

# Bio-analysis and preliminary pharmacokinetics of the experimental antitumour drug LL-D49194 $\alpha_1$ \*

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**Abstract:** LL-D49194 $\alpha_1$  is a recently discovered compound, produced by *Streptomyces vinaceus-drappus*. This micro-organism produces a number of antibiotics, all showing antibacterial and antitumour activity, of which LL-D49194 $\alpha_1$  is one of the main compounds. The compounds' antitumour effectiveness has been proven *in vitro* and the drug is undergoing further tests. For the assay of the drug in plasma a high-performance liquid chromatographic (HPLC) system has been developed, preceded by a clean-up step. The drug is extracted from the biological matrix with ethyl acetate followed by direct HPLC analysis of the organic layer via an analytical RP8 column preceded by a guard column to retain endogenous plasma compounds. Detection of drug and metabolites was carried out by fluorescence with reference to a non-fluorescent internal standard detected by UV absorption. The detection limit was 1 ng ml<sup>-1</sup> plasma (using 1 ml sample; signal-to-noise ratio, 3), i.e. 1 ng on column. The method has been utilized in a preliminary pharmacokinetic study in rat.

**Keywords:** *Experimental antitumour agent; LL-D49194 $\alpha_1$ ; HPLC; bioanalysis; pharmacokinetics.*

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## Introduction

LL-D49194 $\alpha_1$  (NSC 381 856), introduced by Lederle American Cyanamid Laboratories, is a recently discovered antibiotic drug, produced by *Streptomyces vinaceus-drappus*.

This micro-organism produces a number of antibiotic substances, of which 12 different species have now been identified.

LL-D49194 $\alpha_1$ , together with its congener LL-D49194 $\beta_1$ , is the main substance produced. The antibiotics have been shown to possess antibacterial and antitumour activity *in vitro* [1].

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LL-D49194 $\alpha_1$ , is a very complex compound, consisting of an aglycone linked with three sugar moieties via two glycosidic bonds. The compound is chemically related, but clearly distinguishable from the antibiotics of the trioxacarcin class. The structure contains several reactive functions among which the two epoxy groups are prominent (Fig. 1). The mode of action of LL-D49194 $\alpha_1$  has been associated with potent inhibition of DNA and RNA synthesis in P815 mastocytoma cells [2].

The drug is reportedly unstable in solution [1], however, no details of the degradation are given in the literature.

A formulation of the drug is being developed for the first clinical tests. The aim of this study was to develop a suitable bioanalytical assay for LL-D49194 $\alpha_1$  in order to monitor plasma levels during these clinical trials and to establish the pharmacokinetics of the drug given by various routes of administration. This paper is the first report on the bioanalysis of LL-D49194 $\alpha_1$ .

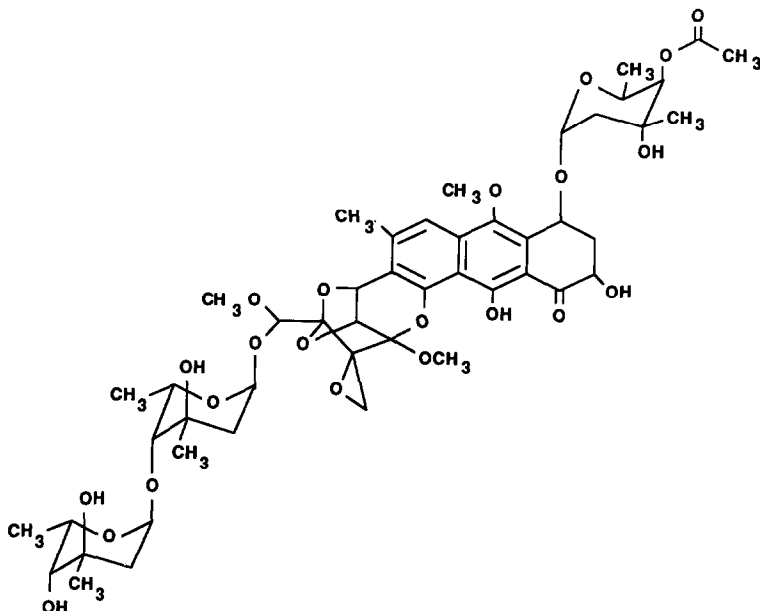
## Experimental

### Chemicals

LL-D49194 $\alpha_1$  was obtained from Lederle, American Cyanamid Laboratories (Pearl River, USA) through the EORTC New Drug Development Office (Amsterdam, The Netherlands) and used as such. All other chemicals were of analytical quality and de-ionized water was used throughout.

