ELSEVIER

Food Chemistry 76 (2002) 225–230

Food Chemistry

www.elsevier.com/locate/foodchem

Analytical, Nutritional and Clinical Methods Section

A physiological approach for preparing and conducting intestinal bioavailability studies using experimental systems

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Received 28 February 2001; received in revised form 1 July 2001; accepted 1 July 2001

Abstract

Various in vitro models, such as digestion procedures, element dialysis across membranes and also the Caco-2 cellular absorptive system, are currently used to study intestinal nutrient bioavailability. These systems were established as an alternative to human and animal studies. The principle requirement for successfully performing such kinds of experimental studies is to achieve conditions, which are similar to in vivo conditions. Therefore, it is absolutely necessary to consider the natural environment in the human gut. Consequently, in vitro studies should be prepared and conducted according to evidence-based gastrointestinal physiology. This concise review summarizes important processes in the human intestine, like peptic and pancreatic digestion, adjustment of pH, peristaltic frequency, osmolality, composition of the serosal (= acceptor) solution, and permeability characteristics of the enterocyte monolayer, which are relevant for bioavailability studies using in vitro models. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Bioavailability; Digestion; Human gut; Gastrointestinal physiology

1. Introduction

Bioavailability can be defined as the proportion of a nutrient that can be utilized for normal physiological functions. The main component of bioavailability refers to the digestion and absorption of nutrients in the gut, which is the main rate-limiting factor. Studying this so-called intestinal bioavailability (Ekmekcioglu, 2000) in humans is very costly and not always easy due to ethical reasons and large interindividual variations. Therefore, experimental models are used to overcome these problems. A variety of in vitro methods were presented in the last half of the twentieth century yielding more or less reliable results. These techniques are mainly based on: (1) In vitro digestion of homogenized foods in a closed system and determination of the soluble nutrient fraction (Narasinga Rao & Prabhavathi, 1978), (2) In vitro digestion and dialyzability of soluble nutrients across a semipermeable membrane with or without removement of the dialyzed nutrients (Wolters, Schreuder, van den Heuvel, van Lonkhuijsen, Hermus, & Voragen, 1993), (3) Usage of Caco-2 cells and either measurement of (a) cellular uptake of nutrients after transmembranous dialysis during intestinal digestion of foods (Glahn, Wien, Van Campen, & Miller, 1996) or (b) Transepithelial transport of solubilized nutrients from digested foods (Ekmekcioglu, Pomazal, Steffan, Schweiger, & Marktl, 1999). The main disadvantages of these models are the lack of the complex mucosal barrier with all of its regulatory processes and also the static transport conditions not allowing an accurate calculation of whole fractional transport and flux rates. A further drawback of these models is often the lack of an evidenced-based approach when planning and performing the studies.

To fill this gap this manuscript will summarize important aspects of human gastrointestinal physiology, which are relevant for bioavailability investigations using experimental models. The major physiological parameters of the human gut, which should be considered in the planning phase of the studies, are presented in Table 1 and discussed subsequently in the different sections of the manuscript.

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PII: S0308-8146(01)00291-6

2. Peptic digestion and incubation time

Peptic digestion of food initiates in vitro bioavailability studies. For this purpose food is normally homogenized in a definite volume of water, afterwards pH is adjusted to 1–2, pepsin suspension (EC 3.4.23.1) is added, and the sample is incubated for 1–2 h holding the pH at 1–2 during the incubation period.

Basal pepsin output in middle-aged humans was shown to be about 1900 IU per 15 min while maximum pepsin output after stimulation with pentagastrin was 4600 IU per 15 min (Feldman, Cryer, McArthur, Huet, & Lee, 1996). Therefore, a minimum amount of 4000–5000 IU of pepsin seems to be necessary for optimal protein digestion, which is anyway realized in most of the bioavailability studies.

