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Dynamic continuous-flow dialysis method to simulate intestinal digestion for in vitro estimation of mineral bioavailability of food

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Abstract

A system for dynamic continuous-flow dialysis during intestinal digestion for an in vitro simulation of gastrointestinal digestion is presented as an alternative to human and animal in vivo methods for estimation of the bioavailability of minerals. The method is based on the in vitro batch dialysis method described by Miller, which was developed into a continuous-flow system of a simple design to perform dynamic dialysis in the intestinal digestion stage. A flow dialysis system has the advantages of simulation being close to in vivo physiological conditions because pH change during dialysis is gradual and dialyzed components are continuously removed. The proposed new design performed dialysis during a continuous flow of dialyzing solution ($NaHCO_3$) around a dialysis bag containing peptic digest, which is placed inside a glass dialysis chamber. Gradual change of dialysis pH, similar to that occurring in the gastrointestinal tract, was obtained by optimization of flow rate and concentration of $NaHCO_3$. The dialysate collected in fractions was analyzed to determine dialyzed minerals and pH change in the course of dialysis. The method was tested by determination of calcium bioavailability of powder milk and calcium carbonate tablets.

Keywords: In vitro method; Bioavailability; Continuous-flow

1. Introduction

The total concentration of a mineral micronutrient in food does not provide information about its bioavailability. Speciation of a micronutrient or the determination of its chemical forms in food and in the gastrointestinal tract is essential to the understanding and the prediction of its availability for absorption [1]. This is often difficult to perform. Nutrient bioavailability has usually been estimated by in vivo human study. In vivo experiments, however, are time consuming and very expensive and often give variable results caused by uncontrollable physiological factors. Laboratory in vivo experiments in animals are sometime used as a model for human. Experiments with animals are less expensive but are limited by uncertainties with regard to differences in metabolism between animals and human. As an alternative to in vivo human and animal studies, nutrient bioavailability has also been estimated through in vitro methods [2–9]. These methods have gained popularity because of their simplicity, precision, speed of analysis and relatively low cost.

Interest in development of in vitro methods for estimating bioavailability of essential mineral elements dates back to at least the early 1930s [2]. These methods provide insights on minerals and trace element nutrition that are not achievable by human or animal experiments. The earliest trial [2] assumed ionizable minerals as potentially available and determined ionizable iron in food by extracting with complexing agents such as α,α'-dipyridyl and bathophenanthroline. Another approach attempted to simulate gastrointestinal digestion conditions and determined soluble or dialyzable minerals [3–9]. Particularly, the in vitro method developed in 1981 by Miller et al. [5] has been reported to provide availability measurements that correlate well with in vivo studies for iron. This method has been the basis for several in vitro methods for estimation of the bioavailability of iron and other minerals such as calcium and zinc [10,11]. The in vitro method

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involves a simulated gastrointestinal digestion with pepsin at pH 2 for 2 h during the gastric stage and with a mixture of pancreatin and bile salts along with a gradual pH change from 2 to 7 during the intestinal stage. The proportion of the compounds diffusing across a semipermeable membrane during the intestinal stage is used as a prediction of the elemental bioavailability.

In Miller's method, equilibrium dialysis is performed to obtain dialyzable compounds during intestinal digestion. The drawback is that dialyzed components are not removed during dialysis, as occurred in the real situation of the digestive tract. This may cause lower dialyzability. Therefore, a modified continuous dialysis in vitro method was developed by Minihane et al. [12] in which dialyzed components were removed continuously. The model developed by Minihane et al. used an Amicon stirred cell for continuous dialysis. The pH was adjusted gradually over a 30 min period from 2.0 to 7.0 before dialysis was started. Minihane's method was further modified by Shen et al. [13] to obtain a gradual pH change during the dialysis instead of adjusting the pH before dialysis. Shen et al. performed continuous dialysis by introducing a gradual pH adjustment using a small dialysis bag filled with an amount of $NaHCO_3$ equivalent to the predetermined titratable acidity of the peptic digest. The dialysis was carried out in a vessel under a pressure of 50 psi.

