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SEPARATION OF FOUR ISOMERS OF LYSERGIC ACID α-HYDROXYETHYLAMIDE BY LIQUID CHROMATOGRAPHY AND THEIR SPECTROSCOPIC IDENTIFICATION

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SUMMARY

A method is described for the separation of four isomers of lysergic acid α-hydroxyethylamide and its decomposition products ergine and erginine using LiChrosorb NH$_2$ as the stationary phase and isocratic elution. The substances under study were determined by ^{13}C and ^{1}H NMR and mass spectroscopy. The relative proportions of individual isomers of lysergic acid α-hydroxyethylamide, ergine and erginine in the fermentation medium are assumed to result from chemical equilibrium reactions. The method is reproducible and suitable for kinetic studies of the isomerization and degradation of lysergic acid α-hydroxyethylamide.

INTRODUCTION

Lysergic acid α-hydroxyethylamide (LAH) is an important substrate of semisynthetic ergot alkaloids. In fermentation production, LAH[1] decomposes spontaneously[2] and is thus found in a mixture with ergine and erginine[3]. These compounds are also formed on heating ergotamine[4]; other products include C(8) and C(2′) epimers. The same isomerization products were found to be formed on heating other cyclol alkaloids with dilute acid[5–8]. The analysis of these compounds was carried out by Bethke *et al.*[4], who determined ergotamine and the products of its isomerization, hydrolytic and addition reactions by liquid chromatography on a reversed phase. Our aim was to elaborate a separation method for the isomerization and decomposition products of LAH. Because of the considerable instability of the compounds under study it was necessary to prepare reference samples from fermentation broth. The elaboration of the high-performance liquid chromatographic (HPLC) method was based on earlier results[9].

EXPERIMENTAL

Fermentation

The method of cultivation of the saprophytic strain *Claviceps paspali* F 2056 and the conditions of submerged fermentation were described earlier[10].

Alkaloid extraction

On the fourteenth day of fermentation the culture broth was adjusted to pH 9 with aqueous ammonia and extracted with chloroform–isopropanol (4:1); the chloroform layer was separated, dried over sodium sulphate and evaporated to dryness under reduced pressure at a temperature below 15°C. The crude extract was partially dissolved in chloroform, undissolved substances were filtered off and the solvent was evaporated. The crude extract enriched in *iso*-compounds was dissolved in chloroform–methanol (4:1) and used directly for semi-preparative liquid chromatography.

Reagents

Ergine, erginine, ergometrine and ergometrinine standards were obtained from Galena (Opava, Czechoslovakia). The solvents diethyl ether, chloroform, methanol, ethanol and isopropanol (Lachema, Brno, Czechoslovakia) were of analytical reagent grade and were distilled before use. The stationary phase was MicroPak NH_2, particle size 10 μm (Merck, Darmstadt, G.F.R.), in a ready packed column (50 cm × 8 mm I.D.) (Varian Aerograph, Walnut Creek, CA, U.S.A.).

Instruments

Semi-preparative liquid chromatography was carried out on an apparatus consisting of a VCM 300 high-pressure micropump and a variable-wavelength UV detector (both from Development Workshops, Czechoslovak Academy of Sciences, Prague, Czechoslovakia). The substances were eluted isocratically with diethyl ether–ethanol (9:1) as the mobile phase.

Mass spectra were measured on a Varian-MAT 311 instrument under the following conditions: energy of ionizing electrons, 70 eV; ionizing current, 1 mA; ion source temperature, 200°C; direct inlet system operated at 110–180°C. The elemental composition of the ions was determined by the peak-matching technique ($\pm$ 5 ppm; perfluorokerosene standard).

^{1}H and ^{13}C NMR spectra were measured on a JEOL FX-60 spectrometer (59.797 and 15.036 MHz, Fourier transform mode, 25°C) in a mixture of deuteriochloroform and perdeuteriomethanol (4:1). The $CDCl_3$ signal was used as a lock. Chemical shifts were referred to internal tetramethylsilane and were calculated with an accuracy of $\pm$ 0.005 and $\pm$ 0.06 ppm for the digitally obtained address differences.

