Note

Triazolines

XXII. Chromatography of 1-phenyl-5-(4-pyridyl)-Δ^2-1,2,3-triazolines and related 1,2,3-triazoles, a new class of anticonsulvant agents

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The conventional antiepileptic drugs contain a dicarboximide or a ureide function in the molecule and these may be responsible for the hypnotic and sedative effects experienced by many patients [1] Pioneering studies by Kadaba [2] have led to the emergence of a new and potentially unique family of anticonvulsant agents with a mechanism of action quite different from the more traditional anticonvulsants. These compounds are the $\triangle^2$-1,2,3-triazolines [3] and the closely related $\triangle^2$-1,2,3-triazoles. Many different substituted compounds have been synthesised in these series; this paper examines the chromatography of the 1-phenyl-5-(4-pyridyl)-substituted derivatives (Fig. 1). Several of these have been shown to possess anticonvulsant activity in animal models, and one compound, the 1-(4-chlorophenyl)-5-(4-pyridyl)-$\triangle^2$-1,2,3-triazoline (ADD17014) was found to be particularly potent in preventing a variety of seizure types in animal models [4]. Several potential metabolites of ADD17014 have also been synthesised; these are the triazole, the aziridine and the imine (Fig. 2). As these heterocyclic-substituted compounds are a novel class, no data on their chromatography have been published. The major difficulty, particularly with development of the high-performance liquid chromatographic (HPLC) method, was the intrinsic instability of
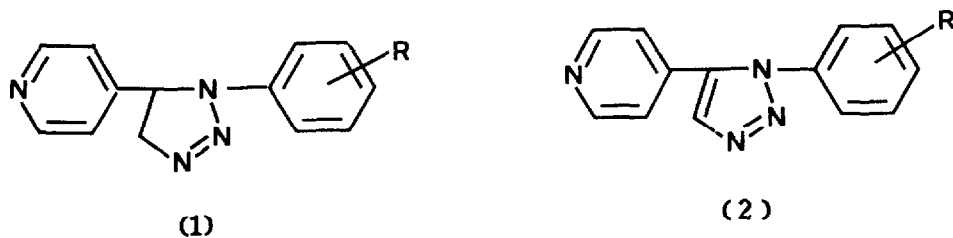
Fig 1 Structures of 1-phenyl-5-(4-pyridyl)-substituted $\triangle^2$-1,2,3-triazolines (1) and 1H-1,2,3-triazoles (2)

two of the four compounds, the triazoline itself and the imine. They are both known to undergo chemical breakdown at acidic or neutral pH values at room temperature [5]. Therefore the analytical challenge here was the development of chromatographic conditions which did not result in any *ex vivo* changes in concentrations of these components during sample analysis.
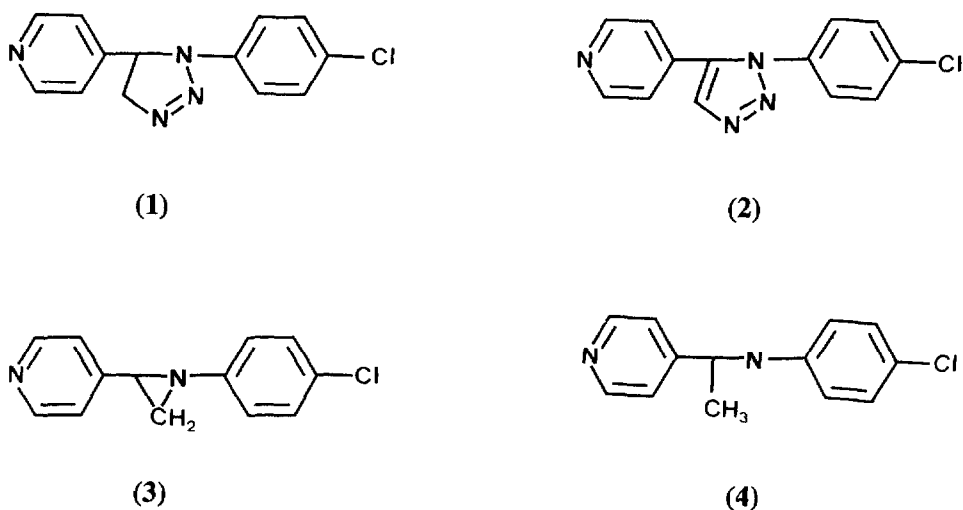