Wolters et al. [9] developed an in vitro method for continuous dialysis of minerals and trace elements based on a hollow-fiber system. The hollow-fiber system for continuous dialysis consists of a reaction vessel placing in a water bath at 41 °C. The food suspension in this vessel is pumped via a peristaltic pump through a suction tube into the hollow-fiber membrane. A fine filter cloth stretched across the inlet of the hollow-fiber and a magnetic stirrer was used to prevent clogging of the hollow-fiber by large particles. Components in the suspension that could pass through the hollow-fiber membrane were dialyzed and collected in a plastic bottle for subsequent analysis. That part of the suspension that could not pass through the hollow-fiber membrane was pumped back into the reaction vessel where these components could be digested further and recirculated into the hollow-fiber for complete dialysis.

A multicompartmental computer controlled simulated gastrointestinal digestion system has been developed [14] and applied [15,16] for evaluation of bioavailability. The system consists of several successive compartments to simulate the digestion in the stomach, duodenum, jejunum and ileum. Compartments are connected by peristaltic valve pumps to regulate the transfer of digestive enzymes. The system was also equipped with rotary pumps, syringe pumps for water pressure and secretion controls. Because the model aimed to mimic the whole GI-tract from stomach to ileum, it was rather complicated and not easy to perform. A simple method to access maximum bioaccessibility based on flow injection leaching of food sample by artificial saliva, gastric juice and intestinal juice was recently developed [17]. The method has the advantages of rapidity and simplicity. However, because leaching was accomplished in only a few minutes, the food sample may only be partially digested and leached.

In the present study, a simple setup for continuous-flow dialysis to perform an in vitro simulated intestinal digestion was developed. Considering that mineral absorption takes place mainly at the intestinal digestion stage [5], this setup was designed for dialysis in the intestinal digestion stage to occur by a continuous flow of dialyzing solution (dilute $NaHCO_3$ solution) around the dialysis tubing containing the gastric digestate. The gastric digestion was performed in a batch manner to effect high sample throughput because a large number of samples could be digested at the same time. In the simulated intestinal digestion stage, gradual change of pH, similar to that occurring in the intestinal tract, was obtained by optimization of flow rate and concentration of $NaHCO_3$. The dialysate collected in fractions was analyzed to determine the amount of dialyzed minerals. The graphical plot of dialyzed minerals with time of dialysis provides kinetic information of the dialysis process. The feasibility of the developed system was tested by applying it to evaluate dialyzability of calcium in calcium carbonate tablets and powder milk.

2. Experimental

2.1. Design and setup of continuous-flow dialysis system

A continuous-flow dialysis system was designed to serve three objectives: a gradual pH change at the early stage of dialysis, a convenient means of addition of enzymes at will and continuous removal of dialyzable components during dialysis.

The proposed dialysis system is presented schematically in Fig. 1. A dialysis chamber was designed to allow containment of a dialysis tubing, around which dialyzing solution could flow during dialysis. The chamber (ca. 20 cm in length and 0.8 cm inner diameter) and its cover were constructed in-house from borosilicate glass. Dialysis tubing MMCO 12,000–14,000 Da (Spectra/Por, Thomas Scientific, USA) was used. To prepare the dialysis chamber, a dialysis tubing

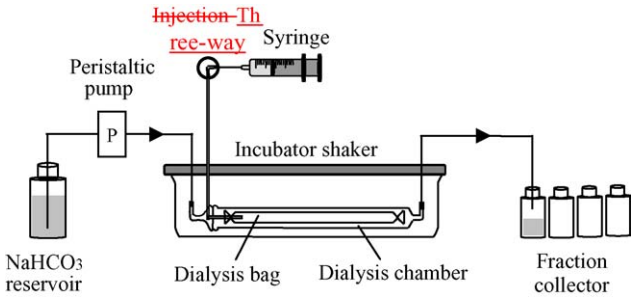

Fig. 1. Schematic diagram of the proposed continuous flow in vitro dialysis setup.

