Pharmacokinetics and organ distribution of fluorescein in experimental pigs: an input study for confocal laser endomicroscopy of the gastrointestinal tract

Martin Kuneš 1, Jaroslav Květina 1, Jana Maláková 2, Jan Bureš 3, Marcela Kopáčová 3, Stanislav Rejchrt 3

1 Institute of Experimental Biopharmaceutics, Joint Research Centre of the Czech Academy of Sciences and PRO.MED.CS Praha a.s., Hradec Králové, Czech Republic
2 Institute of Clinical Biochemistry and Diagnostics, University Teaching Hospital, Hradec Králové, Czech Republic
3 2nd Department of Medicine, Charles University in Prague, Faculty of Medicine in Hradec Králové, University Teaching Hospital, Hradec Kraľove, Czech Republic

Correspondence to: Martin Kuneš, MSc., PhD.
Institute of Experimental Biopharmaceutics, Joint Research Centre of PRO.MED.CS Praha a.s. and the Czech Academy of Sciences, Heyrovského 1207, Hradec Králové, Czech Republic.
tel: +420 495 514 771; fax: +420 495 512 719; e-mail: kunes@uebf.cas.cz

Submitted: 2010-09-15  Accepted: 2010-11-16  Published online: 2010-12-28

Key words: fluorescein; pharmacokinetics; organ distribution; confocal laser microscopy; experimental pigs

Abstract

OBJECTIVE: Confocal laser scanning endomicroscopy (CLSE) is a diagnostic technology that produces virtual histology of the mucosal layer using fluorescence technique. Fluorescein (FSC) is the most commonly used fluorescence agent. Fluorescence light coming from a horizontal special focal plane is detected during confocal laser endomicroscopy of the gastrointestinal tract. FSC causes intensive yellowish discoloration of tissues, including skin and mucous membranes. This pre-clinical study was aimed to evaluate the tissue distribution and pharmacokinetics of FSC after its intravenous administration.

METHODS: The study was performed in an adult experimental pig. A reversed-phase high-performance liquid chromatographic method with fluorescence detection was used for the determination of fluorescein in blood plasma and tissue samples.

RESULTS AND CONCLUSION: The pharmacokinetic study of fluorescein determined the optimum time interval for diagnostic scanning (5–10 min.) The biodistribution study of fluorescein (aimed on the potential organ accumulation) proved the high concentration in the renal system followed by levels in bile > lung > adipose tissue > all other organs (including gastrointestinal wall) and these were relatively similar to each other. Fluorescein has a significantly low distribution in the brain (contrast with the level in adipose tissue indicates the low ability to penetrate the blood-brain barrier).
INTRODUCTION

Confocal laser scanning endomicroscopy (CLSE) is a key endoscopic technique which allows subsurface in vivo microscopic analysis during ongoing endoscopy, using a systematically or topically administered fluorescent agent (Gheorghe et al. 2008). CLSE may make in vivo histological diagnosis by virtual histology possible (Inoue et al. 2005). The principle of CLSE: a laser light source delivers blue excitation light at a wavelength of 488 nm. The fluorescence substance (fluorescein - FSC), being administered systemically, absorbs this light in the tissue and emits a green-yellowish light at a longer wavelength of 510–580 nm by itself. Only fluorescence light coming from a specific focal plane is detected afterwards by the endomicroscopy system. Our research group has successfully used CLSE in previous pre-clinical experiments in pigs. CLSE images corresponded well with those of classic haematoxylin-eosin staining (Kopáčová et al. 2009).

FSC hardly permeate through cell membranes. In humans, a dose of 7–30 mg/kg given intravenously as a bolus produces yellow-brown discoloration of the skin and ocular fundus (O’Goshi & Serup 2006). Despite its increasing use, there are rare data on the kinetics and dynamics of this substance (Becker et al. 2008).

From the toxicological aspect, several cases of adverse reactions are cited in the older literature after intravenous administration of FSC. For example, nausea, pruritus, hoarseness and partial respiratory obstruction as an anaphylactic reaction (LaPiana & Penner 1968), abdominal discomfort and severe retrosternal chest pain radiating into the jaw as an acute myocardial infarction (Deglin et al. 1977), collapse and cardiac arrest, tachycardia and noncardiac pulmonary edema followed by death (Cunningham & Balu 1979; Hefnner 1980) were observed in human after intravenous administration. Also yellow discoloration of the skin and mucous membranes may be for the patient, from a psychological point of view, certain complication.

The aim of our study was to specify the optimum diagnostic level of FSC in the tissue of particular gastrointestinal segments in pigs and to delimitate the potential toxicological risks following the relations of its organ distribution. The study was performed on experimental pigs due to their relatively very similar gastrointestinal and metabolical functions in comparison to man (Kararlí 1995; Boes & Helwigh 2000).

MATERIAL AND METHODS

Animals

Five mature female pigs (Sus scrofa f. domestica), hybrids of Czech White and Landrace breeds, weighing 32.6 ± 2.3 kg (4–5 months old), entered the study. They were kept in air-conditioned rooms, fed twice a day (standard food A1; Cerea a.s., Czech republic) and allowed access to water ad libitum.

