		Means	63.65	2.60	4.19

[a]Hygrine was added to 10-mL aliquots of a working stock extract which was prepared by refluxing 2.0 g of dry E. novo leaves in 100 mL of 95% ethanol. [b]Results are the mean of two or more replicates, standard deviation, and the coefficient of variation (%). [c]Denotes not detectable.

The standard curve for hygrine (Fig. 2) was fitted by the regression equation $f(x) = 0.077 + 3.63x$, with the coefficient of regression (r^2) of 0.994. The detection limit (signal/noise = 3) for hygrine was determined as 0.05 mg/mL, or 250 ng/injection at the 0.05 AUFS sensitivity level.

Analysis of Leaves: Table I shows that the recoveries of hygrine in fortified samples ranged from about 55 to 71% with a mean recovery of 63.7% and a coefficient of variation of 4.19%. Hygrine was below the limit of detection in the 0.5 mg per 10 mL sample. The recoveries of hygrine witnessed here are similar to those observed for cocaine in an earlier study (5). The losses of hygrine witnessed here presumably occurred in the rotary evaporation steps. Significant losses of hygrine occurred when fortified samples went to dryness during rotary evaporation (6).

TABLE II.

Hygrine Contents (%) of Air Dried E. coca and E. novo Leaves.

Species	Avg. Dry Wt.	Hygrine Content (%)	S.D.	C.V. (%)
E. novo	2.00g (n = 2)	0.10	0.01	10.00
E. coca	2.02g (n = 3)	0.06	0.01	16.66
	4.30g (n = 3)	0.05	0.01	20.00
	10.00 (n = 3)	0.17	0.02	11.76
	Mean	0.09	0.013	16.14

[a]Results are the mean of two or more replications, standard deviation, and the coefficient of variation (%).

Table II shows that the hygrine content of air-dried E. coca and E. novo leaves was 0.09 and 0.1 %, respectively. The precision was less for the analysis of the leaf extracts (C.V.=16.14%, n=9) than for the analysis of hygrine in fortified samples (C.V.=4.19 %, n=8). The 0.09% hygrine content found in the present investigation is near the 0.1% hygrine content reported for Colombia E. coca leaves using a CGC method in which hygrine was derivatized with heptafluorobutyric anhydride (4). In a similar study using a CGC method (10), a higher hygrine content of 0.3% was reported for greenhouse cultivated E. coca leaves. Preliminary results obtained from CGC analyses in this laboratory show a higher hygrine content present in coca leaves, i.e., approximately 0.3 % in E. coca and 0.2% in E. novo leaves (6). The variability in the present method apparently masks the difference in the hygrine content of these two plant species. A comparative study of hygrine in E. coca and E. novo using CGC and HPLC analyses will be presented in the near future.

CONCLUSION

The modified extraction procedure and the new HPLC method using the strong cation exchange column proposed in this paper provide a reliable method for quantitating hygrine in coca leaf extracts. The precision of the method is good for hygrine in fortified samples with a C.V. of 4.2% and is acceptable for hygrine

in coca extracts with a C.V. of 16.1%. This HPLC method offers the advantages of being simple and not requiring extensive derivatization of hygrine. Also, this analytical procedure can be easily scaled up to a preparative method for the separation and purification of hygrine and any of the alkaloids that are chromatographed under these reported conditions.

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