### Apparatus

UV-vis absorption spectra were recorded on a Shimadzu UV-200 double beam spectrophotometer (Shimadzu, Corp., Kyoto, Japan) equipped with a Kipp BD 40 recorder.



**Figure 1**  
Structure of LL-D49194 $\alpha_1$ .

Fluorescence excitation and emission spectra were recorded on a Perkin–Elmer 204 fluorescence spectrophotometer.

HPLC analysis was performed using a Model 510 solvent delivery system, a U6K injector, a Model 440 dual wavelength UV detector (all from Waters Associates, Milford, Massachusetts, USA) and a Hitachi F100 fluorescence detector (Hitachi, Tokyo, Japan), connected in series after the UV detector.

The analytical column (125  $\times$  4 mm) was filled with Lichrosorb RP8 (5  $\mu$ m) material. Prior to the analytical column a guard column (20  $\times$  4 mm) was inserted, slurry-packed with Lichroprep RP8 (5–20  $\mu$ m) material.

The solvent consisted of methanol–5 mM potassium phosphate buffer pH 7.0 (55:45% v/v) with a flow rate of 1.0 ml min<sup>-1</sup>.

UV detection was carried out at 254 nm and fluorescence detection at 470 nm with excitation at 400 nm.

### Analytical Procedure

Plasma (100  $\mu$ l) containing LL-D49194 $\alpha_1$  spiked with 50  $\mu$ l of a solution of 150  $\mu$ g ml<sup>-1</sup> ethyl hydroxybenzoate (internal standard (IS)) in methanol was vortex mixed with 1.0 ml ethylacetate for 30 s. The layers were then separated by centrifugation for 5 min (4000 rpm). A 0.5 ml aliquot of the organic layer was evaporated to dryness under a stream of nitrogen at 30°C and the residue redissolved in 50  $\mu$ l of the HPLC solvent in an ultrasonic water bath for 5 min. Then 15  $\mu$ l of this solution was injected into the chromatographic system and the amount of LL-D49194 $\alpha_1$  determined from the peak height ratio to the internal standard, with fluorescence detection of drug and metabolites at 470 nm, and absorbance detection of the IS at 254 nm, respectively.

### Pharmacokinetics

A rat (weight 250 g) received 0.5 mg of LL-D49194 $\alpha_1$ , given as an *i.v.* bolus injection. At appropriate time intervals blood samples of 300  $\mu$ l were withdrawn, collected in polypropylene test tubes containing 10  $\mu$ l heparin (equivalent to 50 IU) solution and immediately centrifuged to obtain the plasma fraction. An aliquot of 100  $\mu$ l plasma was then subjected to further analysis, after addition of the internal standard.

### Results and Discussion

#### *UV–vis and fluorescence spectroscopy*

The absorbance maxima of LL-D49194 $\alpha_1$  are known to be at about 230, 270 and 400 nm [1]. However, the compound also exhibits fluorescence with an emission wavelength of 470 nm.

The optimum excitation wavelength to obtain this emission is 400 nm. The fluorescence intensity at 470 nm is sufficiently high to permit sensitive detection after HPLC separation.

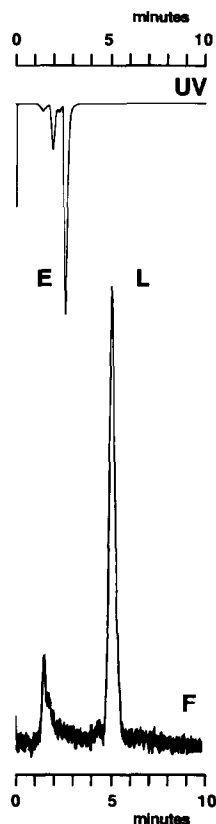
#### *Chromatography*

The ideal internal standard for the HPLC assay has to exhibit not only similar extractive and chromatographic properties but also similar possibilities for detection. However, despite testing of a large number of compounds, no such ideal compound was found to be available. Finally, ethyl hydroxybenzoate was selected, since it has suitable

extractive and chromatographic properties and an intense UV absorption at 254 nm. Its use is only possible with a selective dual detection system, consisting of a UV detector (for the internal standard) followed by a sensitive fluorescence detector (for LL-D49194 $\alpha_1$  and metabolites), connected in series. The ratio of these signals is used for quantification. The relatively high concentration of internal standard used enables detection to be carried out at low UV detector sensitivity (0.5 AUFS), which eliminates to a great extent interferences of endogenous plasma peaks. Figure 2 shows a typical chromatogram of LL-D49144 $\alpha_1$  in plasma. High concentrations of metabolite M2 (see Fig. 4) may interfere with the UV absorbance of E. This can be verified by analysing samples, without addition of IS. When overlap occurs one could use a higher concentration of IS to such an amount that the contribution of M2 to the UV signal of IS becomes negligible. However, interference between IS and M2 was not observed in our pharmacokinetic study in rat.

