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Running Title: Microdialysis study of levofloxacin in lung

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ABSTRACT:

A microdialysis distribution study of levofloxacin in the lung and muscle of rats was conducted from a single point determination after intravenous infusion at steady-state. This approach was presented as an interesting alternative to investigate antibiotics tissue distribution.

Several different methodological approaches are available to asses the distribution of antibiotics in the lung of animals or patients (12). It is now recognized that drug concentrations in whole tissue homogenates are difficult to interpret and therefore not informative (18). In human the most popular experimental approach to characterize antibiotics distribution in lungs relies on bronchoalveolar lavages (BAL) (3, 4, 5) and micro-BAL was recently proposed (2). Microdialysis is an appealing technique that was increasingly used over the last years to investigate antibiotics distribution in the extracellular fluid (ECF) of various tissues including lung, both in rats and humans (6, 11, 15, 17, 19, 23). One of the major advantages of microdialysis over BAL or micro-BAL is that it allows multiple determinations in the same subject and therefore full description of the drug concentrations versus time profile following its administration. However in human lung microdialysis studies are limited to patients with elective thoracic surgery and in rats they require maintenance of the animal anaesthetized with open chest surgery (6, 17). As a consequence for antibiotics with long elimination half-lives, it may be difficult to maintain study duration for a period of time long enough to properly characterize major pharmacokinetic parameters such as total drug concentrations versus time area under curves (AUCs). Comparison of total unbound AUCs in lung ECF and plasma may provide important information such as indirect evidence for the involvement of active efflux transport systems (7). These systems, including P-glycoprotein (P-gp), are present in brain and are responsible for lower unbound AUCs in brain ECF than in plasma as previously demonstrated with several anti-infectious drugs using microdialysis (8, 10, 13, 16). The presence of P-gp in lung is suspected (9, 14, 20, 21) and therefore potentially responsible for restricted drug tissue distribution, although

this has never been documented, at least to our knowledge. In order to do that it should be possible to compare total unbound AUCs in lung tissue ECF and plasma following single dose administration, but interestingly comparing unbound drug concentrations following intravenous infusion at steady-state would provide exactly the same information (7). The advantage of steady-state conditions is to require a single concentration determination in plasma and tissue, which is much more simple and probably more accurate than determining AUCs after single dose administration. The feasibility and optimization of such an alternative approach is now being tested using levofloxacin (LVX) as a representative antibiotic.

Experiments were done in accordance with the Principles of Laboratory Animals Care (NIH Publication #85-23, revised 1985) using male Sprague Dawley rats (Janvier Laboratories, Le Genest-St-Isle, France), weighing 316 ± 49 g. The day before experiments (D-1), between 9:00 pm and 11:00 am, rats were anesthetized with air-isoflurane mixture and equipped with a vein femoral catheter for LVX administration and with microdialysis CMA/20 probes (polycarbonate, cutoff 20000 Da, membrane length 10 mm, CMA microdialysis, Phymep, Paris, France) in blood and muscle for drug concentrations determinations, as previously described (17). After rats wake up and six to seven hours post surgery, infusion of a commercial solution of LVX (5 $mg.mL^{-1}$, Sanofi Aventis, Paris, France) was started at a rate of 1.0 $mg.h^{-1}$, maintained overnight to reach steady-state and until the end of experiment. On the morning of day zero (D0) rats were anesthetized, tracheotomized, mechanically ventilated and torachotomized for lung microdialysis probe insertion (LMP 5.35.35, polyether sulfone, cut off: 6000 Da,

