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Short Communication

Triazolines

XXIII. High-performance liquid chromatographic assay in rat blood for a novel triazoline anticonvulsant (ADD17014)

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ABSTRACT

A sensitive and specific high-performance liquid chromatographic (HPLC) method for the analysis of 1-(4-chlorophenyl)-5-(4-pyridyl)-Δ^2-1,2,3-triazoline (ADD17014, I), a novel anticonvulsant agent, in rat blood is described. Compound I and the internal standard (dipyridamole) were extracted into diethyl ether (5 ml) from alkalinised blood (0.25 ml of blood plus 0.75 ml of pH 10.7 buffer), with extractability nearing 100% under these conditions. The assay is based on reversed-phase HPLC (25 cm × 0.46 cm I.D. Spherisorb 5-ODS) using a mobile phase of methanol–acetonitrile–McIlvaine's citric acid–phosphate buffer (pH 8.0, 0.005 *M*) (30:30:40, v/v) and ultraviolet detection at 290 nm. Calibration curves were linear and reproducible (correlation coefficient > 0.999). Measurement of I in rat blood (250 μl sample size) was linear in the range 0–40 μg/ml and the coefficient of variation was less than 5%. The minimum detectable level was about 0.1 μg/ml; however, a larger blood sample size (1–2 ml) allowed measurement of levels as low as 10 ng/ml, especially for estimation of drug levels in samples withdrawn at later time points (24 h).

INTRODUCTION

ADD17014 is a representative member of a new class of anticonvulsant compounds, the Δ^2-1,2,3-triazolines [1,2]. Although triazolines have been known for some considerable time [3], it is only relatively recently that attention has focused on them as potential therapeutic agents [1,2,4]. ADD17014, 1-(4-chlorophe-

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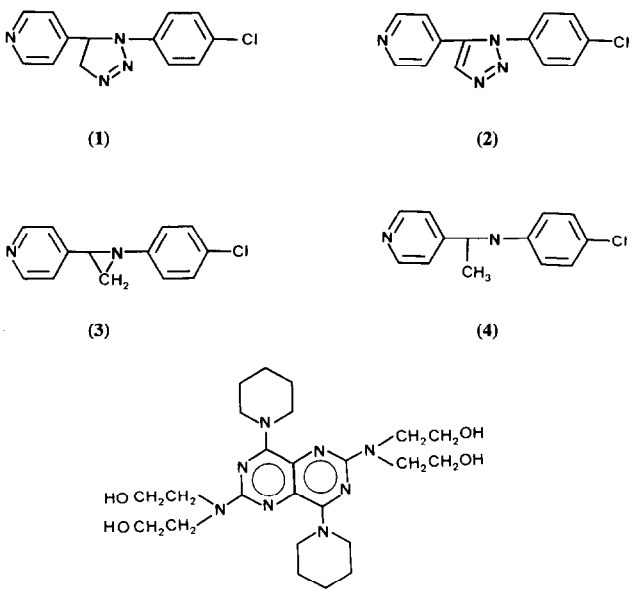

Fig. 1. Structures of I, 1-(4-chlorophenyl)-5-(4-pyridyl)-Δ^2-1,2,3.triazoline (1), and of the related triazole (2), aziridine (3) and imine (4) derivatives, together with the internal standard, dipyridamole (5).

nyl)-5-(4-pyridyl)-Δ^2-1,2,3-triazoline (I, Fig. 1), is one of a series of compounds synthesised by one of us (P.K.K.) [2] that have been shown to possess anticonvulsant activity [4]. The need for new antiepileptic drugs is clear, given that a large number of epileptic patients are not adequately controlled by existing drugs. In addition many of the current treatments rely on compounds that contain a dicarboximide or ureide function, and these may contribute to the inherent hypnotic and sedative properties of these drugs [1]. Compound I, however, lacks these structural moieties and hence could be predicted to be less toxic [1,2]. Indeed, I is the most effective triazoline synthesised to date and affords seizure protection in a variety of animal models [4]. As part of the overall drug safety evaluation, we proposed to study the metabolism and pharmacokinetics of I in the rat; however, no methods for the assay of triazolines in biological samples have been reported.