UV spectra were measured on a Variscan LC instrument immediately during the qualitative determination by a stop-flow method.

RESULTS

Liquid chromatography

Analysis of an alkaloid mixture produced under submerged condition by the fungus *C. paspali* F 2056 revealed eight alkaloids in the extract. The elution times of four of them corresponded to ergine (V), erginine (VI), ergometrine (VII) and ergometrinine (VIII) (Table I, Fig. 1). The other four substances had elution times that did not correspond to any standard at our disposal and had to be isolated by semi-preparative liquid chromatography in order to determine their structures.

Semi-preparative liquid chromatography was carried out under the conditions

TABLE I

RELATIVE CAPACITY FACTORS, $k'_{rel} = k'_i/k'_I$, OF SUBSTANCES i (II–VIII)

Compound i	k'_{rel}
II	0.44
III	1.37
IV	0.53
V	1.28
VI	0.63
VII	1.66
VIII	0.81

given in the legend to Fig. 1. To obtain sufficient amounts of pure standard substances the chromatographic cycle was repeated 20 times; to prevent possible changes in the structures of the substances (isomerization, degradation), the individual frac-
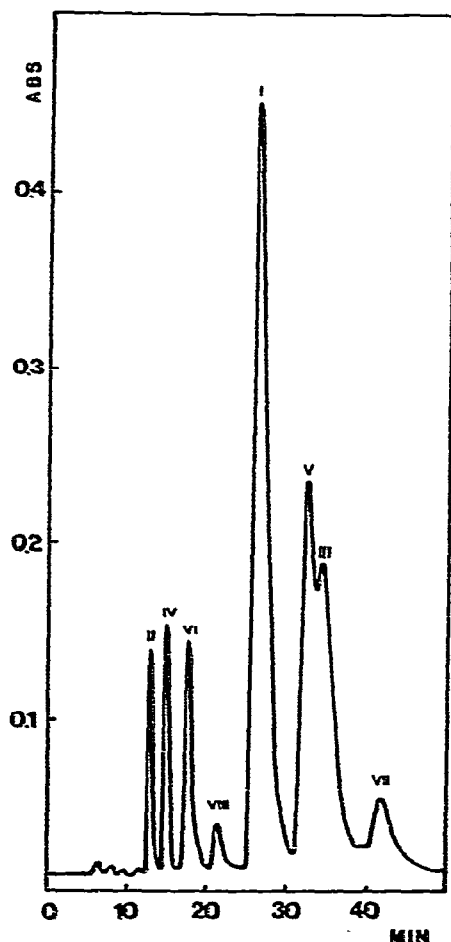

Fig. 1. Chromatogram of alkaloids I–VIII. MicroPak NH₂ (particle size 10 μm), ready-packed column (50 cm × 8 mm I.D.). Mobile phase, diethyl ether–ehtanol (9:1). Flow velocity, 220 ml/h. UV detection: 310 nm, 0.50 A. Injection, 50 μl.