Pharmacokinetics

The pigs were intravenously administered with FSC (15 mg/kg; Fluorescite 10%, Alcon Lab., Texas, USA). Blood samples were withdrawn from the cannulated vena cava cranialis (cannulation one day before blood collection) using a permanent central catheter in the following time intervals: 5, 10, 20, 30, 45, 60, 90 and 120 min. after FSC administration. Blood sampling proceeded in pen with free animal movement. Blood samples were centrifuged (3000 t./min, 10 min). The blood plasma was frozen at −30°C until chromatographic detection.

Cannulation of vena cava cranialis via vena jugularis externa was performed (24 h before the pharmacokinetic study) under general anaesthesia: intramuscular injection of ketamine (20 mg/kg; Narkamon, Spofa, Prague, Czech Republic) and azaperone (2 mg/kg; Stresnil, Janssen-Pharmaceutica, Beerse, Belgium) was used as an introduction, continued by inhalation of nitrous oxide and oxygen in mixture and halothane (during 30 min of surgical procedure).

The pharmacokinetic parameters were evaluated using software Kinetica™ version 4.4.1 (Thermo Electron Corporation, U.S.A.).

Organ distribution

FSC (15 mg/kg) was administered intravenously to the same animals used in the previous pharmacokinetic experiment (7th day after the end of pharmacokinetic study). The animals were sacrificed with an i.v. injection of thiopental and exsanguinated ten minutes after FSC administration. Subsequently, the following tissue samples were collected: brain, thymus, heart, lung, liver, pancreas, kidney, adrenal gland, spleen, oesophagus, stomach, duodenum, jejunum, ileum, caecum, colon, rectum, bladder, lymph-node, skin, adipose tissue, muscle, blood plasma and bile. Tissue samples were frozen at −30°C until analysis.

Fluorescein detection

A reversed-phase high-performance liquid chromatographic method with fluorescence detection was used for the determination of total FSC (free and bound to plasma proteins) in blood plasma and other tissues. Plasma samples and tissue homogenates (homogenization of the tissues in a phosphate buffer 0.05 mol/L pH 7.4) were purified using protein precipitation with an acetonitrile and zinc sulphate solution. FSC and internal standard 5-(bromomethyl) fluorescein were separated on a Zorbax Eclipse XDB-C18 column (Agilent, 250 mm × 4.6 mm I.D.) at a flow rate of 0.8 ml/min at 30°C. The mobile phase consists of 25% acetonitrile and 75% of aqueous ammonium acetate (10 mmol/L, pH 6.8). HPLC analysis was performed on a 2695 Waters Separations Module equipped with Waters 2475 fluorescence detector operated at excitation and emission wavelengths of 485 nm and 535 nm.
Ethics
The study was approved by the Institutional Review Board of the Animal Care Committee of the Institute of Experimental Biopharmaceutics, Czech Academy of Sciences. Animals were held and treated in accordance with the European Convention for The Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Council of Europe 1986).

RESULTS
The elimination of FSC from blood is rapid (mean \( t_{1/2} = 38 \) min) (Figure 1). On that account, the following distribution study was carried out at 10 min after intravenous FSC administration. The values of clearance and volume of distribution are high as well as variances in the parameter of areas under the curve (Tab). Quite monotonous decrease (monoexponential) can be seen in the interval of blood sampling, without significantly faster phase.

In the distribution study, the levels of FSC (\( \mu g/g \) of tissue) were several times higher in the kidneys and slightly increased in the lungs and liver compared to other organs (heart, pancreas, spleen, thymus) and the digestion tract tissues (oesophagus, stomach, intestinal wall) (Figure 2). Results indicate that FSC was poorly distributed into the brain.

DISCUSSION
CLSE is a diagnostic technique allows a unique look at cellular structures and functions at and below the surface of the gut that has recently been introduced into live endoscopy (Hoffman et al. 2006; Kiesslich et al. 2006; Kopáčová et al. 2007).

The intensive yellow marking of the skin after system FSC administration for CLSE of the digestive tract has a significant negative impact on the psycho-social interactions between medical staff and the patient. FSC is a small molecule that is highly water soluble, rapidly diffuses out of capillaries and into the extravascular tissue. The uptake and distribution pattern of FSC reflects both local blood flow and capillary permeability (Perbeck et al. 1987; Jager et al. 1997). The experimental proof of the caducity of these attributes and eventual selective organ FSC accumulation was the reason for argumentation for use of experimental animal species metabolically close to man in this pre-clinical study.

Besides FSC, there are other several agents that can be used in order to obtain the confocal endomicroscopy images. For example, acriflavine (administered topically) stains only the superficial layers of the mucosa, including the cell nuclei. On the other hand, the advantage of FSC is its distribution from the capillaries through the entire mucosa, showing the microvascular network and the connective tissue architecture (Hoffman et al. 2006). Becker et al. (2008) aimed their investigation to determine the ideal time period for the best CLSE imaging in pig when using FSC. They concluded the best contrast and image quality within first 8–10 minutes after injection of FSC. We obtained high quality CLSE images within the first 30 min in our previous experiments (Kopáčová et al. 2009).

