Journal of Chromatography, 456 (1988) 183–190
Elsevier Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 772

RADIO HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF ^{14}C-LABELLED LF 2-0254, A 1,4-DIHYDROPYRIDINE CALCIUM ANTAGONIST, IN RAT AND DOG PLASMA USING OFF-LINE LIQUID SCINTILLATION COUNTING

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SUMMARY

LF 2-0254 is a 1,4-dihydropyridine calcium antagonist with a slow onset of action. The pharmacokinetics of [^{14}C]LF 2-0254 were studied in rats and dogs. A sensitive high-performance liquid chromatographic method using liquid scintillation counting was developed for the quantitation of labelled LF 2-0254 in plasma. The peak height of the internal standard in the chromatogram was measured by UV detection and the mobile phase containing the chromatographic peak of [^{14}C]LF 2-0254 was collected and counted for radioactivity. The concentration of labelled drug in the plasma was then determined using a calibration graph constructed from the determination of [^{14}C]LF 2-0254 of known specific activities. The limit of determination was dependent on the specific activity of the drug administered. This method permits the measurement of the radioactive drug in biological fluids.

INTRODUCTION

LF 2-0254, [8-aza-8-(4-chlorophenyl)-1,4-dioxaspiro-4,5-decan-2-yl]methyl-1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)pyridine-3,5-dicarboxylate (Fig. 1), is a calcium antagonist with a slow onset of action *in vitro*. Unlike nifedipine and nicardipine, LF 2-0254 displays a long duration of action[1]. Dihydropyridine calcium antagonists are a potent group of drugs that exert their desired effects *in vivo* at concentrations of 10^{-9}–10^{-7} M[2]. Sensitive techniques are therefore required to detect and quantitate the levels of these drugs and their metabolic products in body fluids.

The most common techniques employed are packed-column gas chromatography (GC) with electron-capture detection (ECD)[3–6] and capillary GC with either nitrogen-selective detection or ECD[7–11]. Quantification by stable isotope dilution and electron-capture negative-ion chemical ionization has been applied to nitrendipine[12]. Many workers have utilized reversed-phase high-performance liquid chromatography (HPLC) to separate and determine 1,4-dihydropyridine in plasma. With the exception of one electrochemical HPLC method to determine nifedipine[13], the application of UV detection appears to be common[13–20]. The principles of reverse isotope dilution have been applied to quantitate a new 1,4 dihydropyridine[21].

For the determination of LF 2-0254 in plasma, an HPLC method with UV

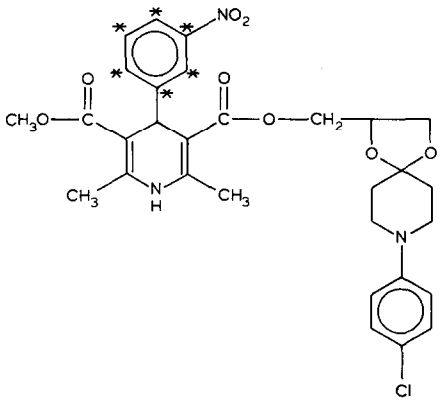
0021-9673/88/$03.50

detection at 239 nm has been developed in our laboratory. Its sensitivity (2 ng/ml) did not allow us to analyse plasma from animals administered low doses of the drug (0.1 mg/kg). A more sensitive method using liquid scintillation counting was then set up for the quantitation of labelled LF 2-0254 in plasma by counting the radioactivity of the [^{14}C]LF 2-0254 chromatographic peak. The limit of determination in plasma was 0.24 ng/ml for a 0.1 mg/kg dose (specific activity 136.95 μCi/mg).

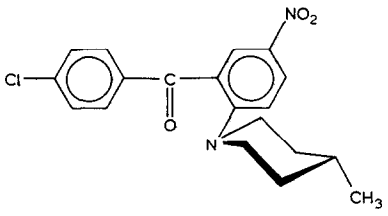
EXPERIMENTAL

Chemicals

[^{14}C]LF 2-0254, specific activity 136.95 μCi/mg (3030.30 MBq/mmol), radiochemical purity >95% by thin-layer chromatography and HPLC, was a custom synthesized by Isotopchim (Mougins, France). LF 2-0254 was labelled at all six carbons of the nitrophenyl ring. The internal standard, LF 1608, synthesized in our Department of Organic Chemistry, was chosen for its good HPLC and extraction properties and for its stability (Fig. 1). Hexane and diethyl ether (RPE, unstabilized) were obtained from Burdick and Jackson (Muskegon, MI, U.S.A.) and Carlo Erba (Milan, Italy). Sodium hydrogencarbonate was of analytical-reagent grade (Fluka, Buchs, Switzerland). Acetonitrile was of HPLC grade S (Rathburn, Walkerburn, U.K.). Other solvents, of the best grade available, were triethylamine (Fluka), perchloric acid (Prolabo, Paris, France) and deionized water (Milli Q system, Millipore, St. Quentin en Yvelines, France).