#### *Clean-up procedure*

For the extraction of LL-D49194 $\alpha_1$  from plasma samples a number of organic solvents have been tested, such as chloroform, ether, ethyl acetate and chloroform-isopropanol (4:1, v/v). In general recoveries were low and reproducibility was poor, except for ethyl acetate. With this extraction solvent recoveries were determined. For that purpose plasma samples were spiked with appropriate amounts of LL-D49194 $\alpha_1$ , extracted with



**Figure 2**  
HPLC chromatogram of plasma spiked with LL-D49194 $\alpha_1$  (30 ng ml $^{-1}$ ), with ethyl hydroxybenzoate (75  $\mu$ g ml $^{-1}$ ) as the internal standard. L, LL-D49194 $\alpha_1$  after fluorescence detection (F) at 470 nm; E, ethyl hydroxybenzoate after UV detection (UV) at 254 nm. For chromatographic conditions see text.

ethyl acetate, IS added and the extract analysed. Comparison with standard solutions LL-D49194 $\alpha_1$  in plasma revealed recoveries of 98% at 100 and 5000 ng ml $^{-1}$  with a standard deviation of 3.8% ( $n = 8$ ) so that complete recovery of the drug could be achieved with this procedure. Calibration curves for the assay of LL-D49194 $\alpha_1$  in plasma in the region 10–40  $\mu\text{g ml}^{-1}$  to 10–100 ng ml $^{-1}$  show good linearity (Table 1). The detection limit appears to be 1 ng ml $^{-1}$  plasma (using 1 ml plasma samples). Spiked plasma samples (100 ng ml $^{-1}$ ) were stable for at least two weeks when stored at  $-20^\circ\text{C}$ .

#### Pharmacokinetics of LL-D49194 $\alpha_1$

The bio-analytical method has been used in a single pharmacokinetic study in a rat.

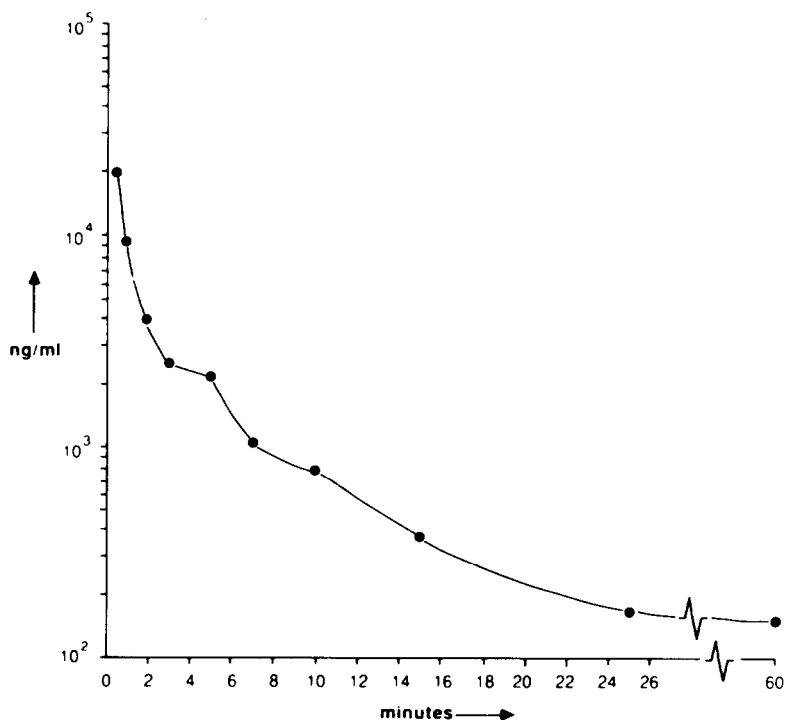
The plasma concentration–time curve is depicted in Fig. 3. A two-compartment model was used to calculate the pharmacokinetic parameters (Table 2).