Proof of the dominant and fast FSC transport from blood circulation into the kidney (rapid bioelimination) results from its hydrophilic properties in a
slightly basic organism environment. In humans, Dollery (1999) describes that peak plasma concentration occurs immediately after intravenous injection of FSC. Thereafter, the concentration declines rapidly. The fast decline in plasma FSC suggests a multicompartmental model of distribution, with a rapid drop in concentration within the first 10 min because of equilibration with extravascular fluid compartments. Subsequently, levels decline slowly because of elimination of FSC from the circulation predominantly determined by the kidneys. The excretion rate is more than the glomerular filtration rate, and active secretion in the renal convoluted tubules is likely to occur. That agrees with previous data (Conway 1985; Blair et al. 1986; Knudsen et al. 1992) described biexponential decline of blood FSC concentrations after administration of similar dose (14 mg/kg).

On the other hand, our results in pigs suggest that pharmacokinetics of FSC has mono-exponential character. According to these findings, it is the linear pharmacokinetics and elimination appears to be consistent with first-order kinetics.

In the liver, FSC is metabolised to fluorescein glucuronide (Blair et al. 1986). This metabolite also contributes substantially to the plasma fluorescence after intravenous or oral fluorescein administration (Grotte et al. 1985). Glucuronidation is active already 2 min after intravenous injection and at 60 min., over 80% is glucuronated (Dollery 1999; Chahal et al. 1985). On the other hand, many studies have called attention to the necessity of measuring free plasma fluorescein (Conway 1985; Mota & Cunha-Vaz 1985; Blair et al. 1986), which is available for transport across the barrier. Palestine and Brubaker (1981) describe that only unbound FSC highly permeate blood-retinal barrier and thus plasma binding must be considered as a variable that may significantly affect the level of fluorescence. In our study, only total FSC (free FSC and FSC bound to plasma protein together) were measured (not glucuronide metabolite of FSC). It is therefore not possible to unambiguously interpret the essence of a high volume of distribution. A mono-exponential concentration/time dependence of FSC corresponding to the one-compartment model of distribution. High value of distribution volume (149.8 L/kg) suggests an important role in binding or biotransformation processes. In the case of FSC is it probably a combination of both. In human, FSC is highly bound to plasma proteins (about 80–90%) (Dollery 1999).

The FSC elimination liver first-pass effect is apparently about one position value lower in comparison to bile concentration. Nevertheless, the bile FSC level is high and exceeds the concentrations in other tissues and corresponds with FSC levels in the duodenal wall (in which the accumulation is higher compared the other gastrointestinal segments). Although, selective FSC uptake in diagnosed tissues (in gastrointestinal wall) will be desirable, the FSC levels are comparable with most of the evaluated tissues. The highest FSC levels were found in the lungs in comparison to other parenchymatic tissues. In spite of the fact that we macroscopically observed yellow marking of the skin, the detected concentration of FSC in tissue samples was very low and was comparable with the level in muscle (probably due to the same extent of vascularisation).

To some extent there are discrepancies (differences) in the indicated fluorescein ability to migrate through the various barriers in the body. It was observed that FSC crosses into mother’s milk in human (Mattern & Mayer 1990). In the toxicity study on rats, it has been found (Salem et al. 1979) that FSC freely crosses the placental barrier and is distributed throughout the amniotic fluid and the fetus within 15 min after intravenous injection. In spite of that fact, FSC is considered relatively non-toxic, with high doses for LD50 (mice 4738 mg/kg, rat 6721 mg/kg) (Yankell & Loux 1977) and does not appear to pose any significant risk when administered during pregnancy. FSC does not produce embryotoxic, teratogenic (Salem et al. 1979) or carcinogenic effect (Dollery 1999).

Martinez & Koda (1988) states that FSC penetrate into the brain in rat and that exists the sex differences in measured concentrations. Our findings in pigs revealed low FSC concentration in the brain. It is interesting, not only from the kinetic-distribution perspective but also with regards to toxicologic aspects. When comparing the relatively high FSC concentration in subcutaneous adipose tissue we can judge its handhold in the blood-brain barrier. It corresponds with the findings Malmgren & Olsson (1980) which indicates that sodium fluorescein does not cross the blood-brain barrier. On the other hand, they describe very rapid penetration of FSC into peripheral ganglia and into the epineurium and perineurium of large peripheral nerves.

ACKNOWLEDGEMENT

The study was supported by grant of Czech Science Foundation No. 305/08/0535 and by research plan MZO 00179906 from the Ministry of Health, Czech Republic. Authors would like to thank Dr. Jaroslav Chládek for pharmacokinetic analysis.

REFERENCES

Pharmacokinetics and organ distribution of fluorescein in experimental pigs