of 10 mm flat width and ca. 17.5 cm in length was tied at both ends, one end with a silicone tube (2 mm inner diameter and 5 cm long) inserted for the injection of a peptic digest sample and required enzymes. The other end of this silicone tube is pierced through an aperture in the chamber cover to allow convenient addition of a peptic digest aliquot and PBE mixture via a three-way valve by a 3 ml disposable syringe (both were purchased from a local medical equipment supplier). The cover was tightly sealed onto the chamber with a silicone gasket and a rubber band. The dialysis chamber was placed in a shaking water bath at $37 \pm 1\,^{\circ}C$. The dialyzing solution ($NaHCO_3$) from the reservoir was pumped through the chamber using a peristaltic pump (Eyela, Model MP-3N, Japan). The dialysis flow can be adjusted between 0.5 and 10 ml min^{-1} but 1.0 ml min^{-1} was found to be optimal. Dialyzable components in the peptic digest suspension could pass through the dialysis membrane and collected in plastic collectors.

To perform dialysis, the prewashed [18] dialysis tubing is prepared as above. Before adding the peptic digest, the dialysis tubing is flattened to remove any air bubbles or liquid inside using a syringe connecting to the silicone tube insert. Peptic digest aliquot of 2.5 ml is then injected through the same silicone tube. The dialyzing solution of optimum concentration is flowed through at 1.0 ml min^{-1} or at the required flow rate.

2.2. Instrument and equipment

Determination of calcium by flame atomic absorption spectrometer (FAAS) was performed using a Perkin-Elmer Model 3100 equipped with deuterium background correction (CT, USA), providing a background corrected signal. The operating parameters for measurement of calcium were 422.7, 0.7 nm band width and air–acetylene flame. The calcium contents of the dialysate and digested samples were determined using standard addition method.

A pH meter of Denver Instrument Model 215 (USA) with a glass combination electrode was used for all pH measurements. Commercial standard buffers (Damstadt, Germany) of pH 4.00 ± 0.01 and 7.00 ± 0.01 were employed for the pH meter calibration.

An incubator shaker from Grant Instrument, Model SS40-D2 (Cambridge, England), was used to shake and incubate samples at $37 \pm 1\,^{\circ}C$.

2.3. Chemicals and test materials

Enzymes pepsin (P-7000, from porcine stomach mucosa), pancreatin (P-1750, from porcine pancreas) and bile extract (B-6831, porcine) were from Sigma (St. Louis, MO, USA). Ca standard solution (1000 mg l^{-1}) was a certified NIST standard.

A pepsin solution was prepared by dissolving 0.16 g pepsin (P-7000, from porcine stomach mucosa) in 1 ml of 0.1 M of hydrochloric acid.

A pancreatin–bile extract (PBE) mixture was prepared by weighing 0.004 g pancreatin and 0.025 g bile extract into a beaker and dissolving in 5 ml of 0.001 M sodium bicarbonate. The concentration of sodium bicarbonate in PBE solution was prepared at 0.001 M so that addition of PBE (after 30 min of intestinal digestion) will not disturb the pH change already optimized.

Calcium carbonate tablets (dietary supplement) were from Vitamin World (New York, USA). Milk samples were obtained from a local supermarket.

2.4. Determination the total calcium content in test materials and in residues after dialysis

To determine the total calcium content of each calcium source, the sample (250 mg for tablet and 10.0 g for milk powder) was dissolved by wet digestion with nitric acid to clear solution and diluted to 100.0 ml with pure water. For residues after dialysis, the food suspension after dialysis was transferred from the dialysis tube into a beaker (100 ml) and rinsed with two aliquots (3 ml each) of 0.01 M EDTA washing and two aliquots (10 ml each) of 2% HNO_3 washing before subsequent digestion to clear solution. The calcium contents were determined by flame atomic absorption spectrometry using standard addition method.

2.5. Peptic digestion and determination of titratable acidity

Peptic digestion was performed according to the procedure of Miller [5]. For calcium carbonate tablet, 90 ml of pure water was added to one tablet (250 mg) and the pH was adjusted to 2.0 with diluted HCl. For milk samples, powder milk of 10.0 g and pure water were added into a 100 ml flask and the mixture was shaken to obtain a suspension of 90 ml. The pH was adjusted to 2.0 by addition of a dilute HCl solution. To each sample suspension, 1.5 ml of pepsin solution was added and pH was adjusted again to 2.00 before the total volume was adjusted to 100.0 ml with pure water and the sample was incubated in a shaking water bath at $37\,^{\circ}C$ for 2 h. The pH was adjusted to 2.00 every 30 min.

