Journal of Chromatography, 169 (1979) 446–452

CHROM. 11,454

Note

High-performance liquid chromatographic separation and trace determination of the antitumour derivatives of ellipticine and related quaternary ammonium compounds

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(Received September 6th, 1978)

Ellipticine and 9-methoxyellipticine are alkaloids found in several plants of the family *Apocyanaceae* that have stimulated much interest owing to their antitumour properties[1–4]. Many synthetic derivatives have been proposed[5,6], among which quaternary ammonium compounds show antitumour properties of particular interest[7]. One of these derivatives is presently undergoing clinical trials[8]. The study of these compounds has raised two analytical problems: the separation of ellipticine and 9-methoxyellipticine from the plant extracts has always been extremely tedious[9], and the trace determination of the quaternary ammonium derivatives in biological fluids has not previously been possible. As shown in this paper, ion-pair partition chromatography permits these two difficulties to be overcome.

EXPERIMENTAL

Chemicals

Water distilled twice in quartz and analytical-reagent grade methanol were used after micropore filtration and degassing. 1-Heptane- and 1-pentanesulphonic acid sodium salts, were obtained from Eastman-Kodak, Rochester, N.Y., U.S.A. Ellipticine derivatives were kindly supplied by Dr. Nguyen Dat-Xuong and Dr. E. Lescot, and olivacine derivatives by Dr. H. P. Husson. Other reagents were of analytical-reagent grade.

Apparatus

The method was developed using a Waters Model ALC 204 liquid chromatograph, equipped with two Model 6000A high-pressure pumps, a U6K universal injector and a Model 660 solvent programmer. The column was a 30 cm × 4 mm I.D. μBondapak C_{18} (Waters Assoc., Milford, Mass., U.S.A.) [10-μm particle size ODS-type (reversed-phase) packing material]. Detection was performed with a Model 440 UV absorbance detector (Waters Assoc.), with double monitoring at 254 and 313 nm, and an FS 970 spectrofluorimeter (Schoeffel, Westwood, N.J., U.S.A.) equipped with a low-volume flow cell (5 μl). Fluorescence excitation was performed at 305 ± 5 nm, and fluorescence emission was measured through a cut-off filter transmitting

wavelengths above 470 nm. Chromatograms were recorded on an OmniScribe Model 5210 dual-pen apparatus (Houston Instruments, Gistel, Belgium).

Procedure

High-performance liquid chromatography (HPLC) in the reversed-phase mode was performed in methanol–water mixtures containing counter ions. The ionic composition of the three eluent systems used were as follows:

(i) 0.005 *M* sodium 1-heptanesulphonate–0.032 *M* acetic acid;

(ii) 0.005 *M* sodium 1-pentanesulphonate–0.032 *M* acetic acid;

(these two systems are identical with Waters Assoc. PIC B-7 and PIC B-5 reagents, respectively); and

(iii) 0.02 *M* ammonium acetate.

The elution flow-rate was 1.5 or 1.2 ml/min.

Procedure for acetylation of 9-hydroxymethylellipticinium acetate. A 0.15-ml volume of dry pyridine and 0.05 ml of acetic anhydride were added to 1 ml of an ethyl acetate extract of 9-hydroxymethylellipticinium acetate. After incubation for 5 min, the solution was evaporated to dryness under a stream of nitrogen and the residue was dissolved in 0.1 ml of methanol.

RESULTS AND DISCUSSION

Separation of ellipticine derivatives

Fig. 1 shows a typical chromatogram of a mixture of six ellipticines under isocratic elution with methanol–water (PIC B-7). The structures of these compounds are given in Table I. To illustrate the effect of counter ions on this separation, the retention times measured with or without counter ions were compared (Table I): with simple isocratic elution in the reversed-phase systems studied, most of these ellipticine derivatives are well resolved, and are eluted in about 10 min, provided that counter ions are present in the methanol–water. This suggests that ion-pair partition phenomena contribute to the resolution of such compounds[10,11].