LF 2-0254



LF 1608

Fig. 1. Structure of LF 2-0254 and LF 1608 (internal standard). ^{14}C labels are indicated by asterisks.

Animals and dosing

Male Wistar rats (body weight 175–232 g) (Iffa Credo, l'Arbratsle, France) were given [^{14}C]LF 2-0254 in polyethylene glycol (PEG 400) and distilled water [1:1 (v/v), dose 0.1 mg/kg, i.v.; or 2:1 (v/v), dose 3 mg/kg, p.o.]. Beagle dogs (body weight 9.7–13.5 kg) (Carriere, Villeneuve/Lot, France) received [^{14}C]LF 2-0254 (0.1 mg/kg, i.v.) in a solution of PEG 400–water (2:1, v/v).

Blood samples were taken at regular intervals after administration into heparinized tubes. Plasma was immediately separated by centrifugation and kept at −20°C until analysis.

Radioanalysis

^{14}C in excreta and solutions was assayed by liquid scintillation spectrometry using a Packard Tri-Carb Instrument Model 2000 CA (Packard, Rungis, France). Aliquots were counted in glass vials with a counting efficiency of greater than 93% using Packard Instagel liquid scintillant. Quench correction was achieved with reference to an external standard using a calibration graph. The quench correction curve was established with a set of manufacturer's quenched standards and stored in the memory of the counter. Subtraction of the background counting rate was performed by the counter's built-in software, using blank vials counted at the start of each batch. Machine performance was verified at frequent intervals with reference to a sealed standard. For radio-HPLC a Gilson Model 201 fraction collector (Gilson, Villiers le Bel, France) was used to collect 0.5- or 1.5-ml fractions of column eluent in minivials, which were counted for ^{14}C following the addition of a scintillant.

Chromatographic procedure

For HPLC a Spectra-Physics Model SP 8780 XR autosampler (Spectra-Physics, La Verpillière, France), Kratos Model 400 pumps, a Kratos Model 783 detector and a Kratos Model 450 solvent programmer (Kratos, Fontenay/Bois, France), were used. The column was Altex C_{18} (5 μm), 150 × 4.6 mm I.D. (Beckman, Gagny, France). This system was linked to a Shimadzu CR3-A integrator (Shimadzu, Kyoto, Japan). The mobile phase was filtered and degassed on a Millipore GVWP 0.45-μm membrane. It consisted of acetonitrile–water (containing 0.02% triethylamine, pH 3.5, adjusted with concentrated perchloric acid (70:30, v/v) during the first 12 min of analysis, followed by a column wash with 100% acetonitrile for 7 min (Fig. 2). This column wash successfully eliminated peaks with long retention times and maintained column performances.

Sample treatment

Plasma samples from five rats in each dose group were pooled by time point. Plasma samples from dogs ($n=6$) were analysed individually. The extraction procedure was performed using amber glassware or tubes wrapped with aluminium foil in order to prevent photo-oxidation of the dihydropyridine ring. A 50-ng amount of LF 1608 (internal standard) dissolved in 50 μl of acetonitrile was added to 1 ml of plasma in Corning tubes with PTFE screw-caps. The solution was made alkaline by addition of 1 ml of 0.5 *M* sodium hydrogencarbonate and the aqueous phase was extracted once with 8 ml of hexane–diethyl ether (75:25, v/v). After centrifugation, the organic phase was evaporated to dryness under a stream of nitrogen. The residues were

