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Noninvasive fluorescent study *in situ* and in real time of glucose effects on the pharmacokinetic of calcein

Sylvie Bégu
Jean Marie Devoisselle
U.F.R. des Sciences Pharmaceutiques
Laboratoire de Technique Pharmaceutique Industrielle
15 avenue Charles Flahault-BP 14 491
34093 Montpellier Cedex 5-France

Serge Mordon
INSERM-EA 2689 IFR 22
Pavillon Vancostenobel, CHU
59037 Lille Cedex-France

Abstract. This study was undertaken to compare the effect of glucose injection on the pharmacokinetic behavior of a soluble dye in normal and tumoral tissues. The measurements were done using a noninvasive fluorescent spectroscopy *in situ* and in real time. The experiments were performed on three groups of animals with calcein as a soluble *p*H-insensitive fluorescent dye combined or not with glucose. Glucose solution was injected 5 or 30 min before calcein. Fluorescence emission intensity was recorded on normal and tumor tissues with an optical multichannel analyzer. Calcein concentration was also measured in blood using repetitive blood sampling. In the control group (without glucose injection), calcein is rapidly cleared from the blood, with a slow tissue clearance. Fluorescence of normal tissue was higher than fluorescence measured in tumor tissue. When glucose is injected 5 min before calcein, there was a rapid increase of tissue fluorescence followed by a plateau remaining during the whole experiment. No difference between tumor and normal tissue fluorescence intensity was observed. When glucose was injected 30 min before calcein, the plateau phase was reduced to 50 min in normal tissue. Tumor tissue fluorescence displays no distinct plateau phase. These results clearly showed the effect of glucose injection *in situ* and in real time, by a noninvasive method, on the pharmacokinetic of a soluble dye in a tumor tissue compared to a normal tissue. Differences between blood compartment and tissues kinetic profiles were also clearly demonstrated. [DOI: 10.1117/1.1501559]

Keywords: calcein; glucose; spectrofluorometer; pharmacokinetic; *in vivo*.

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1 Introduction

Our group has already demonstrated that it is possible to measure *p*H using fluorescence spectroscopy or imaging with a *p*H sensitive fluorescent probe such as 5,6-carboxyfluorescein (5,6-CF) or 4,5-biscarboxyethylcarboxyfluorescein (4,5-BCECF).[1–3] In order to validate this new and innovative approach, the studies were usually performed on CDF mice bearing lymphoid leukemia P388 (NCI) tumors. During these experiments, *p*H inside the tumor was depressed using intraperitoneal glucose administration according to the technique previously described by Jain.[4] However, little is known about the influence of glucose on the biodistribution of the fluorescent probes inside the tissue (normal or tumoral) and also how it may modify their pharmacokinetic behavior and their fluorescence emission characteristics. Our measurement technique is based on the monitoring by a noninvasive spectroscopic method of the fluorescence intensity emitted by the fluorescent probe. When using 5,6-CF, BCECF or C-SNAFL-1, it is not possible to distinguish the effect of glucose administration on their pharmacokinetic behavior from the effects of changes in tissue *p*H on their fluorescence emission. Consequently, in order to evaluate the pharmacokinetic behavior of the probe with the spectroscopic method, we must select a fluorescent probe whose fluorescence intensity is only affected by its pharmacokinetic and not by two parameters pharmacokinetic and *p*H which is the case of 5,6-CF and similar probes. This problem can be overcome by using a *p*H-insensitive fluorescent probe displaying properties similar to 5,6,-CF. In this context, calcein appears to be an ideal candidate since several studies have demonstrated that calcein exhibits fluorescence that is essentially *p*H independent between 6.5 and 9.[5,6] Calcein is a polyanionic fluorescein derivative with six negative and two positive charges at *p*H 7, as well as BCECF and 5,6-CF. Unlike the other fluorescein derivatives, calcein is a preferred reagent for following volume changes because its fluorescence is not particularly sensitive to either *p*H or physiological concentrations of other ions. This study aimed to determine the pharmacokinetic behavior of a soluble fluorescent dye (calcein) in normal and tumoral tissues after previous intraperitoneal glucose administration by using *in vivo* fluorescence spectroscopy.