Analysis by chromatography is difficult because of the inherent chemical instability of I and the imine derivative (a potential metabolite) (Fig. 1) at neutral and acidic pH [5]. Although both chemical and biological degradation are likely to occur *in vivo*, it is essential that the chemical integrity of the sample is retained prior to and during analysis. Consequently, it is important to establish that no *ex vivo* alteration in the concentration of drug and/or metabolites occurs during sample storage and assay work-up. This is achieved by raising the pH of the

sample by the addition of Delory and King's carbonate–bicarbonate buffer (pH 10.7) immediately after sample collection. Preformulation chemical stability studies [5], conducted in our laboratories, have led to the development of thin-layer chromatographic and high-performance liquid chromatographic (HPLC) methods for the analysis of I and its potential metabolites, the aziridine, imine and triazole (Fig. 1) [6]. Under the given conditions there is no chemical breakdown of any of the four compounds during sample storage, extraction or chromatography. We now report on a method for the quantitative recovery of I from biological fluids and its quantification, together with a full validation of the assay.

EXPERIMENTAL

Chemicals

Methanol, acetonitrile and diethyl ether were HPLC grade from Fisons (Loughborough, U.K.). All salts for the preparation of buffers were of analytical-reagent grade and obtained from British Drug Houses (Poole, U.K.). The buffers were made by dissolving all salts in filtered, distilled water. McIlvaine's citric acid–phosphate buffer, pH 8.0, comprised 2.75 ml of 0.1 *M* citric acid (21.0 g $C_6H_8O_7 \cdot 1H_2O$ per l) and 97.25 ml of 0.2 *M* disodium hydrogenphosphate (35.6 g $Na_2HPO_4 \cdot 2H_2O$ per l) and Delory and King's carbonate–bicarbonate buffer, pH 10.7, was prepared from 21.3 ml of 0.2 *M* anhydrous sodium carbonate (21.2 g per l), 3.8 ml of 0.2 *M* sodium bicarbonate (16.8 g per l) and 74.9 ml of distilled water. All buffers were stored at 4°C. Dipyridamole was obtained from Sigma (Poole, U.K.). Compound I was synthesised by one of us and was fully characterised by spectrometric and elemental analysis.

Standards

Compound I was made up to 1 mg/ml in HPLC methanol and stored at −20°C. From this stock solution, working solutions of 10 and 100 μg/ml in HPLC methanol were prepared and these too were stored at −20°C for use as HPLC standards or for use in preparation of calibration curves. Dipyridamole was made up to 200 μg/ml in HPLC methanol and stored at 4°C and used as internal standard for the drug assay.

HPLC apparatus

The HPLC apparatus consisted of a ConstaMetric® pump, a variable-wavelength UV SpectroMonitor® detector and a CI4000 computing integrator (all from LDC, Stone, U.K.). The column was a reversed-phase Spherisorb 5-ODS type (25 cm × 0.46 cm I.D.) (HPLC Technology, Wilmsley, U.K.) with a 5 cm × 0.2 cm I.D. pellicular ODS guard column fitted (Whatman, Maidstone, U.K.). Samples were introduced into the system via a Rheodyne injector fitted with a 20-μl loop. Detection was by UV absorption at 290 nm and all chromatograms were recorded and peak areas and retention times calculated by electronic integration.

Recovery from biological samples

Control rat blood was obtained from male Wistar rats (King's College Animal Unit) by cardiac puncture, transferred into heparinised tubes and used immediately. To determine the recovery of I from blood, aliquots (20 μl, $n = 5$) of the drug (100 μg/ml) were injected directly onto the HPLC column. Similar aliquots (20 μl, $n = 5$) were spiked into clean extraction tubes containing control blood (250 μl) and Delroy and King's carbonate–bicarbonate buffer (750 μl). The buffer was added to prevent any breakdown of I which is known to occur at physiological pH 7 [5]. The blood–buffer mixture was then taken through the extraction procedure, which consisted of rocking with diethyl ether (5 ml) for 10 min followed by centrifugation (400 *g* for 5 min). The organic phase was then carefully transferred into clean, dry tubes, evaporated to dryness and the residue resuspended in methanol (50 μl) prior to analysis. By comparing peak areas of the drug before and after extraction it is possible to calculate the recovery. The aqueous phase remaining after extraction was also analysed for I and a further 5-ml extraction with diethyl ether undertaken to confirm that no additional drug could be extracted. Analogous procedures were followed to determine the recovery of the potential metabolites and the internal standard.