Fig 2 Structures of ADD17014, 1-(4-chlorophenyl)-5-(4-pyridyl)-$\triangle^2$-1,2,3-triazoline (1), and potential metabolites, triazole (2), aziridine (3) and imine (4)

EXPERIMENTAL

Materials

Citric acid ($C_6H_8O_7 \cdot 1H_2O$) and disodium phosphate ($Na_2HPO_4 \cdot 2H_2O$) (both SLR grade) were obtained from Fisons (Loughborough, U.K.). The buffers were made by using various compositions of 0.1 M citric acid (21 g/l) and 0.2 M disodium phosphate (35.6 g/l). All solvents were HPLC grade from Fisons, with the exception of ammonia solution (0.88 S.G.) which were of SLR grade (Fisons). Dipyridamole was obtained from Sigma (Poole, U.K.). Aluminium-backed thin-layer chromatography (TLC) plates (20 × 20 cm) precoated with 0.2-mm silica gel 60 GF_{254} were from E. Merck (Darmstadt, F R.G.).

Standards

Standard solutions of 1 mg/ml of the compounds in HPLC-grade methanol were prepared for the TLC work and stored at −20°C. From these stock solutions, 100 μg/ml solutions in methanol were prepared for HPLC and these were stored at −20°C. Standard samples were stable under these conditions for several months.

Determination of molar extinction coefficients

From the 1 mg/ml stock solutions of each compound a series of dilutions were prepared in duplicate. These were in the range 0–100 nmol/l for ADD17014 and the aziridine and 0–12 nmol/l for the triazole and the imine. The absorbance of each of the dilutions were recorded on a Uvikon 860 spectrophotometer (Kontron, Watford, U.K.) at the previously determined absorption maxima (λ_{max}) for the compound in question. The absorbances obtained were plotted against concentration and the molar extinction coefficient calculated from the Beer–Lambert equation.

HPLC apparatus

The HPLC system consisted of a ConstaMetric® pump 3000, a variable-wavelength UV SpectroMonitor® 3100 detector and a computing integrator CI4000 (all from LDC, Stone, U.K.) The column was a reversed phase Spherisorb 5-ODS column (25 cm × 0.46 cm) (HPLC Technology, Wilmsley, U.K.). A Pellicular ODS guard column (5 cm × 0.2 cm) (Whatman, Maidstone, U.K.) was used to protect the analytical column. Samples were introduced into the system *via* a Rheodyne injector fitted with a 20-μl loop. Detection was by UV absorption at 254 nm and all chromatograms were recorded and peak areas and retention times calculated by electronic integration using the computing integrator CI4000.

TLC analysis

Approximately 10 μl of the stock solutions of each of the standards were applied onto the TLC plate 2 cm from the bottom and 1 cm apart using dis-

posable glass capillary tubes and dried in warm air. The plate was then developed in a glass TLC tank (25 × 25 × 10 cm) containing 100 ml of an appropriate solvent system. After the solvent had risen to 15 cm the plate was removed, dried in warm air and examined under UV light at 254 nm

RESULTS AND DISCUSSION

UV spectroscopic data

Table I shows the absorption maxima (λ_{max}) and molar extinction coefficients obtained for the four compounds. Both ADD17014 and the triazole have considerably lower molar extinction coefficients than the imine and the aziridine. This can be explained by the two extra nitrogen atoms in the structure of the ADD17014 and the triazole; an increase in nitrogen content of a chromaphore is known to decrease the intensity of absorption due to forbidden $\pi \rightarrow \pi^*$ transition [6]. ADD17014 and the triazole also have their absorption maxima at longer wavelengths (290 and 250 nm, respectively) than the aziridine and imine (235 and 236 nm, respectively). This can be explained by the greater conjugation on the former which produces a bathochronic shift [6].