**Table 1**  
Equations of calibration data

Concentration range*	Equation	$r^\dagger$	$n$
10,000–40,000	$y = 2.45 \times 10^{-5}(\pm 1.6 \times 10^{-6})x + 6.8 \times 10^{-2}(\pm 3.6 \times 10^{-2})$	0.996	5
500–10,000	$y = 2.32 \times 10^{-4}(\pm 3.7 \times 10^{-6})x - 5.4 \times 10^{-5}(\pm 2.7 \times 10^{-2})$	0.9996	6
50–500	$y = 1.65 \times 10^{-3}(\pm 1.4 \times 10^{-4})x + 1.4 \times 10^{-1}(\pm 4.8 \times 10^{-2})$	0.993	5
10–100	$y = 5.6 \times 10^{-3}(\pm 2.8 \times 10^{-4})x + 1.0 \times 10^{-1}(\pm 1.6 \times 10^{-2})$	0.997	5

\* Concentration in ng ml $^{-1}$ ;  $y$  is the peak height ratio of LL-D49194 $\alpha_1$  to internal standard;  $x$  the LL-D49194 $\alpha_1$  concentration in ng ml $^{-1}$ .

$^\dagger r$ , Correlation coefficient.



**Figure 3**  
Plasma concentration–time curve of LL-D49194 $\alpha_1$  in a rat.

**Table 2**  
Pharmacokinetic parameters of LL-D49194 $\alpha_1$  using a two-compartment model

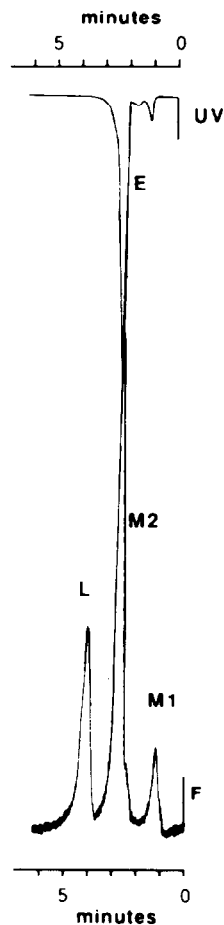
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$C(t)$	$= A e^{-\alpha t} + B e^{-\beta t}$
AUC	$= A/\alpha + B/\beta$
$Cl_{tot}$	$= \text{Dose}/\text{AUC}$
$A$	$42 \mu\text{g ml}^{-1}$
$B$	$4.4 \mu\text{g ml}^{-1}$
$t_{1/2}(\alpha)$	0.35 min
$t_{1/2}(\beta)$	4.18 min
AUC	$47.6 \mu\text{g min ml}^{-1}$
$Cl_{tot}$	$10.5 \text{ ml min}^{-1}$

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**Figure 4**

HPLC chromatogram of a rat plasma sample ( $t = 10$  min). L, LL-D49194 $\alpha_1$ ; M1 and M2, putative metabolites after fluorescence detection (F) at 470 nm; E, ethyl hydroxybenzoate after UV detection (UV) at 254 nm. For chromatographic conditions see text.



A typical HPLC chromatogram of a plasma sample ( $t = 10$  min) is shown in Fig. 4. Apart from the LL-D49194 $\alpha_1$  peak, two other peaks were present in the chromatograms. The major peak (M2) reached its maximum at 15 min; M2 and ethyl hydroxybenzoate have almost the same  $k'$  value but analysis of plasma samples without addition of the internal standard showed that M2 does not interfere with the ethyl hydroxybenzoate signal at 254 nm.

A minor peak (M1) appeared in the chromatograms in the course of time. These fluorescent compounds are considered to be metabolites of LL-D49194 $\alpha_1$  although the structures have not yet been established.

It is concluded that the present method for the bioanalysis of the new experimental antitumour antibiotic LL-D49194 $\alpha_1$  gives reliable quantitative data and can be used for pharmacokinetic analysis in Phase I studies.

## References

- [1] M. D. Lee, A. A. Fantini, D. P. Labeda, W. M. Maiese, R. T. Testa and D. B. Borders, US Patent 4.626.503, December (1986).
- [2] J. P. Thomas, H. L. Lindsay, R. E. Wallace, R. V. Citarella, M. B. Zee and F. E. Durr, *Recent Advances in Chemotherapy* (J. Ishigami, Ed.), pp. 523–524. Tokyo Press, Tokyo (1985).

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