The gastric sojourn time of food homogenates varies dependent on the meal composition. Factors like hyper- and hypoosmolality, and pH of the duodenal fluid can influence gastric emptying. Especially meals rich in fibre and fat can delay gastric emptying. In recent studies, full gastric emptying was shown to last up to 4 h, dependent primarily on the meal composition but also on gender. For example Tougas et al. (2000) found median values for gastric retention of 69, 24, and 1,2% at 60, 120 and 240 min, respectively after ingestion of a low fat meal. In another study it was shown that the half gastric emptying time was about 108 min for a high fat meal and 83.8 min for a high carbohydrate meal (Robertson & Mathers, 2000). However shorter gastric emptying times were also reported by others (Bennink et al., 1999), suggesting that the composition of meals and also the different study methods could probably affect the results. With regard to these human studies, longer times for peptic digestion, e.g. up to 4 h, would be most probably reasonable.

3. Pancreatic digestion and incubation time

For pancreatic digestion, usually a pancreatic-bile extract mixture is added to the food suspension containing major pancreatic enzymes, such as lipase, amylase, trypsin, ribonuclease and protease and also bile salts. The activities of the protease, lipase and amylase in the pancreatin powder are at least equivalent to 3–4× of USP specifications (US Pharmacopeia) leaving a safety margin for protein- lipid- and carbohydrate digestion. In humans the food stimulated output of amylase is approximately 30–55 U per kg body mass per 15 min after intake of 40 or 160 kcal/h infusions (Holtmann, Kelly, & DiMagno, 1996) while cholecystokinin stimulated peak output of amylase was reported to be 39 kU/h (Lam, Gielkens, Coenraad, Souverijn, Lamers, & Masclee, 1999). The cholecystokinin induced trypsin output in humans lies between 5 and 10 kU in 30 min over 4 h registration time (O'Keefe, Bennet, Zinsmeister, & Haymond, 1994). Finally lipase and bile salt outputs to intra-intestinal infusions of liquid food with 4.5 kcal/min were demonstrated to be about 4 kU and 20 μM per min, respectively in healthy people (Landry et al. 1995).

4. Adjustment of pH

In experimental studies pH is held at 1–2 for digestion in the stomach and then elevated to approximately 6.5–7.5

Table 1
Anatomical, physical and physiological variables in the human gastrointestinal tract relevant for experimental bioavailability studies

Variables	Data
Stomach	
Fasting pH	≈ 1.5 (range 1–3.5)
Postprandial pH	Up to a median value of 5, then declining time dependently to 1–2
Sojourn time of meals	up to 4h
Basal pepsin output	≈ 1900 IU /15 min
Maximum pepsin output	≈ 4600 IU/15 min
Peristaltic frequency	≈ 3/min
Small intestine	
Transport area (without microvilli) in cm^2	≈ 100 000
Luminal fluid volume (ml)	≈ 9000 entering duodenum per 24 h,mean 5250
Fluid load $\mu l/(cm^2 \times h)$	≈ 2.2
Transepithelial resistance (R_T) Ω/cm^2	≈ 25–50 in jejunum
Effective pore radius of tight junctions (Å)	≈ 6.7–8.8 in jejunum
Transepithelial potential difference ψ^{ms} (mV)	≈ 0–3 in jejunum, ≈ 4–6 in ileum
Peak amylase output	≈ 39 kU/h
Peak trypsin output	≈ 5–10 kU/30 min
Food stimulated lipase output	≈ 4 kU/min
Food stimulated bile salt output	≈ 20 μM/min
pH	≈ 5.7–6.4 in the duodenum, up to ≈ 7.4 in the jejunum, up to ≈ 7.7 in the ileum
Peristaltic frequency	≈ 11.7/min in the duodenum, 8.9–9.8/min in the jejunum and ileum
Luminal osmolality (mosmol/kg H_2O)	≈ 290–300

for pancreatic digestion. These pH values are roughly also valid for the situation in the human gastrointestinal system. However, since the pH affects the activity of digestive enzymes and could also modulate chemical properties and solubility of nutrients, like iron, it is essential to adjust the pH values as similar as possible to human conditions. The following points should be considered when adjusting pH.