tions were immediately evaporated to dryness after each cycle under reduced pressure at 5–10°C. Analyses of the individual fractions showed that compounds I, II, IV and VI (Fig. 2) were obtained in pure form whereas compounds III and V were mixtures. The resulting standard compounds were used for identification.
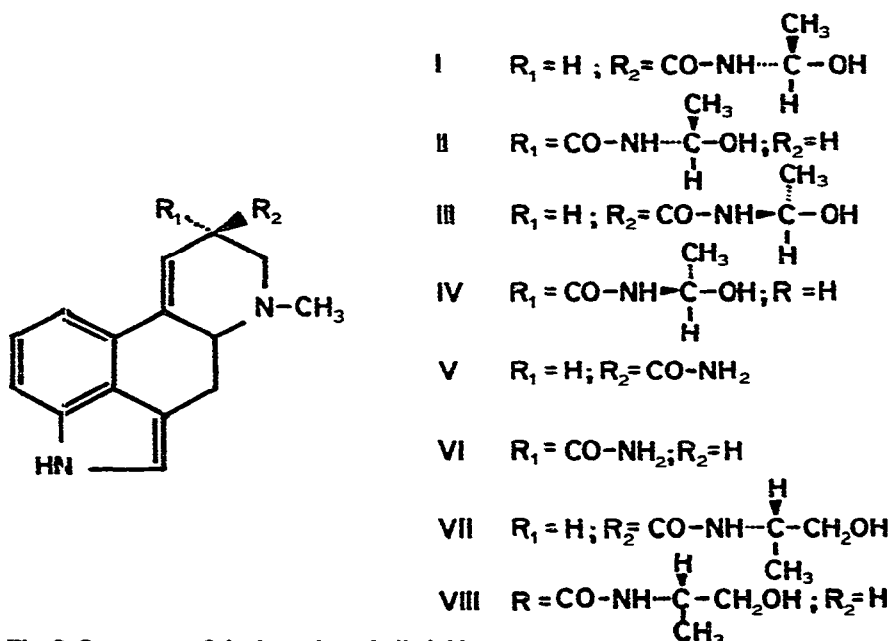
$$I \quad R_1 = H \; ; R_2 = CO-NH\cdots\overset{\overset{\displaystyle CH_3}{|}}{\underset{\underset{\displaystyle H}{|}}{C}}-OH$$

$$II \quad R_1 = CO-NH\cdots\overset{\overset{\displaystyle CH_3}{|}}{\underset{\underset{\displaystyle H}{|}}{C}}-OH ; R_2 = H$$

$$III \quad R_1 = H \; ; R_2 = CO-NH\mathbf{\rightarrow}\overset{\overset{\displaystyle CH_3}{|}}{\underset{\underset{\displaystyle H}{|}}{C}}-OH$$

$$IV \quad R_1 = CO-NH\mathbf{\rightarrow}\overset{\overset{\displaystyle CH_3}{|}}{\underset{\underset{\displaystyle H}{|}}{C}}-OH ; R = H$$

$$V \quad R_1 = H; R_2 = CO-NH_2$$

$$VI \quad R_1 = CO-NH_2; R_2 = H$$

$$VII \quad R_1 = H; R_2 = CO-NH-\overset{\overset{\displaystyle H}{|}}{\underset{\underset{\displaystyle CH_3}{|}}{C}}-CH_2OH$$

$$VIII \quad R = CO-NH-\overset{\overset{\displaystyle H}{|}}{\underset{\underset{\displaystyle CH_3}{|}}{C}}-CH_2OH ; R_2 = H$$

Fig. 2. Structures of the investigated alkaloids.

Identification of substances

All components of the mixture analysed provided suitable UV spectra of $\Delta^{9,10}$-ergolene derivatives. The mass spectra (Table II) always exhibited an ion of m/z 267 ($C_{16}H_{17}N_3O$) and ions of the fragmentation series typical of ergine (V)[11]. They are therefore substances derived from either ergine or erginine. The observed type of

TABLE II

MASS SPECTRA OF COMPOUNDS I–VI

Compound	m/z (rel. int. in %, composition)
I	295 (14, $C_{18}H_{21}N_3O$), 293 (9, $C_{18}H_{19}N_3O$), 267 (100, $C_{16}H_{17}N_3O$), 249 (37), 224 (49), 223 (60), 221 (100), 207 (86), 196 (46), 192 (43), 180 (69), 167 (46), 154 (66)
II	293 (4, $C_{18}H_{19}N_3O$), 267 (100, $C_{16}H_{17}N_3O$), 249 (16), 224 (26), 223 (30), 221 (56), 207 (54), 196 (36), 192 (20), 180 (46), 167 (26), 154 (40)
III + V	295 (4, $C_{18}H_{21}N_3O$), 267 (100, $C_{16}H_{17}N_3O$), 249 (26), 224 (40), 223 (37), 221 (67), 207 (66), 196 (37), 192 (26), 180 (46), 167 (31), 154 (43)
IV	293 (9, $C_{18}H_{19}N_3O$), 267 (100, $C_{16}H_{17}N_3O$), 249 (22), 224 (36), 223 (36), 221 (52), 207 (68), 196 (45), 192 (26), 180 (67), 167 (28), 154 (45)
VI	267 (100, $C_{16}H_{17}N_3O$), 249 (22), 224 (37), 223 (35), 221 (67), 207 (72), 196 (46), 192 (29), 180 (57), 167 (32), 154 (56)