Address all correspondence to Sylvie Bégu. Tel: +33-(0) 4.67.63.54.31; Fax: +33 (0) 4.67.04.88.74; E-mail: begu@cit.enscm.fr

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2 Materials and Methods

2.1 Fluorescent Markers

Calcein is a fluorescein carboxylated derivative (3,6-dihydroxy-2,3-bis[N,N′-di(carboxymethyl)-aminomethyl) fluoran]. It is largely used as a calcium titrant[7] and in liposome research as fluorescent marker of the internal aqueous phase.[5] This dye is highly fluorescent and soluble.[6] It was purified according to the procedure of Ralston.[8] Briefly, the dye was precipitated on active charcoal and then purified on Sephadex LH-20 column (2.5 cm×40 cm) in phosphate buffer solution (PBS:150 mM, 145 mM NaCl, 145 mM Na_2HPO_4, *p*H 7.4). The dye purity was assessed by high-performance liquid chromatography. Samples were eluted at room temperature with a linear gradient of methanol in 0.5% acetic acid. The gradient (25%–100% methanol) was run in 20 min at a flow rate of 2 ml/min on a C 18 reverse phase column (Lichrosphere). Calcein had emission spectral properties similar to 5,6-carboxyfluorescein and displays very little fluorescence intensity variation in the 6–7.4 *p*H range. The effect of *p*H on calcein fluorescence characteristics was determined on a Shimadzu spectrofluorometer in PBS at a 5 μM concentration.

2.2 Instrumentation

A xenon lamp equipped with a focusing system and narrow band interference filter centered at 490 nm with a full width at half maximum of 10 nm was used as the excitation source. Light was transmitted through a glass optic fiber (600 μm diameter). The delivered light power density was very low (3 mW). A second optic fiber of the same characteristics was used to measure fluorescence emission and was connected to an optical multichannel analyzer. It consisted of a CP-200 spectrograph (Jobin-Yvon, France) equipped with a computer controlled shutter. This spectrograph was connected to a liquid nitrogen cooled charge coupled device (CCD) camera (Spectraview-2D CCD, Jobin-Yvon, France). An interface unit supplies power for the CCD and amplifies and digitizes the CCD signal. Full spectral scans (300–800 nm) were performed in order to evaluate (i) the autofluorescence of normal and tumoral tissue and (ii) the emission peak of the marker. This system had a very high signal to noise ratio. By placing the probing optic fiber successively on tumor and normal tissue area, spectra were collected on either normal tissue or the tumor. The fiber bundle was fixed at a distance of 10 mm from the tissue surface, giving an 8-mm-diam illuminated area. Autofluorescence of both areas was determined prior to each experiment. Emission spectra were recorded with the use of 490 nm excitation wavelength during 210 min.

2.3 Animals and Tumors

Culture conditions of tumors cells: Lymphoïd leukemia P388 (National Cancer Institute). The culture cells were realized in three steps. A first cells line was obtained after injection of 1 million cells subcutaneously on anesthetized CDF mice (Ketalar and Largactil). Fifteen days after the injection, the animal was sacrificed and the second cells line was realized by implantation of a sample tumor under the skin in the abdomen. The tumor grew during 12 days in order to weigh 2.5 g. The tumor was grafted subcutaneously on CDF anesthetized mice. This second mouse was sacrificed, the tumor was taken up and cut in same pieces and implanted on five mice. Due to their fast-growing property, reproducible tumors of 5 mm diameter were obtained eight days after grafting. Three groups of animals were studied: group 1: calcein injected alone ($n=5$), group 2: glucose injected 5 min before calcein injection ($n=5$), group 3: glucose injected 30 min before calcein injection ($n=5$). Anesthetized mice were injected in the tail vein with 250 μl of 10^{-3} M calcein physiological saline solution (6.5 mg/kg) after previous intraperitoneal glucose administration (600 μl of a 10% w/v glucose solution, 6 g/kg) 5 min (group 2) or 30 min (group 3) before. As we want to do this study *in vivo* and real time, the mice are continuously anesthetized throughout the experiment with Ketalar and Largactil. We did not take into account the effect of the anesthesia on the blood flow because all the mice used for the experiments are anesthetized in the same way. Mice were shaved before spectra collection on the tumor area and on normal tissue corresponding to an underlying muscle tissue.