4.1. Peptic digestion

Fasting mean pH in the gastric fluid of humans is about 1.5 (range 1–3.5; Fallingborg, 1999). Ingestion of food, milk and eventually other basic liquids can increase the pH up to 7 (Russell et al., 1993) which then time dependently declines to acidic values. For example, Russell et al. (1993) measured the postprandial deviation of pH after ingestion of a standard meal (consisting of hamburgers, bread, potatoes, ketchup, mayonnaise, tomato, lettuce, and milk, total of 4180 kJ) in healthy humans. They found that after ingestion of the meal the pH rose to a median value of 5 and then declined to pH 4 after 14 min, to pH 3 after 42 min, and finally to pH 2 after a total of 100 min. With regard to these time-dependent pH modulations, the following procedure for gastric pH adjustment in bioavailability studies is recommended: (1) suspend the homogenized food sample in watery solution having a pH of 1–2, (2) afterwards measure actual pH and slowly titrate the pH (with HCL) to values of 1–2. The term slowly is rather empirical and could mean that the time to decline one pH value is about 10–20 min, so that the whole time to titrate the pH from, for example, 6 to 2 would take 40–80 min. This procedure would be the most evidenced based approach.

4.2. Pancreatic digestion

In most of the bioavailability studies pH is adapted to 6.5–7.5 with $NaHCO_3^-$ (amount equivalent to titratable acidity) during pancreatic digestion. This is obviously a valid approach. However, some further considerations, especially regarding the absorption site of the studied nutrient, should be made to obtain similar pH conditions as in vivo. The intraluminal pH in the small intestine of humans gradually increases from values between 5.7–6.8 in the proximal small intestine to 6.8–7.7 in the middle to distal part of the ileum (Fallingborg, 1999). Since most of the nutrients are absorbed in the jejunum and ileum it is reasonable to adjust the pH in the test solutions to values around 7. One major exception is iron, whose main absorption site lies in the duodenum (Rucker, Lönnerdal, & Keen, 1994). As the pH in the human duodenum varies between 5.7–6.4 (Fallingborg, 1999) or 6.2–6.7 during a meal (age dependent; Russell et al., 1993), it would be rational to adjust the pH in the luminal test solution in studies investigating iron bioavailability to values around 6.2–6.7. Since the solubility and therefore the transport rate of ferric iron is especially dependent on the luminal pH it is likely that the H^+-concentration could affect the fractional transport rates of this trace metal.

5. Frequency of peristaltic contractions

In many in vitro studies the food samples are incubated in shaking water during peptic and pancreatic digestion. However, it is seldom mentioned which shaking frequency was used. The shaking frequency should correspond to the peristaltic frequency in the intestine during the digestive processes. Gastric pacesetter potentials or slow waves depolarize at a frequency of approximately 3 cycles per minute (Koch, 1999) whereas the peristaltic frequency in the small intestine was shown to be 11.7 per minute in the duodenum and 8.9–9.8 per minute in the ileum (Kellow, Borody, Phillips, Tucker, & Haddad, 1986). Therefore, it is reasonable to adjust the shaking frequency during peptic or pancreatic digestion to values of about 3/min and 10/min, respectively.

6. Adjustment of osmolality

If two aqueous solutions with different osmolalities (mosmol/kg H_2O) are separated by a semipermeable membrane, water will move from the solution with the lower osmolality to the solution with the higher particle concentration. This process is called osmosis. Osmosis can lead to an alteration in nutrient transport across membranes and could especially modulate the nutrient concentration in the test solution. Therefore, osmosis and osmolality may play crucial roles in experimental studies using models where two compartments, e.g. the luminal (donor) and serosal (acceptor) compartment, are separated by a semipermeable membrane.