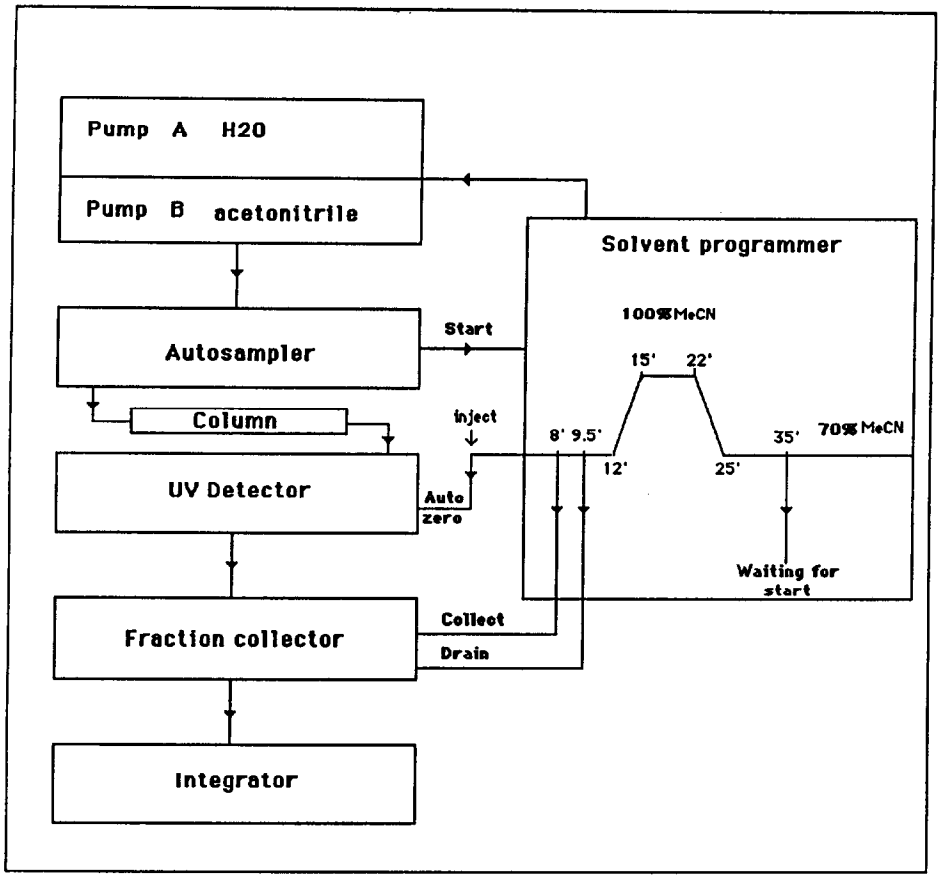


Fig. 2. Scheme of the chromatographic system. After injection of the sample, the programmer, started by the autosampler, begins the solvent gradient and controls the collection of the peak between 8 and 9.5 min. At 12 min into the run, the column wash starts. Acetonitrile (MeCN) begins ramping from its initial value of 70% to 100% in the next 7 min, then ramps back to 70%. A period of 10 min is enough to establish the initial conditions. At 35 min, the programmer is waiting for the next cycle of analysis.

dissolved in 80 μl of acetonitrile and an aliquot of 50 μl was injected on to the HPLC column.

The LF 2-0254 peak, located by its retention time at 239 nm, was collected between 8 and 9.5 min in a glass scintillation vial and counted for 20 min for ^{14}C after the addition of 20 ml of scintillant. It was established by collecting 0.5-ml fractions of mobile phase that no interfering labelled metabolites co-eluted during this interval of time (Fig. 3). As shown with synthetic standards, the pyridine metabolite did not interfere with the [^{14}C]LF 2-0254 assay. This collection of 1.5 ml of eluate was sufficient to obtain the whole peak of [^{14}C]LF 2-0254 in the case of high concentrations of drug. Calibration graphs and quantification were effected by correlating the dpm value obtained after counting the [^{14}C]LF 2-0254 fraction with the peak height of the internal standard detected by UV absorption at 239 nm (Fig. 4).