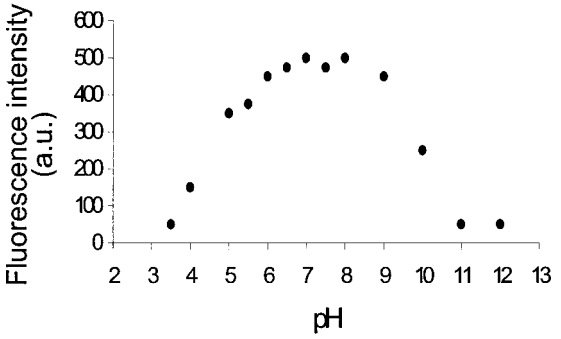

Fig. 1 Evolution of the calcein fluorescence intensity in function of *p*H ($n=3$).

2.4 Blood Samples

Blood samples were withdrawn by the orbital bleeding technique.[9] Typically, 20 μl of blood were taken into a heparinized capillary. This capillary was immediately placed in 4 ml of isosmotic PBS solution at 4 °C and centrifuged at 3000 rev/min for 10 min. An aliquot of the supernatant was settled into a 1 cm×1 cm quartz cuvette and assayed for fluorescence. Fluorescence intensities at 515 nm emission wavelength were measured on a rf-5000 SHIMADZU (Japan) spectrofluorometer using 490 nm excitation wavelength.

3 Results

Calcein is a fluorescent marker which has the advantage of displaying very little fluorescence intensity variation in the 6–7.4 *p*H range (Figure 1). A characteristic *in vivo* fluorescence spectrum of calcein collected 120 min after calcein injection was shown in Figure 2. This spectrum recorded in normal tissue was similar to the spectrum recorded in tumor tissue. The maximum emission was centered at 515 nm. The autofluorescence of normal and tumor tissues was negligible. When calcein was injected alone (group 1), the dye clearance from the blood (Figure 3) was very rapid (half time = 7 min). The tissue kinetic profile was quite different (Figure 4). Fluorescence intensity increased up to 30 min and was followed by a slow decrease. At the end of the experiment

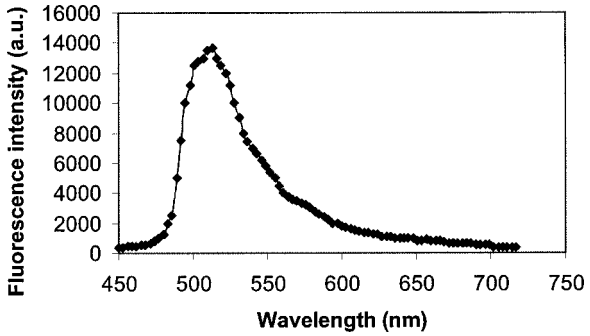

Fig. 2 Fluorescence spectrum of calcein in normal tissue. The mouse received 250 μl of 10^{-3} M calcein in physiological saline buffer solution.

(210 min), fluorescence intensity was close to the autofluorescence value. Sixty five minutes after dye injection, the tissue fluorescence intensity (for normal and tumor tissue) was half the original value. Fluorescence intensity was always higher in normal tissue than in the tumor (15 000 and 11 000 a.u. for normal and tumor tissues, respectively) but normal and tumor tissues exhibited a similar kinetic profile. When glucose was injected 5 min before the dye (group 2), kinetic profiles of calcein fluorescence in tissue were drastically modified (Figure 5). The tissue fluorescence increased and reached a maximum 90 min after dye injection followed by a plateau phase that was maintained until the end of the experiment. No significant difference can be observed between normal and tumor tissue fluorescence kinetic profiles. Fluorescence intensities were the same at the plateau phase (about 15 000 a.u.). Blood clearance showed an increase of fluorescence with a maximum at 50 min immediately followed by a slow decrease of fluorescence. The maximum intensity was 500 a.u. at 50 min. With a 30 min delay time after glucose injection (group 3), fluorescence intensity kinetic profiles of tissues showed an increase of fluorescence with a maximum at 50 min followed by a limited plateau phase followed by a decrease during the measurement time (Figure 6). The fluorescence intensity of