membrane length: 10mm, outer diameter: 0.6 mm, Microbiotech, Stockholm, Sweden) as previously described (17). Probes recoveries were estimated by the retrodialysis by calibrator method. A solution of ciprofloxacin (CIP) (2 $\mu g.mL^{-1}$) was perfused into the three probes at a flow rate of 2 $\mu L.min^{-1}$ for 15 min. A flow rate equal to 0.5 $\mu L.min^{-1}$ was then maintained for 60 min before starting dialysates collection, and until the end of the experiment. Dialysates were collected from blood, muscle and lung probes every 45 min over 270 min, corresponding to 6 samples. The relative recovery by loss of the calibrator (CIP) was calculated for each interval of collect according to the following equation: $RL_{CIP} = (Cin - Cout)/ Cin$, where Cin and Cout correspond to the CIP concentrations in perfusate and in dialysates collected. Actual LVX concentrations were estimated by correcting measured concentrations in dialysates by the recovery by loss of CIP determined during the same interval of collect. For each rat and medium ECF concentrations at steady-state are presented as a mean ± S.D value derived from the 6 consecutive determinations. The *in vitro* recoveries by gain and by loss of CIP and LVX were evaluated from three probes (two CMA 20 probes and one linear probe) during 240 min at a flow rate of 1 $\mu L.min^{-1}$ and concentrations equal to 3 $\mu g.mL^{-1}$. *In vivo* recoveries by loss of CIP and LVX were compared in 3 dedicated rats: A, B and C. A mixture of the 2 compounds, each at a concentration of 2 $\mu g.mL^{-1}$, was perfused in each probe at a flow rate of 0.5 $\mu L.min^{-1}$ during 270 min and dialysates were collected by fractions every 45 min. Simultaneous analyse of LVX and CIP in dialysates were performed by HPLC as previously described (16). Dialysates were injected directly after dilution in phosphate buffer (pH=7) (1/1, vol/vol). Both compounds were analyzed at the same wavelengths: λex=285 nm, λem=490 nm. The between-day variability of the assay was characterized

each day of the analysis and was respectively less than 20% and 10% at two concentrations levels (0.125 and 1 $\mu g.mL^{-1}$).

Steady-state conditions preclude the retrodialysis by drug method to estimate probes recoveries, and considering that our objective was to conduct an experiment within a limited period of time, the retrodialysis by calibrator method was the best method for that. CIP had been previously validated as an appropriate calibrator for another fluoroquinolone antibiotic, norfloxacin (NOR) (16). It was therefore tested again as a potential calibrator for LVX. *In vitro* recoveries by gain and by loss of CIP and LVX were not statistically different (data not shown). Differences between LVX and CIP *in vivo* recoveries by loss in blood, muscle and lung, were always lower than 7%, with only an exception for Rat C in lung (Table 1).

Individual values of CIP recoveries by loss determined in rats receiving LVX (Rats 1-8) are presented in Table 2. These values were generally consistent with those obtained during the validation phase (Rats A-C), although relatively higher recoveries were occasionally observed especially in blood. Recovery by loss of CIP in lung was 3 to 4 folds lower on average than in muscle or blood (Table 2), again consistent with observations made in Rats A-C, as well as with previous experiments (6, 17). These relatively low recoveries in lung are most likely due to the different nature and cut-off of the membrane compared to CMA/20 probes. The between rats variability in lung probes recoveries, with extreme values ranging between 9.2 ± 3.6% and 31.5 ± 7.5% was also larger than in other media (Table 2). Altogether these data suggest that CIP may be

considered as an appropriate calibrator for LVX, although discrepancies between the estimated recovery by loss of the calibrator and the actual recovery of the tested compound may occasionally occur, especially in lung, possibly leading to outliers values in the estimated LVX concentrations.

Individual steady-state unbound concentrations of LVX in each medium are presented on Figure 1. The between rats as well as the between medium variability was rather limited and average unbound steady-state concentrations in the various rats and medium compared favorably with only one exception (lung data in Rat 3). Errors bars corresponding to standard deviations associated to these estimated average concentrations (n=6) were most often higher in lung than in other medium, illustrating the greater uncertainty on concentrations estimates in this tissue compared to plasma or muscle. This observation which is again consistent with previous findings (6, 17) is probably a consequence of the lower lung probes recoveries. Because flow rate was already quite low (0.5 $\mu L.min^{-1}$), it does not seem possible to substantially increase lung probes recoveries by further reducing the flow. Using different probes with increased recovery should potentially be of greater benefit.