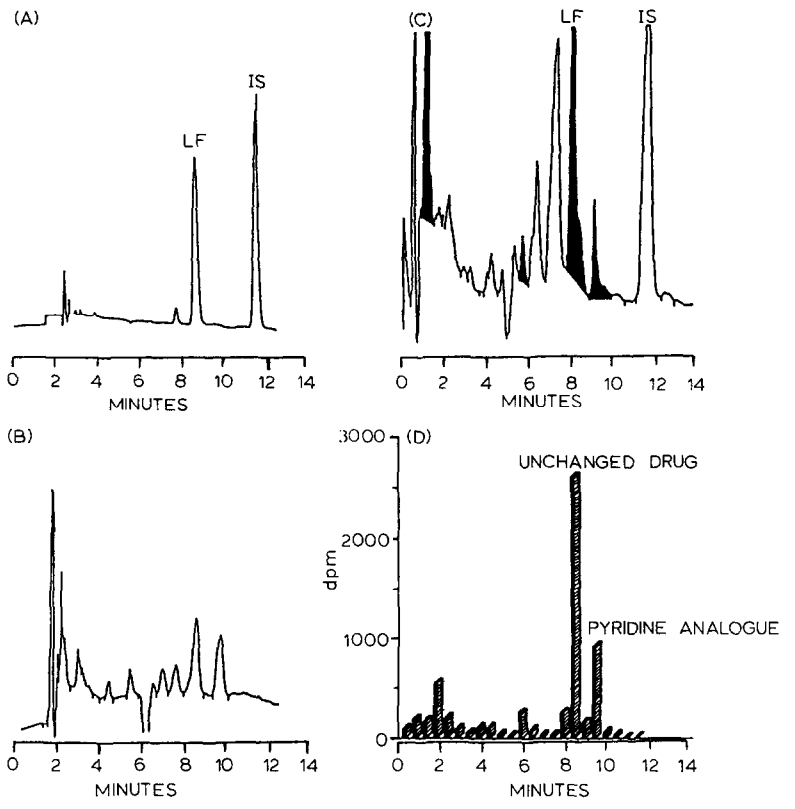


Fig. 3. Chromatograms of rat blank plasma, standards and rat plasma after dosing. (A) HPLC separation of a mixture of LF 2-0254 and LF 1608 (IS) with UV detection at 239 nm ($1.6 \cdot 10^{-3}$ a.u.f.s.). (B) Separation of rat blank plasma with UV detection at 239 nm ($1.6 \cdot 10^{-3}$ a.u.f.s.). (C) HPLC analysis of rat plasma withdrawn, 1.5 h after administration of [^{14}C]LF 2-0254 (3 mg/kg, p.o.) with UV detection at 239 nm ($1.6 \cdot 10^{-3}$ a.u.f.s.). (D) Radiochromatogram of labelled unchanged drug and labelled pyridine analogue of rat plasma withdrawn 1.5 h after administration of [^{14}C]LF 2-0254 (3 mg/kg, p.o.).

RESULTS

Accuracy and precision

Control plasma samples (1 ml) were spiked with 72–172 000 dpm of [^{14}C]LF 2-0254 with a specific activity of 136.95 μCi/mg (0.24–565 ng/ml). The calibration-graphs correlating the dpm counted with the peak height of the internal standard allowed us to determine [^{14}C]LF 2-0254 in plasma knowing the initial specific activity of the dose administered (Fig. 4). The intra-day coefficient of variation was in the range 1.2–11.9% for five extractions per concentration with an accuracy of 0.4–13.3% (Table I). The inter-day coefficient of variation was in the range 5.6–16.3% for 5 days with an accuracy of 0.2–9.6%. For concentrations of LF 2-0254 above 5 ng/ml, a good correlation between the radioactivity calibration graphs and the UV calibration graph was found (data not shown). The limit of determination (0.24 ng/ml) showed a precision of 8% with an accuracy of 17% for twelve extractions.

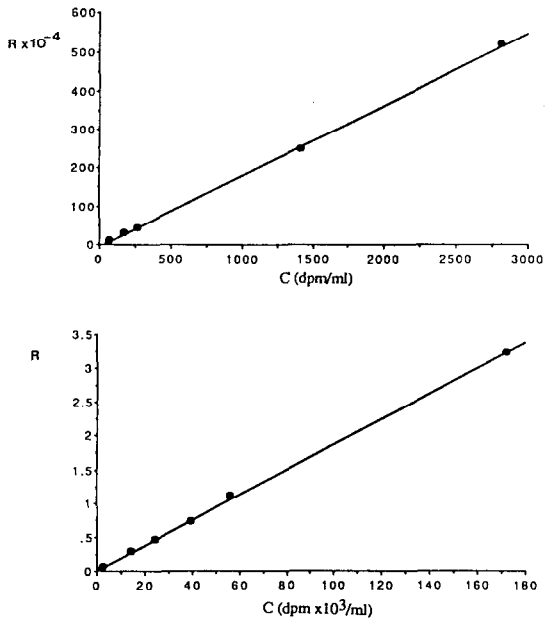


Fig. 4. Intra-day linear regression. Top, calibration graph for the low concentrations; bottom, calibration graph for high concentrations. *R* represents the ratio between the amount of radioactivity in the [^{14}C]LF 2-0254 peak fraction and the peak height of the internal standard detected by UV absorption.