Stability of I in rat blood

To determine the stability of I in blood before extraction, aliquots (50 μl) (100 μg/ml) of I were spiked into extraction tubes containing heparinized rat blood (250 μl) and Delory and King's carbonate–bicarbonate buffer (pH 10.7) (700 μl). The tubes were stored at room temperature or −20°C before analysis. The tubes at room temperature were analysed at various intervals up to 8 h whereas those at −20°C were analysed over a longer time period (up to twelve weeks) to determine long-term stability. Immediately prior to analysis the tubes were spiked with the internal standard, dipyridamole (20 μl) (200 μg/ml) and taken through the analysis procedure outlined above.

Calibration curves

Preliminary unpublished work had shown that compound I is cleared from the rat fairly rapidly, with a very short half-life. In order to measure the triazoline levels in blood accurately at both the early time points after administration, when concentrations would be high, and also at later time points, when the concentrations would be very much lower, it was deemed necessary to construct two standard curves. These were over the concentration ranges 0–4 and 0–40 μg/ml. It is thus possible to follow the disappearance of the drug over a much longer time period and much more accurately than if only one calibration curve were employed.

The calibration curves were constructed by spiking varying amounts of I (10 or 100 μg/ml in methanol) into extraction tubes containing control blank rat blood (250 μl) and Delory and King's carbonate-bicarbonate buffer (pH 10.7) (750 μl).

The internal standard, dipyridamole (20 μl of 20 μg/ml for lower-range standard curve or 20 μl of 200 μg/ml for higher-range standard curve), was added to the blood–buffer mixture prior to extraction as described above. Peak-area ratios were then plotted against concentrations of I. Fig. 2A shows a typical HPLC trace of a diethyl ether extract of control rat blood spiked with dipyridamole.

Assay validation

To determine the precision and accuracy of the assay known concentrations of I were spiked into 250 μl of blood plus 750 μl of pH 10.7 buffer and taken through the extraction procedure. The study was carried out five times and the coefficient of variation (C.V.) was taken as a measure of assay precision and the mean percentage difference (M.D.) as a measure of accuracy.

Pharmacokinetic studies

Preliminary pharmacokinetic experiments have been conducted in concious rats. The carotid arteries and jugular veins were cannulated under light anaesthesia and exteriorised, the animals were then left to recover overnight. Compound I was administered intravenouly and samples collected via the indwelling cannula in the artery. The pH of the blood sample (250 μl) was immediately raised by the

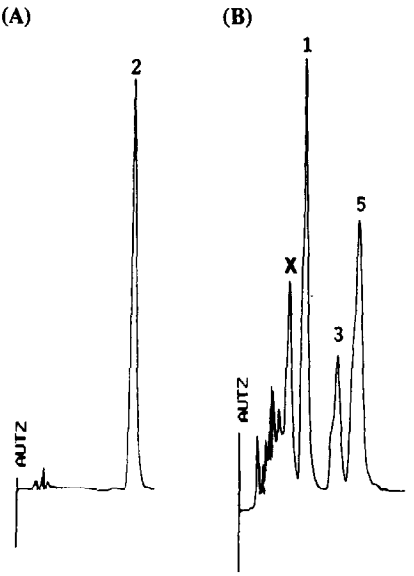

Fig. 2. (A) High-performance liquid chromatogram of a diethyl ether extract of control rat blood (250 μl) plus Delory and King's carbonate–bicarbonate buffer (pH 10.7) (750 μl); peak 2 is the internal standard, dipyridamole. (B) High-performance liquid chromatogram of an extract of rat blood taken at 2 h after dosing I (20 mg/kg intravenously). The extraction and analysis is as described in Results and discussion. Peaks: 1 = I (t_R = 7.5 min); X = unknown metabolite (t_R = 4.5 min); 3 = aziridine (t_R = 11.0 min); 5 = dipyridamole (t_R = 13.0 min).

addition of Delory and King's carbonate–bicarbonate buffer (pH 10.7) (750 μl) to prevent any *ex vivo* alterations in drug and metabolite concentrations due to known chemical instability of the triazoline and imine [5] and the potential instability of other unknown metabolites. Samples were stored at 4°C for a maximum of 2 h before extraction.