fragmentation and the character of the UV spectra preclude the presence of amides of $\Delta^{8,9}$-ergolenic acid (paspalic acid).

It is possible to distinguish the above derivatives by ^{1}H and ^{13}C NMR spectroscopy. Compounds V and VI differ in the orientation of substitutent on C(8) (Fig. 2); in lysergic acid the substituent is pseudo-equatorial whereas in isolysergic acid is pseudo-axial. The dihedral angle H(8)–C(8)–C(9)–H(9) in the former instance is close to 90°C, causing a small or zero value of $J_{8,9}$; in the latter instance the protons are nearly staggered and the coupling constant may be larger. Using this rule, components II, IV and VI can be classified into an *iso*-series whereas I, III, and V belong to the normal series. The coincidence of the elution time of compound VI with that of an authentic erginine preparation (VI) confirms the correctness of this deduction.

Comparison of the ^{13}C NMR spectra of the pairs ergometrine (VII)– ergometrinine (VIII)[12] and ergine (V)–erginine (VI) (Table III) indicates that the largest differences in chemical shifts are observed on C(7), C(8) and C(9). In the *iso*-series the C(8) always resonates in a lower field and C(7) and C(9) in a higher field than in the normal series. The observed values (Table III) agree with the above classification. The non-stoichiometric ratio of the intensities of some signals in the ^{1}H NMR spectrum of fraction III–V indicates that this is a mixture of substances insufficiently separated by HPLC. Comparison with an authentic standard showed that compound V was ergine.

TABLE III

COMPARISON OF ^{13}C CHEMICAL SHIFTS OF COMPOUNDS I–IV WITH MODEL COMPOUNDS

Position	Ergine*	Erginine*	Ergometrine*	Ergometrinine*	I**	II**	III**	IV**
C(7)	54.7	53.9	55.6	54.0	57.8	54.8	58.0	53.8
C(8)	41.8	43.3	42.8	42.6***	42.6	43.4	42.6	43.4
C(9)	119.9	119.3	120.3	119.2	110.8	110.5	110.7	110.4
C_z	–	–	–	–	71.0	70.6	71.7	71.3
C_β	–	–	–	–	21.4	21.6	21.6	21.4

* In d_6-DMSO.
** In $CDCl_3 + CD_3OD$ (4:1).
*** Data from ref. 13, corrected on the basis of spectra measured by the technique in ref. 14.

In the ^{1}H NMR spectra of compounds I, II, IV and mixture III–V (Table IV) the double resonance proved the presence of the $CH_3CH(OH)NH$–moiety. The carbon atoms of this group exhibit resonances at 21 and 70 ppm (off-resonance: quartet and doublet) in ^{13}C NMR spectra (Table III). These findings allow one to interpret the peak with the highest m/z 293 in the mass spectrum as an $M - H_2O$ peak (Table II). The compounds are therefore isomers of α-hydroxyethylamide of the isolysergic and lysergic acids. Compounds II and IV differ in the magnitude of the coupling constant between the NH proton and the secondary alcoholic group of the methine (< 1 Hz and 6.1 Hz); they are therefore epimers on C_z of the side-chain. Compounds I and III form a similar pair. The carbon atom C_z in isomers with higher J_{NH,H_z} value (III and IV) resonates 0.7 ppm downfield of their counterparts I and II. Examples of epimers of lysergic acid dialkylamides hydroxylated in the side-chain

were described by Ishii *et al.*[13]. Although they gave no values for the appropriate coupling constants, the recorded spectrum shows that the epimers also differ in the magnitude of the vicinal constant of the OCH proton. This is probably due to the different population of rotamers caused by different possibilities for hydrogen bond formation. The average conformation observed in the NMR spectra has similar values of chemical shifts for epimer pairs but different magnitudes of the coupling constants.