ECF steady-state unbound tissue to blood concentrations ratios were respectively equal to 1.00 ± 0.15 in muscle and 1.06 ± 0.40 in lung, suggesting passive tissue distribution of LVX (7). These values are in partial agreement with data previously obtained in human and published mostly by researchers from the same group (1, 11, 22, 24). The first LVX microdialysis distribution study in human was conducted in the skeletal muscle tissue of

patients with sepsis (22). Unbound plasma concentrations were derived from measured total plasma concentrations assuming an average fraction bound equal to 35%. Ratio of the tissue to plasma unbound AUCs from time 0 to 8h (AUC_{0-8}) was used to characterize LVX distribution and was estimated to 0.85. The second study was conducted in subcutaneous adipose tissue using similar procedure, except that AUCs were estimated from time 0 to 10h and plasma protein binding was assumed to be equal to 25% (1). Average tissue to plasma unbound AUCs ratios were again close to unity (1.1 ± 0.6 for healthy and 1.2 ± 1.0 for inflamed subcutaneous adipose tissue). The third article describes the first microdialysis distribution study in lung (11) and lead to the conclusion that LVX penetration in this tissue was lower than in muscle (22) or subcutaneous adipose tissue (1). However in this study unbound AUCs of LVX in lung were compared to total plasma AUCs, with a mean ratio equal to 0.6. Therefore after correcting plasma AUC for protein binding as previously done by these authors, and considering that 30% of LVX is bound in plasma, one would obtain a mean tissue to plasma unbound AUCs ratio equal to 1.9, that is actually higher than the previously reported values close to unity (1, 22). The last study was then conducted to clarify the apparently conflicting data between muscle or subcutaneous tissue and lung (24). Unfortunately distinct subjects were enrolled in the study with muscle and subcutaneous distribution being investigated in healthy volunteers and lung distribution in patients undergoing elective lung surgery. Furthermore unbound tissue concentrations determined by microdialysis were again compared with total plasma concentrations making AUCs ratios interpretation difficult, especially since LVX plasma protein binding may vary between healthy volunteers and patients. But interestingly in this last study total AUCs were used to characterize LVX

tissue distribution. Unfortunately microdialysate samples were collected over a 8 h period of time, which considering the delayed peak in tissue (between 1h and 3h) and the relatively long elimination half-life of LVX (estimated to 5- 6h in this study), does not allow precise estimation of total AUCs. This study lead to the conclusion that LVX levels in the ECF of soft tissues cannot serve as a surrogate for predicting its pharmacokinetics in lung (24), whereas the results of our study that was conducted in rats but measuring LVX concentrations in both tissues of each animal, suggest the opposite.

Microdialysis tissue distribution studies following single doses administrations should rely on the comparison between total unbound AUCs in tissue and plasma (7), but as exemplified by this series of studies conducted with LVX this is not always done, and it may be difficult to accurately estimate these total AUCs. On many occasions AUCs between two consecutive administrations at steady-state should be equal to total AUCs after single doses, but they may not be more practical to estimate. Therefore comparison of tissue ECF and plasma unbound concentrations following intravenous infusion at steady-state appears as an interesting alternative to characterize drug tissue distribution, in particular to look for active efflux transport phenomenon. Although a single concentration measurement in tissue and plasma would be enough from a theoretical standpoint, six consecutive series of determinations were conducted for this initial study. Yet this was without major benefit since these individual values were always consistent and the number of determinations should be reduced for the next studies. Yet as opposed to microdialysis studies conducted with multiple drug determinations after single dose administrations, the major limitation of this proposed approach based on single points

determination at steady-state, is that it does not provide any information on the tissue rate of distribution, that can be modified in the presence of various patho-physiological conditions such as altered blood flow.

In conclusion lung microdialysis investigations following drug infusion at steady-state appear as an interesting alternative to the same type of experiments conducted after single dose administration for the characterization of lung distribution of antibiotics.