Nutrients are absorbed from an approximately isotonic intraluminal fluid in the small intestine. The osmolality in the contents of human jejunum and ileum lies approximately between 290–300 osmol/kg H_2O, respectively (Chang & Rao, 1994; Fordtran & Locklear, 1966; Phillips & Summerskill, 1967). It was recently shown in human volunteers that the osmolalities of hypotonic, isotonic, and hypertonic solutions are adjusted to values around 300 osmol/kg H_2O in the upper intestine postprandially (Gisolfi, Summers, Lambert, & Xia, 1998). Unfortunately, in most of the studies measuring in vitro bioavailability it is seldom mentioned whether the osmolality in the test solutions were adjusted to isotonic values prior to the assays. This is a

drawback and primarily could lead to inaccurate determinations of the fractional transport rates compared to human data, especially when the osmolalities between the luminal and serosal compartments differ largely form each other.

7. Composition of the serosal (acceptor) fluid compartment

A further aspect which should be considered in bicameral in vitro systems is the composition of the acceptor, basal or serosal compartment. Nutrients are transported in vivo from the intestinal lumen by a transcellular and/or paracellular pathway to the interstitial fluid from which they are transferred to the capillaries (Granger, 1981). Therefore, it seems reasonable to compose the acceptor solution according or similar to the content of the interstitial fluid, which has, approximately, the following composition (in mM): 136–143 Na^+; 4 K^+, 1.2–1.3 Ca^{2+}, 0.5-0.7 Mg^{2+}, 115 Cl^-, 28 HCO_3^-, 1 HPO_4^{2-}, 0.5 SO_4^{2-}, 5 organic acids, proteins < 1 (albumin 0.19; Fogh-Andersen, Altura, Altura, & Siggaard-Andersen, 1995; Lang, 1996). This is important since firstly nutrients are transported in vivo against a gradient, and secondly, it is well known that nutrients can compete at the absorption site or can modulate mutually their transport rates. Consequently, efforts should be made to adapt the acceptor solution as similar as possible to in vivo conditions. However, in cases where the transport of non-radioactive nutrients are investigated by using conventional analytical methods, like atomic absorption spectrometry, gradient driven transport cannot be accurately investigated, because in these cases it is impossible to discriminate between luminal-serosal or vice versa transport.

8. Transport area

The human small intestine has an absorptive area of approximately 100 000 cm^2 (without considering microvilli-area). The luminal fluid volume of the human intestine is about 9000 ml per day resulting in a fluid load of about 3.75 $\mu l/(cm^2 \times h)$. Filter grown Caco-2 cells or dialysis membranes are usually used as tools in experimental models for studying the transport of nutrients between two compartments. The membrane area for nutrient transport in these studies is limited and far below the values present in vivo resulting in a considerably higher fluid loads as for example 317.80 $\mu l/(cm^2 \times h)$ in a Caco-2 filter system with an area of 4.72 cm^2. These anatomical discrepancies could lead to different fractional transport rates. The differences in fluid loads can partially be overcome by using less test volume or prolonging the incubation times, though this has only a relevance in dynamic transport conditions, since in static conditions equilibrium is reached after a relatively short period of time.

9. Membrane characteristics

In the human gut, nutrients are transported by a transcellular and/or paracellular route from the intestinal lumen to the interstitial fluid and afterwards to the blood. The paracellular transport is accomplished by passive diffusion which is mainly dependent on the concentration gradient between the intestinal lumen and the interstitium. Additionally, other factors like the transepithelial potential difference but to a great extent also the effective pore radius of the tight junctions at the apical pole of the cells influence the passive, paracellular transport rates of nutrients. Previous studies, for example, reported a significant correlation between molecular size (cross-sectional diameter) of substances and their intestinal epithelial permeation rate (Hollander, Ricketts, & Boyd, 1988; Ma, Hollander, Bhalla, Nguyen, & Krugliak, 1992).