RESULTS AND DISCUSSION

The chromatograms showed single, sharp peaks for both I and the internal standard, with complete baseline separation between the two compounds. Recovery of I from blood was almost 100% when extracted into diethyl ether (5 ml) (Table I). The analysis of the aqueous phase after extraction showed no I indicating total extraction; indeed a further 5-ml extraction with diethyl ether yielded no additional compound in the organic phase. The stability of I in alkalinised blood at room temperature over 8 h is shown in Table II. From this it can be seen that samples left for up to 4 h at room temperature showed little breakdown. For increased confidence that no *ex vivo* breakdown was occurring experimental samples were stored at 4°C and for a maximum of 2 h before extraction and analysis. Table III shows the recovered concentrations of I for the tubes stored at −20°C for several weeks. It can be seen that no significant breakdown occurred up to eight weeks. The calibration curves for both the 0–4 μg/ml and the 0–40 μg/ml ranges exhibited excellent linearity (linear correlation coefficient >0.999), and the assay was reproducible with low inter- and intra-assay variation. It can be seen that the precision of the assay as indicated by the C.V. for five separate determinations was very good, and the accuracy based on M.D. was satisfactory (Table IV).

Although the detection limit in blood as described is ~ 0.1 μg/ml, this could be increased by the collection of larger blood samples from the animal. For example, a sample size of 2 ml would allow measurements as low as 10 ng/ml, which may be needed for quantification of I at later time points after drug administration.

TABLE I

EXTRACTION RECOVERY OF I, ITS POTENTIAL METABOLITES AND THE INTERNAL STANDARD FROM RAT BLOOD (250 μl)

Compound	Recovery (mean ± S.D., $n = 3$) (%)	C.V. (%)
I	95 ± 5	5.3
Triazole	95 ± 9	9.5
Aziridine	98 ± 7	7.1
Imine	95 ± 8	8.4
Internal standard	86 ± 2	2.3

TABLE II

STABILITY OF I IN ALKALINISED RAT BLOOD AT ROOM TEMPERATURE

Time (h)	Concentration of I (mean ± S.D., n = 3) (μg/ml)	Percentage breakdown
0	20.00 ± 0.35	0
1	20.42 ± 0.42	0
2	19.05 ± 0.31	5
4	19.06 ± 0.43	5
8	15.45 ± 0.84	23

TABLE III

LONG-TERM STABILITY OF I IN ALKALINISED RAT BLOOD FROZEN AT −20°C

Time (weeks)	Concentration of I (mean ± S.D., n = 3) (μg/ml)	Percentage breakdown
0	20.00 ± 0.35	0
1	19.10 ± 0.62	4
2	19.54 ± 0.28	2
3	19.59 ± 1.94	2
4	19.68 ± 1.57	2
8	19.51 ± 1.21	2
12	17.49 ± 0.72	13

TABLE IV

PRECISION AND ACCURACY OF THE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR I IN RAT BLOOD

Spiked concentration (μg/ml)	Found concentration (mean ± S.D., n=5) (μg/ml)		C.V.[a] (%)	M.D.[b] (%)
0.40	0.38	<0.01	0.86	−3.08
1.60	1.56	0.07	4.48	−2.50
12.0	11.29	0.29	2.56	−6.73
24.0	22.89	0.48	2.11	−5.02

[a] C.V. = (S.D./mean) × 100.

[b] M.D. = (mean-spiked concentration)/spiked concentration × 100.

Preliminary pharmacokinetics have shown that the drug is cleared from the animal at a very high rate. Following administration of a 20 mg/kg intravenous bolus dose, the β-elimination half-life was 58 min with a peak concentration of 7 μg/ml and a 6-h measurement of 0.05 μg/ml. Fig. 2B shows a chromatogram of an extract of rat blood taken at 2 h after dosing compound I.

In conclusion, a sensitive HPLC assay has been developed for the novel triazoline anticonvulsant compound, ADD17014, in rat blood. The assay is reproducible with low inter- and intra-assay variation and has been fully validated using known concentrations of I. We are currently using this method to assay I in rat blood in order to elucidate the full metabolic and pharmacokinetic profile of this drug. This method should be amenable to the analysis of I in rat urine and other biological samples and provide valuable information on urinary metabolites.

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