Under acidic conditions, hydroxyellipticine and its corresponding methyl

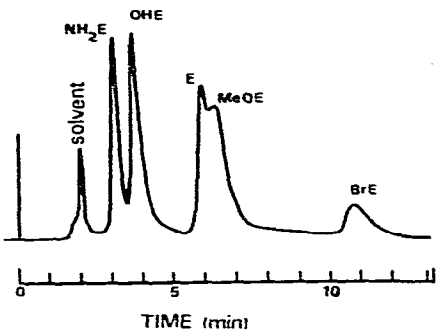
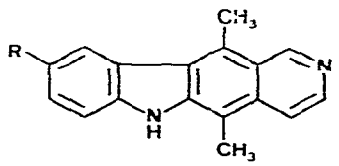
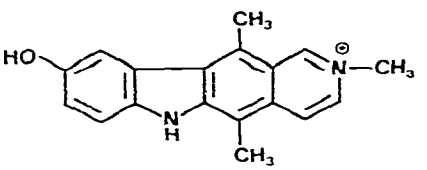


Fig. 1. Separation of six ellipticines on μBondapak C_{18}. Mobile phase: methanol–water (70:30), PIC B-7. Isocratic elution. Flow-rate: 1.5 ml/min. UV detection (254 nm). For structures of compounds, see Table I.

TABLE I

STRUCTURES AND RETENTION TIMES OF SIX ELLIPTICINES

Column: 30 cm × 4 mm I.D. μBondapak C_{18}. Mobile phase: methanol–water with or without counter ions. Flow-rate: 1.2 ml/min.

Compound	*R*	t_R *(min)*				
		MeOH–H_2O (70:30)			*MeOH–H_2O (75:25)*	
		No counter ions	*PIC B-7*	*PIC B-5*	*PIC B-7*	*CH_3COONH_4*
	NH_2	>30	3.8	3.5	3.5	4.9
	OH	>30	4.5	3.8	4.0	5.5
	H*	>30	7.1	5.2	5.4	9.3
	OCH_3	>30	7.4	5.7	5.7	9.5
	Br	>30	13.5	8.2	8.2	—
	—**	>30	4.6	3.9	4.1	4.2

* Ellipticine.

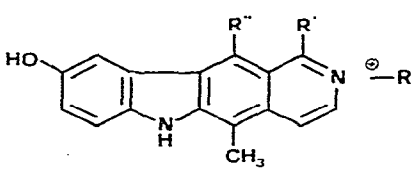
** 9-Hydroxy-2-methylellipticinium.

quaternary ammonium derivative are not resolved. At neutral pH, the elution of deprotonated hydroxyellipticine is retarded, as expected, while the elution of the quaternary ammonium remains almost unchanged. Neutral elution conditions will then be preferred for separating methyl quaternary ammonium derivatives from their corresponding weak bases. As an illustration of this principle, Fig. 2 shows the separation of a mixture of two ellipticine analogues (hydroxyellipticine and hydroxyolivacine) and corresponding quaternary derivatives (Table II). It can be seen that the two base isomers are well resolved.

TABLE II

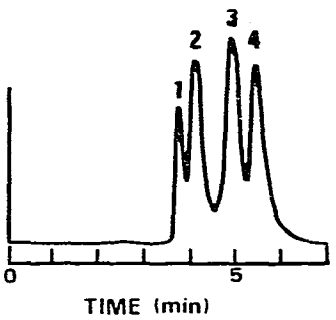
STRUCTURES AND RETENTION TIMES OF FOUR HYDROXYLATED ELLIPTICINE ANALOGUES

Chromatographic conditions are given in Fig. 2.

Compound	*R*	*R'*	*R''*	*Peak No.*
	CH_3	H	H	1
	CH_3	H	CH_3	2
	—	CH_3	H	3*
	—	H	CH_3	4**

* Hydroxyolivacine.

** Hydroxyellipticine.



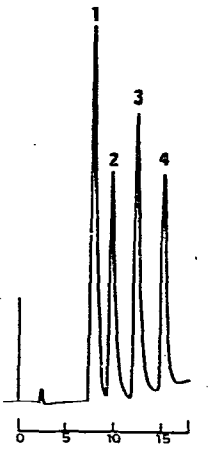


Fig. 2. Separation of four hydroxylated ellipticine analogues. Mobile phase: methanol–0.1 *M* ammonium acetate (75:25). Flow-rate: 1.2 ml/min. UV detection (313 nm).

Fig. 3. Separation of four 9-hydroxy-*n*-alkylellipticinium acetates (1, R = CH_3; 2, R = C_2H_5; 3, R = *n*-C_3H_7; 4, R = *n*-C_4H_9) using a gradient elution programme (Waters, curve No. 7). Mobile phase, methanol–water (PIC B-7): A, 50:50; B, 90:10. 20% B to 60% B in 12 min. Flow-rate: 1.2 ml/min.