In experimental studies based on membrane dialysis, passive diffusion is the only way for nutrients to move between the luminal and serosal chamber (Miller, Schricker, Rasmussen, & Van Campen, 1981). The dialysis membranes used in these kind of investigations have a particular permeability defined by the term molecular cut-off (MCO), which equals the molecular weight of the largest globular protein that can pass through the pores of the membrane. In general, proteins that weigh more than the MCO-value will be retained by the membrane. The MCO-value is roughly proportional to the pore radius of the membranes. The permeability characteristics of the artificial membranes mainly determined by the MCO values should correspond to in vivo conditions. For example, the effective pore radius of the human jejunum was reported to be 6.7–8.8 Å (Soergel, 1993) while in Caco-2 cell monolayers, which are also used for bioavailability studies, the radius of the tight junction was shown to be slightly higher with approximately 12 Å (Adson et al., 1994). For example, the paracellular permeability of Caco-2 cell monolayers for fluorescein isothiocyanate dextran (FD)-4 with a molecular weight of 4400 Da and a molecular radius of 14 Å was shown to be very low while the epithelial layer was nearly impermeable to FD 20 (MW: 19 400 Da, molecular radius: 31 Å; Duizer, Penninks, Stenhuis, & Groten, 1997). Since the effective pore radius of the tight junctions in the human intestine is lower than those reported for Caco-2 cells it can be expected that the molecular cut-off point in vivo will be less than those shown for Caco-2 monolayers. For example, it was recently shown that lactulose (MW: 342 Da) and ^{51}Cr-

EDTA (MW: 341 Da) with molecular diameters of 9.5 Å and 11.5 Å, respectively, have a very low intestinal permeability in humans (Maxton et al., 1986). There is in general an association between the molecular weight and the molecular size of substances, although is some cases no correlations can be observed (Ma, Hollander, Bhalla, Nguyen, & Krugliak, 1992). Therefore, considering the pore diameters and permeability characteristics of the human intestine, it seems that the usage of dialysis membranes with a molecular cut-off value of less than 5000 Da would most probably approach to the conditions in vivo. However, before conducting the assays, it would be worth fully comparing permeability data across dialysis membranes with those of human studies to find out which membrane is appropriate.

10. Conclusions and future perspectives

When planning, preparing and conducting transport studies in experimental systems it is essential to consider cellular and physiological factors, like digestive enzyme activities, sojourn time, osmolality, pH, composition of acceptor solution, transport area, and membrane characteristics, to obtain study conditions which are as similar as possible to in vivo conditions. A major drawback of non-cellular and also cellular in vitro models are the static transport conditions mainly influencing data derived from passive transport mechanisms. To overcome this problem dynamic experimental models were developed recently. These systems are multi-compartmental and computer controlled mimicking the successive kinetic processes in the gastrointestinal tract. (Minekus, Marteau, Havenaar, & Huis in't Veld, 1995; Zeijdner & Havenaar, 2001). With these models it is able to continuously monitor and regulate parameters during digestion of compounds, such as body temperature, kinetics of the gastric and intestinal pH values, gastrointestinal enzyme secretion and activities, bile salt concentrations, and also peristaltic movements. It was demonstrated that the bioavailability data obtained from studies using these dynamic models were shown to be reproducible and comparable with those of in vivo conditions; for example, as for glucose (Minekus et al., 1995), iron and phosphorus (Larsson, Minekus, & Havenaar 1997), calcium (Zeijdner & Havenaar, 2001), and the analgetic drug paracetamol (Venema, van Nuenen, Smeets-Peeters, Minekus, & Havenaar 2000). All in all, it seems that these models are valuable for studying the bioavailability of nutrients and also drugs. An extension of these dynamic models by combining them with cellular models, like the Caco-2 in vitro system (Ekmekcioglu et al., 1999), may also be conceivable especially to achieve similar mucosal transport conditions as in vivo.

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