Optimisation of the TLC system

The method chosen to enable separation of the various compounds is known as Prisma [7] It classifies possible developing solvents into groups based on similarities between their chemical structures, related to their dipole moments. Each solvent is assigned a solvent strength value with respect to hexane which is given a value of zero. As the major interest was the separation of ADD17014 from its potential metabolites, standards of ADD17014 and the corresponding imine, aziridine and triazole were chromatographed in several solvents selected from the various groups. It was found that ethyl acetate gave the best separation, but ADD17014 chromatographed as two distinct spots, indicating breakdown of this compound during the chromatographic process. In light of the known instability of ADD17014 it was thought that only one of these spots was due to the triazo-

TABLE I

ABSORPTION MAXIMA AND MOLAR EXTINCTION COEFFICIENTS OF ADD17014 AND ITS CORRESPONDING TRIAZOLE, AZIRIDINE AND IMINE IN METHANOLIC SOLUTIONS

Compound	Absorption maximum (nm)	Log molar extinction coefficent
ADD17014	289	3 97
Triazole	250	4 17
Aziridine	238	4 24
Imine	235	4.37

line itself and that the other was a chemical breakdown product brought about due to the acidic nature of the silica surface. Several different volumes of ammonia solution (S.G. 0.88) were tried as additions to the solvent system to prevent this breakdown on the TLC plates, and the final solvent composition chosen was ethyl acetate–ammonia (98.6:0.4, v/v). Total separation of the mixture into the four components was achieved on several different plates on different days and the system is therefore deemed to be suitable. The various triazolines and triazoles were then chromatographed in this solvent and the results shown in Table II. The intrinsic instability of the triazoline ADD17014 and the respective imine is not a problem associated with the TLC under the given conditions. The pure standards chromatograph as single compact spots, provided the TLC plates are developed as soon as sample application is complete. The stability of these compounds on TLC has been confirmed by their removal from silica plates and mass spectrometry.

TABLE II

TLC AND HPLC SEPARATION OF SOME TRIAZOLINES, TRIAZOLES, AZIRIDINES AND IMINES

R-group	TLC R_F value[a]	HPLC retention time[b] (min)
Triazolines (Fig 2-1)		
-4-Cl	0.36	7.49
-4-OCH$_3$	0.36	5.45
-4-F	0.40	5 97
-H	0 40	5 72
-3-Cl	0 41	8 43
Triazoles (Fig 2-2)		
-4-Cl	0 53	4 48
-H	0 53	4 58
-4-CH$_3$	0 55	5 42
-3-CH$_3$	0 54	5 48
-4-F	0 50	4 67
-4-OCH$_3$	0 43	4 81
-3,4-di-Cl	0 51	8 07
-4-Br	0 56	6 83
Aziridine (Fig 2-3)		
-4-Cl	0 61	9 50
Imine (Fig 2-4)		
-4-Cl	0 65	11 02

[a]   TLC was performed on normal-phase silica plates (0 25 mm thickness) and a solvent system of ethyl acetate–ammonia (99 6 0 4, v/v)

[b]   HPLC was performed on a reversed-phase 5-ODS column with a mobile phase of acetonitrile–methanol–McIlvaines citric acid–phosphate buffer (pH 8.0, 0 005 M) (30:30 40, v/v) at a flow-rate of 1 ml/min

Development of an HPLC system

As in the case of TLC, separation of ADD17014 and its potential metabolites was the major aim, and reversed-phase HPLC with methanol–distilled water (70:30, v/v) as the mobile phase was initially investigated for the analysis of these compounds. Whereas separation of the four compounds could be achieved with this mobile phase, the peaks were too broad and too near the solvent front to be of use for the analysis of biological samples. After trying mixtures of solvents of various compositions, the best mobile phase appeared to be methanol–aceto-nitrile–distilled water (30:30:40, v/v) but this lead to on-column breakdown of two of the four compounds, the triazoline itself and the corresponding imine It was clear that the addition of a buffer would be required to prevent this break-down, and therefore several different pH buffers were tried. As can be seen in Table III the triazole and the aziridine were stable at all pH values including distilled water alone, whereas pH 8.0 buffer was needed to stabilise the imine and the triazoline. This is consistent with the findings of chemical stability studies carried out in our laboratories [5].