Recovery

Seven extractions at concentrations of 280 dpm/ml (0.9 ng/ml) and 54 600 dpm/ml (180 ng/ml) showed recoveries of 73.7% and 72.6%, respectively (specific activity 136.95 μCi/mg).

TABLE I
INTRA-DAY REPRODUCIBILITY

Concentration added (dpm/ml)	*Detection* (dpm/ml)*	*Precision (%)*	*Accuracy (%)*	*Slope and r***
72.5	82 ± 5	6.6	13.3	Slope = 0.185; r = 0.993
180	200 ± 13	6.4	11.3	
267	265 ± 21	8.2	0.6	
1424	1377 ± 118	9.5	3.3	
2811	2833 ± 268	9.5	0.8	
14217	15960 ± 958	6.0	12.0	Slope = 0.188; r = 0.991
24261	24580 ± 285	1.2	1.3	
39325	38771 ± 2140	5.5	1.4	
56334	54131 ± 3796	7.0	3.9	
172033	172690 ± 20483	11.9	0.4	

* Mean ± S.D. (n=5).
** r = correlation coefficient.

TABLE II

PHARMACOKINETIC PARAMETERS OF LF 2-0254 IN RAT AND DOG PLASMA

Plasma samples from five rats in each dose were pooled by time point. Plasma samples from dogs were analysed individually. Results are expressed as mean ± S.D. Parameters were calculated using mean concentration–time data for five rats, because five rats were killed at a designated time for blood sampling.

Species	*Route*	*Dose (mg/kg)*	*No. of animals and sex*	$t_{\frac{1}{2}}$ *(h)*	C_{max} *(ng/ml)*	t_{max} *(h)*
Rat	I.V.	0.1	5M	2.2	–	–
	Oral	3	5M	3.8	30.8	4
Dog	I.V.	0.1	3M	7.3 ± 1.8	–	–
			3F	11.1 ± 10.8	–	–

Limit of determination

The limit of detection was set at 33 dpm (twice the background). Vials were counted for 20 min for ^{14}C with an efficiency of 93% and a precision of 10%. Following the procedure described above (see *Sample treatment*), this gave a limit of determination in plasma of 72 dpm/ml. The limit of determination in plasma was dependent on the specific activity of the drug administered; it was 0.24, 0.51 and 0.37 ng/ml for the doses 0.1 mg/kg, i.v. (specific activity 136.95 μCi/mg) and 3 mg/kg, p.o. (specific activity 64.17 μCi/mg) in the rat and 0.1 mg/kg, i.v. (specific activity 8.59 μCi/mg) in the dog.

Application

This radio-HPLC method gave reproducible results and was sensitive enough for the determination of [^{14}C]LF 2-0254 in plasma samples. It was used to establish the pharmacokinetics of LF 2-0254 after oral and i.v. administration to rats and dogs (Table II).

DISCUSSION

The method shows high specificity and the assay is sensitive and reproducible. It allowed us to determine the concentration of [^{14}C]LF 2-0254 in rat and dog plasma for pharmacokinetic studies. The limitation of many methodologies is their inability to separate, detect and quantitate the main metabolites of 1,4-dihydropyridines in biological fluids. Most methods measure the parent drug only and a minority can determine major metabolites. This assay could allow the detection of labelled metabolites in a simple chromatographic run. Major metabolites could be collected, identified by mass spectrometry and quantified using synthetic standards. This work is in progress in our laboratory.

The use of radio-HPLC offers several advantages over conventional techniques. With a sensitivity higher than that of UV detection, it is more selective and allows discrimination between co-eluting peaks or peaks with close retention times, which could interfere with UV detection. The chromatographic conditions are easier to set up than with UV detection because labelled drug may co-elute with unlabelled UV-detectable impurities. Compared with UV detection, the time of analysis is shortened. The limitation of this procedure is that a labelled drug with a relatively high

specific activity (>50 μCi/mg) is needed. The use of a ^{3}H-labelled drug with a higher specific activity could be of interest for lowering the limit of determination in plasma.

ACKNOWLEDGEMENTS

We thank M. C. Martinez, R. Levy Prades Sauron, D. Loisy, F. Souverbie and J. Caldwell for helpful discussions and their contribution to this work.

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