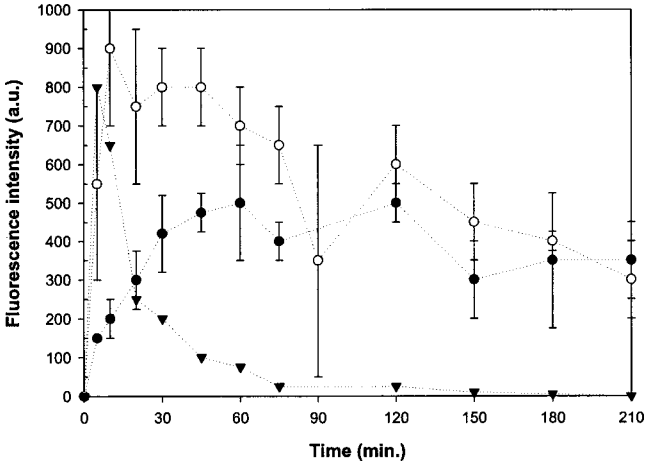

Fig. 3 Kinetic profiles of calcein in blood obtained from dilutes plasma samples as a function of time (▼) calcein alone; (●) glucose injection 5 min before calcein injection ($n=5$); (○) glucose injection 30 min before calcein injection ($n=5$).

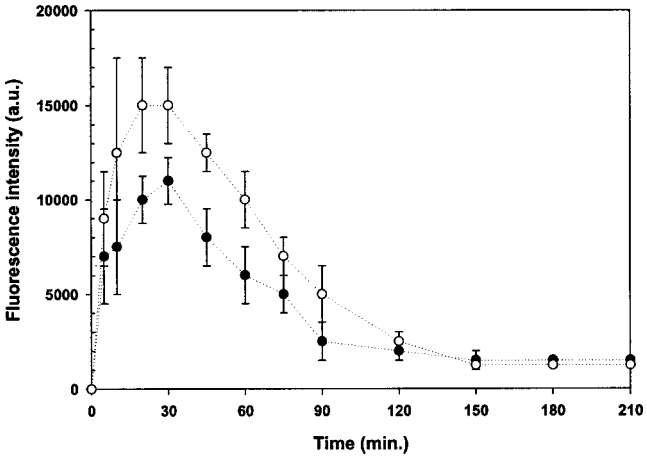

Fig. 4 Fluorescence kinetic profiles in normal and tumor tissues obtained as a function of time. Calcein is injected alone, without glucose injection: (○) normal tissue; (●) tumor tissue ($n=5$).

tumor tissue did not show any appreciable plateau region and was always less than the normal tissue fluorescence. The tissue fluorescence half time was about 110 min for both tissues. The blood fluorescence increased very rapidly with a peak at 5 min with a fluorescence of about 800–900 a.u. After 5 min, the fluorescence intensity decreased and reached 300 at 210 min (half time = 180 min).

4 Discussion

Calcein was used for this study as a *p*H-insensitive fluorescent probe in order to evaluate the influence of glucose administration on the pharmacokinetic behavior of a soluble injected dye. This dye is a carboxylated derivative of 5,6-CF which was used in our previous studies concerning the measurement and imaging of *p*H in normal and tumor tissues.[1] When this dye was injected alone, its pharmacokinetic behavior was quite similar to 5,6-CF pharmacokinetic profiles in blood or tissues (normal and tumor). The clearance rate was slower in tissue compared to blood. This observation could be