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TABLE 1. *In vivo* recoveries by loss of CIP and LVX (mean ± SD, n=6) perfused as a Ringer solution (CIP / LVX mixture at 2 µg.mL^{-1}each) at a flow rate of 0.5 µL.min^{-1} during 270 min, in the blood, muscle and lung probes of 3 rats (A-C)

		***In vivo* recoveries by loss (%) (n=6)**		
		Blood	**muscle**	**Lung**
Rat A	**LVX**	43.6 ± 5.6	38.9 ± 1.7	11.4 ± 3.8
	CIP	42.9 ± 5.9	35.9 ± 1.5	11.3 ± 3.8
Rat B	**LVX**	48.0 ± 3.0	36.8 ± 0.7	12.8 ± 3.0
	CIP	49.1 ± 3.1	36.1 ± 1.8	10.9 ± 4.0
Rat C	**LVX**	51.0 ± 2.1	36.1 ± 3.9	6.9 ± 2.4
	CIP	56.6 ± 2.0	39.1 ± 4.4	11.9 ± 2.4

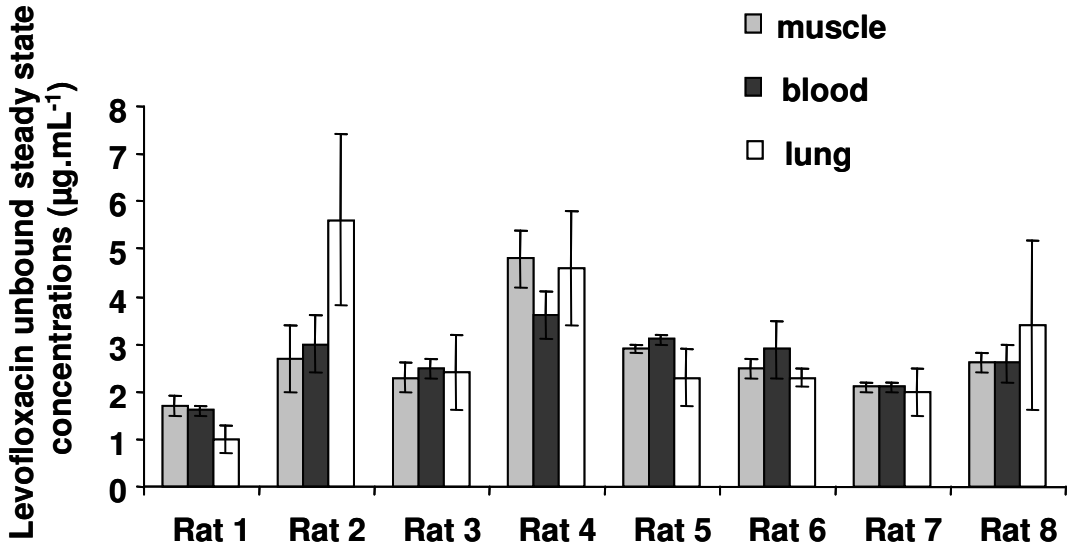
TABLE 2. *In vivo* recoveries by loss of CIP (mean ± SD, n=6) perfused as a Ringer solution (2 µg.mL^{-1}, 0.5 µL.min^{-1}) in the blood, muscle and lung probes of 8 rats receiving an intravenous infusion of LVX (1.0 mg.h^{-1}) at steady state .

	CIP i*n vivo* recoveries by loss (n=6)		
	blood	**muscle**	**lung**
Rat 1	44.6 ± 3.1	45.0 ± 3.9	31.5 ± 7.5
Rat 2	41.0 ± 3.6	38.4 ± 4.4	9.4 ± 2.5
Rat3	32.6 ± 2.2	48.4 ± 2.9	22.8 ± 10.0
Rat 4	74.6 ± 1.1	30.8 ± 1.4	18.8 ± 3.5
Rat 5	38.4 ± 1.1	45.0 ± 1.4	10.4 ± 3.3
Rat 6	46.3 ± 4.3	46.6 ± 2.4	12.1 ± 8.2
Rat 7	73.4 ± 1.2	48.5 ± 1.7	15.9 ± 3.9
Rat 8	73.4 ± 1.3	48.5 ± 1.8	15.9 ± 3.1

FIG. 1. Mean unbound concentrations of LVX (n=6) in muscle ECF, blood and lung ECF, of 8 rats receiving an intravenous infusion of LVX (1.0 $mg.h^{-1}$) at steady state .

1

2


Levofloxacin unbound steady state concentrations (µg.mL-1)
muscle
blood
lung
0
1
2
3
4
5
6
7
8
Rat 1
Rat 2
Rat 3
Rat 4
Rat 5
Rat 6
Rat 7
Rat 8