The PIC B-7 reversed-phase system appears to be particularly suitable for the separation of various quaternary ammonium derivatives. Table III shows the structures and retention times of such analogues in the ellipticine series, for isocratic elution. The methyl-, ethyl-, *n*-propyl- and *n*-butyl-9-hydroxyellipticinium derivatives can be completely resolved using a proper gradient elution programme, as shown in Fig. 3. These results are of particular interest as other chromatographic procedures are unable to resolve these quaternary ammonium derivatives with such facility. Gas chromatography, for instance, cannot be used because these substances are not volatile.

As shown in Fig. 1, ellipticine and methoxyellipticine are poorly resolved. This is not surprising as the separation of these two alkaloids from plant extracts has always been extremely laborious[9]. Fig. 4 shows that almost complete resolution of these two compounds can be obtained simply by use of gradient elution.

Determination of ellipticines and analogues

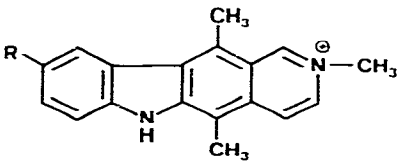
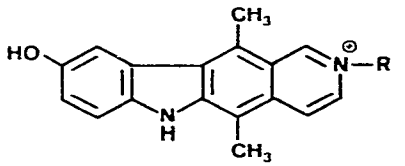
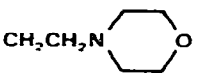
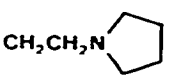
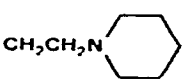
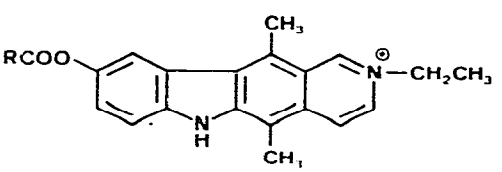
Ellipticine derivatives have an absorption maximum around 300 nm with extinction coefficients of the order of 30,000. With the Waters UV detector set at the 313-nm mercury line, as shown in Fig. 5A, the injection of 5 ng of ellipticine gives a peak the height of which is 10 times the noise value (width of the baseline).

HPLC with UV detection gives a very simple and general procedure for the determination of these compounds. However, their determination in biological fluids often requires a more sensitive method. Fluorescence detection was therefore investigated: several ellipticine derivatives are highly fluorescent, with quantum yields greater than 0.1. As shown in Fig. 5B, the injection of 50 pg of olivacine gives a peak the height of which is 13 times the noise value (width of the baseline). The linearity of the response was checked by injecting increasing amounts of the compound. The results are shown in Fig. 6 for both olivacine and ellipticine.

TABLE III

STRUCTURES AND RETENTION TIMES OF SOME ELLIPTICINE QUATERNARY AMMONIUM DERIVATIVES

Mobile phase: methanol–water (75:25), PIC B-7. Flow-rate: 1.2 ml/min.

Compound	*R*	t_R *(min)*
	H	5.7
	NH_2	3.7
	OCH_3	6.0
	$OCOCH_3$	5.1
	NO_2	5.6
	Br	8.4
	CH_3	4.1
	C_2H_5	4.5
	n-C_3H_7	5.1
	n-C_4H_9	5.9
	n-C_6H_{13}	7.6
	$CH_2CH{=}CH_2$	4.7
	CH_2CH_2OH	3.4
		4.3
		4.6
		4.8
	CH_3	5.4
	n-C_3H_7	8.7
	n-C_5H_{11}	16.0
	C_6H_5	11.8

The detection limit is defined as the amount of compound that yields a peak height twice the baseline width. This limit for UV detection, which is about the same for all compounds in this series, is of the order of 1 ng. In contrast, the detection limit with fluorescence detection varies with the compounds according to their quantum yield in the eluent. For instance, olivacine and ellipticine can be detected in amounts down to 8 and 25 pg, respectively. Unfortunately, substituted derivatives (amino, hydroxy, bromo, etc.) are poorly fluorescent, and UV detection is the most sensitive technique in these instances.