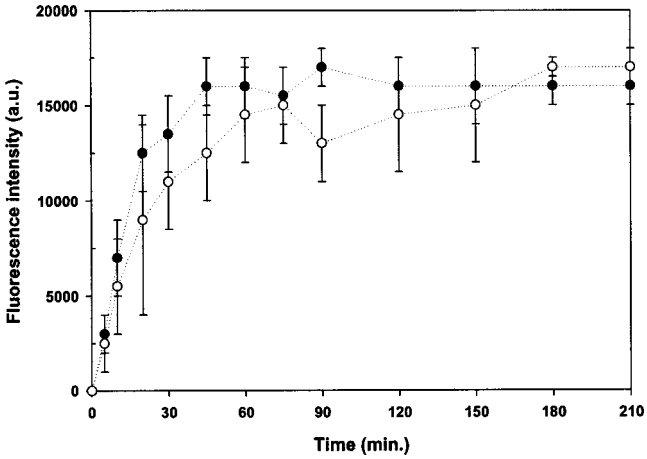

Fig. 5 Fluorescence kinetic profiles in normal and tumor tissues obtained as a function of time. Glucose was injected 5 min before calcein injection: (○) normal tissue; (●) tumor tissue ($n=5$).

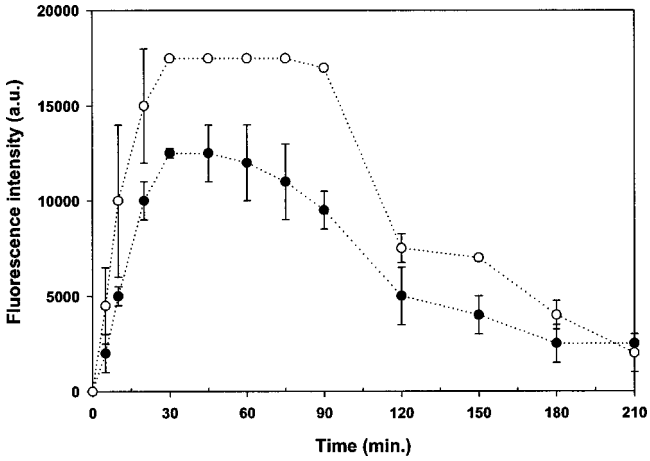

Fig. 6 Fluorescence kinetic profiles in normal and tumor tissues obtained as a function of time. Glucose was injected 30 min before calcein injection: (○) normal tissue; (●) tumor tissue ($n=5$).

due to the diffusion rate of calcein through the endothelium toward the interstitium fluid[10] and the flow of the dye toward very small superficial capillaries. When glucose at 6 g/kg dose was injected via intraperitoneal administration 5 min before calcein, a difference was clearly observed. The dye concentration in superficial tissues remains stable after reaching a plateau over the whole experiment. Blood clearance was similar to the one observed in skin. This observation was confirmed by Kalmus, Okunieff, and Vaupel[11] with a 5 g/kg glucose dose. With a 30 min delay time between glucose and calcein injections, it could be noted that the plateau phase was shorter. The fluorescence intensity of the dye in the tumor tissue was inferior to the one of the dye in normal tissue. The same difference was measured in the absence of glucose injection. These observations confirmed that the delay time between glucose and dye injection seems to play an important role in achieving the plateau phase. It has been demonstrated that the effect of the route of administration and the dose of glucose led to drastic variation of hemodynamics and blood flow because a significant osmotic water shift from the vascular compartment to the intraperitoneal cavity occurs.[12,13] Moreover, this process led to an important hemoconcentration and consequently to a dye concentration in the vascular compartment. The plateau phase observed could be explained by the reduction of the blood flow after glucose injection. The effect of glucose injection had shown that it was possible to keep a high concentration of the dye in normal and tumor tissues with the same extent. Moreover, some studies have demonstrated that glucose administration increases *in vivo* uptake and phototoxicity of hematoporphyrin derivatives in tumor.[14]

5 Conclusion

This study demonstrated the usefulness of noninvasive fluorescence spectroscopy in pharmacokinetic studies. The effect of glucose on the pharmacokinetic behavior of a soluble fluorescent dye led to dye concentration and diminution of blood flow preventing the dye from being cleared from the tissue. Differences between blood compartment and tissue kinetic profiles were demonstrated. Further studies are needed in order to evaluate the potential of this technique as a pharmacokinetic tool *in vivo* in a noninvasive way. Studies are under investigation concerning the ability of this technique to calculate *in situ* the pharmacokinetic parameters of fluorescent probes.

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