TABLE IV

COMPARISON OF SELECTED ¹H NMR DATA OF COMPOUNDS I–IV AND VI

Spectra measured in a mixture of $CDCl_3$ + CD_3OD (4:1), δ-scale; coupling constants in Hz given in parentheses. Abbreviations: s = singlet; d = doublet; q = quartet.

Compound	$H_{(2)}$	$H_{(9)}$	$N–CH_3$	$N_{(1)}–H$	$CONH–$	$CHOH$ α	CH_3 β
I	6.90 s	6.39 s	2.67 s	8.15 s	8.78 s	5.48 q (6.1)	1.40 d (6.1)
II	6.94 s	5.98 d (5.5)	2.61 s	8.17 s	8.68 s	5.41 q (6.1)	1.28 d (6.1)
III	6.94 s	6.45 s	2.67 s	8.39 s	8.82 d (4.9)	5.50 dq (6.1, 4.9)	1.40 d (6.1)
IV	6.92 s	6.56 d (6.1)	2.57 s	7.96 s	8.80 d (6.1)	5.43 dq (6.1, 6.1)	1.30 d (6.1)
VI	6.94 s	6.57 d (4.1)	2.59 s	8.11 s	8.64 s		

Isomerization of LAH

Isomerization of LAH isolated by semi-preparative liquid chromatography was accomplished according to Schlientz *et al.*[5,6]. After alkalization with ammonia solution and extraction with chloroform–isopropanol (4:1), the reaction mixture yielded a sample that was analysed further by liquid chromatography. It contained approximately equal amounts of compounds II, IV and VI (8–10 % each) and about double the amounts of I, III and V (about 20 % each). The elution times of all substances corresponded to the elution times of compounds obtained from naturally occurring material. The results confirm earlier data[2,3] indicating that ergine and erginine are probably only artifacts arising from LAH.

DISCUSSION

Isomerization of simple lysergic acid derivatives on C(8) is known to proceed readily even under mild conditions. With LAH, the reaction is more complex (as in cyclol alkaloids) as it also includes epimerization on asymmetric carbon atoms of the side-chain. Lysergic and isolysergic acid α-hydroxyethylamides (I, II, III and IV) are hemiacetals derived from acetaldehyde and ergine (V) or erginine (VI). The aldolization reaction is reversible. Mutual transformation of II to IV was observed during a 15-h ¹³C NMR measurement; the spectrum displayed signals of both C_x atoms. The formation of isomers II, III and IV can likewise be observed during acid-catalysed

isomerization of the parent LAH. The relative proportions of individual isomers of LAH (I, II, III and IV), ergine (V) and erginine (VI) in the cultivation medium during fermentation[10] can be interpreted as the result of chemical equilibrium reactions.

The chromatographic behaviour of compounds I, II, III and IV is determined by the configuration on asymmetric carbon atoms C(8) and C_x. The pseudo-axial position of the side-chain on carbon C(8) may lead to the formation of an intramolecular hydrogen bond N(6) ···· H–N(20) which causes a marked decrease in the basicity of alkaloid molecules (II and IV); this is demonstrated by the reduced interaction with the basic nitrogen atom of the stationary phase. For this reason compounds II and IV are less retained on the column. The ^1H and ^{13}C NMR spectra of individual diastereomeric pairs of alkaloids (I and III, II and IV) indicate that these substances differ in the population of rotamers with different possibilities of hydrogen bond formation and with different magnitudes of Van der Waals interactions, which is demonstrated by the different basicities of the molecules and thus in different interactions with the stationary phase.

The developed HPLC method can be used to study the kinetics of isomerization reactions and degradation reactions of LAH and for the control of the purity of preparations.

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