However, suitable derivatization can lead to compounds with higher fluorescence quantum yields. 9-Hydroxy-2-methylellipticinium acetate is presently under clinical investigation[8] and it was necessary to measure small amounts of this derivative in biological fluids. It can be selectively extracted with ethyl acetate by ion-pair

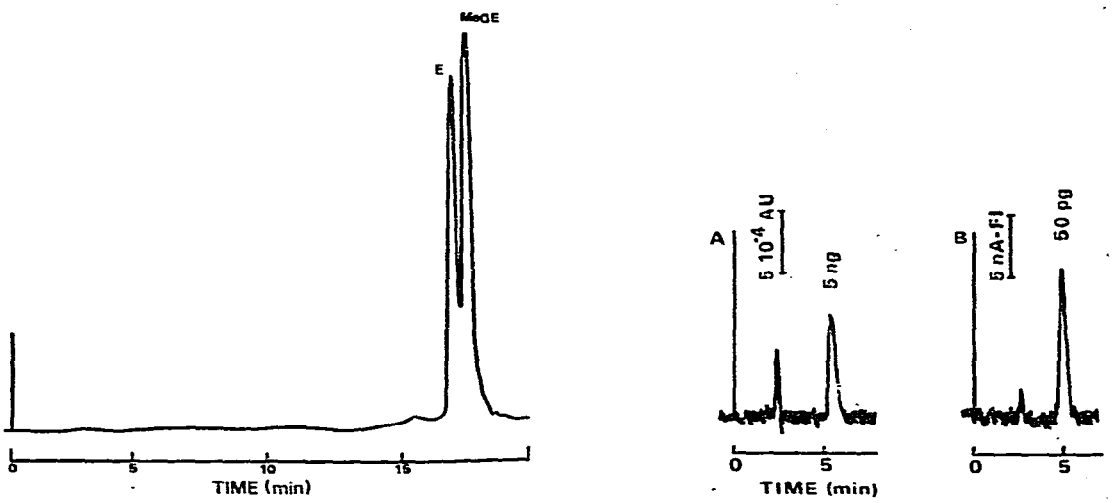


Fig. 4. Separation of ellipticine (E) and 9-methoxyellipticine (MeOE) using a gradient elution programme (Waters, curve No. 9). Mobile phase, methanol–water (PIC B-7): A, 50:50; B, 90:10 10% B to 50% B in 10 min. Flow-rate: 1.2 ml/min.

Fig. 5. Comparison of detection limits of ellipticine derivatives with UV and fluorescence detection. (A) UV detection of 5 ng of ellipticine. Injection of a 10-μl sample of a 0.5 μg/ml solution. UV (313 nm), 0.01 A.U. (B) Fluorescence detection of 50 pg of olivacine. Injection of a 10-μl sample of a 5 ng/ml solution. Excitation, 305 nm; emission >470 nm; 0.1 μA. Mobile phase: methanol–water (75:25), PIC B-7. Isocratic elution. Flow-rate: 1.2 ml/min.

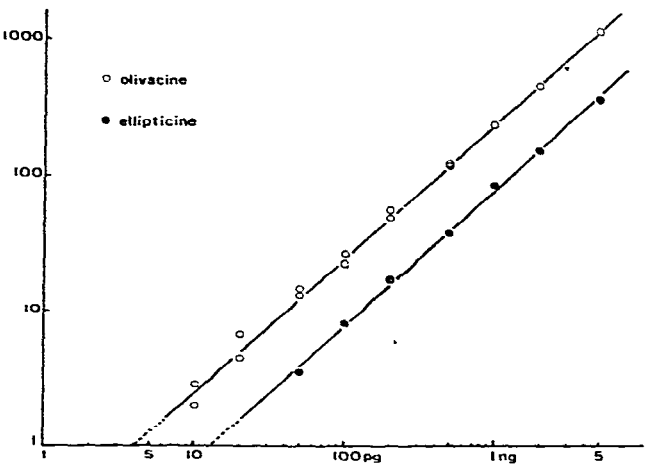


Fig. 6. Fluorescence detection of ellipticine (●) and olivacine (○). Calibration graph of ratio of peak height to baseline width *versus* amount of compound injected. Chromatographic and fluorescence detection conditions as in Fig. 5B; injections of 10-μl samples of solution of increasing concentration.

formation with tetraphenylborate[12]. As it is not fluorescent in methanol–water mixtures, it was transformed quantitatively into its fluorescent acetoxy derivative as described under Experimental. Under these conditions, the detection limit of this derivative is of the order of 50 pg. It was then possible to study the disposition of this antitumour drug in man; the results will be reported elsewhere.

ACKNOWLEDGEMENTS

The authors are grateful to Drs. Nguyen Dat-Xuong, E. Lescot and H. P. Husson for providing samples of the ellipticine derivatives and analogues, and to M. T. Adeline and M. C. Bouger for technical assistance. This work was supported by grants from the Université Pierre et Marie Curie (Paris VI), Délégation à la Recherche Scientifique et Technique (D.G.R.S.T.) and Fondation pour la Recherche Médicale. One of the authors (G.M.) was supported by a Fellowship (I.A.M.O.V.) of the D.G.R.S.T.

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