As a result of this study, an optical ternary mobile phase composed of aceto-nitrile–methanol–McIlvaine's phosphate buffer (pH 8.0; 0.005M) in the ratio 30:30:40 (v/v) was developed, which at a flow-rate of 1 ml/min, gave retention times as follows: ADD17014 (triazoline), 7.5 min, triazole, 4.5 min; aziridine, 9.5 min; imine, 11.0 min. A typical chromatogram is shown in Fig 3. Also shown is a HPLC trace of rat blood taken at 2 h after dosing ADD17014 (30 mg/kg) in-travenously and taken through the extraction procedure (5 ml diethyl ether) The recoveries approach 100% for all four compounds and the proposed internal standard, dipyridamole. The other triazolines and triazoles were subjected to the same HPLC conditions and the retention times shown in Table III. All the com-pounds gave single sharp peaks on chromatographic traces indicating no chem-ical breakdown had occurred. This is important as in later *in vivo* experiments it

TABLE III

STABILITY OF ADD17014 AND ITS POTENTIAL METABOLITES WHEN CHROMATO-GRAPHED IN MOBILE PHASES OF DIFFERING pH

Figures represent the percentage breakdown of each compound during a chromatographic run of 13 min

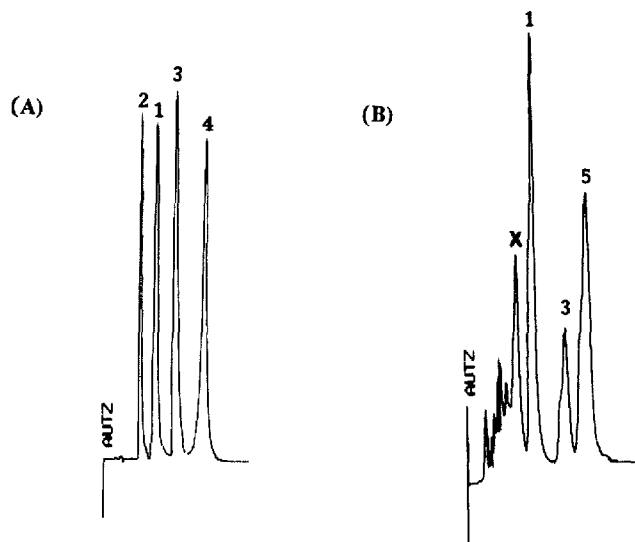| pH	Percentage breakdown during chromatography			
	ADD17014	Triazole	Aziridine	Imine
7.0	4 0	Nil	Nil	6 0
7 4	3 7	Nil	Nil	2 9
7 8	Nil	Nil	Nil	3 1
8 0	Nil	Nil	Nil	Nil

Fig 3 (A) HPLC separation of ADD17014 (1) and potential metabolites, triazole (2), aziridine (3) and imine (4). Retention times: ADD17014, 4 5 min; triazole, 7 5 min, aziridine, 9 5 min, imine, 11 0 min. Conditions as described in Results and discussion. (B) HPLC of an extract of rat blood taken at 2 h after dosing ADD17014 (30 mg/kg, intravenously) The extraction and analysis are as described in Results and discussion Peaks 1 = ADD17014; X = unknown metabolite, 3 = aziridine; 5 = dipyrimadole (internal standard)

will be vital to prevent any *ex vivo* chemical breakdown that may occur after withdrawal of a biological sample from the body. It is important that no alterations in the pattern of metabolites occur during analysis or sample storage.

REFERENCES

1 P K Kadaba, *J. Pharm Sci* , 73 (1984) 850
2 P K Kadaba, *J. Med Chem* , 31 (1988) 196.
3 P K Kadaba, B Stanovnik and M. Tisler, *Adv. Heterocyclic Chem* , 37 (1984) 217
4 P K Kadaba and J T. Slevin, *Epilepsia*, 29 (1988) 330
5 M A F Hamelijnck, P J. Stevenson, P. K Kadaba and L. A Damani, *J Pharm Sci*., submitted for publication
6 J C P Schwarz (Editor), *Physical Methods in Organic Chemistry*, Oliver and Boyd, New York, 1964, p 22
7 S Z Nyiredy, C A J Erdelmeir, B Meir and O. Sticker, *Planta Med* , 51 (1985) 241