Titratable acidity of peptic digest was determined by titrating a 2.5 ml aliquot to which 625 μl of PBE mixture was added, using standard 0.01 M NaOH as a titrant to a pH of 7.5.

2.6. In vitro equilibrium dialysis method

A 2.5 ml portion of the peptic digest was added into the dialysis bag in the dialysis chamber. Then, 3.0 ml of dialyzing solution containing an amount of $NaHCO_3$ equivalent to the titratable acidity of the peptic digest was injected into the dialysis chamber to fill the space in the chamber outside of the dialysis bag. The sample was incubated in a shaking water bath at $37\,^{\circ}C$ for 30 min before 625 μl of PBE mixture was added and incubation continued for an additional 2 h.

The dialysate was collected for subsequent determination of calcium content.

2.7. Optimization of flow rate and concentration of sodium bicarbonate for continuous-flow dialysis

Firstly, standard calcium solution in 0.01 M HCl was subjected to continuous-flow dialysis with varying flow rate of sodium bicarbonate. The sample (2.5 ml) was introduced into the flattened dialysis tubing. The dialysis was performed using 0.001 M sodium bicarbonate and the dialysate fractions were collected continuously for calcium determination. The flow rate that gave a complete dialysis in a short time without too much dilution effect of dialysate was considered as optimal.

Then, the optimal sodium bicarbonate flow rate was used to study the effect of its concentration on pH change. Peptic digest samples of varying titratable acidities, including peptic digest containing 0.01 M HCl, peptic digest containing 0.01 M HCl with 0.04 M ascorbic acid, and peptic digest containing 0.01 M HCl with 0.09 M ascorbic acid, were used. These peptic digests had pH values and titratable acidities of 2.0, 0.01 M; 2.0, 0.05 M and 2.0, 0.10 M, respectively. Each sample (2.5 ml) was subjected to the simulated intestinal digestion.

2.8. In vitro dialysis method with continuous flow

To start the simulated pancreatic intestinal digestion, a segment of dialysis tubing was prepared and placed in the dialysis chamber as described earlier. A silicone gasket was placed on the outlet, and the chamber cover was securely clamped. The chamber was connected to the sodium bicarbonate reservoir and the collector containers using tygon tubings and placed in a water bath. A 2.5 ml pepsin digest sample was injected into the dialysis tubing via the silicone tube insert using a syringe. The bath temperature was maintained at $37 \pm 1\,^{\circ}C$. The peristaltic pump was switched on to start the dialysis. The dialysis flow rate was 1 ml min^{-1}. The dialysate was collected at 10 ml intervals in plastic containers for 30 min before a 625 μl freshly prepared PBE mixture was added and dialysis was continued for an additional 2 h. The dialysate fractions were subjected to FAAS measurement after all fractions were collected. Then, the dialyzed amount of an element was calculated by summation of the amounts in all dialysate fractions.

2.9. Calculation of dialyzability

The amount of dialyzed calcium is expressed as a percentage of the total amount present in the sample as follows:

$$\text{Dialyzability}\,(\%) = \frac{(D - B) \times 100}{W \times A}$$

where D and B are the total and blank amounts (μg), respectively, of mineral dialyzed, W the dry weight (g) of sample used for dialysis and A is the concentration of calcium in the dry sample (μg g^{-1}). For equilibrium dialysis, the dialyzed calcium is calculated as twice of the amount dialyzed when the volumes of the peptic digest and the dialyzing solution were equal because the dialyzed amount accounted for only one half of the dialyzable amount in equilibrium dialysis. When the volumes were not equal, correction was made accordingly.

3. Results and discussion

3.1. Design of continuous-flow system for in vitro determination of mineral bioavailability

As the absorption of minerals and trace elements is taking place in the earlier part of the small intestine, simulation of the conditions prevailing in the small intestine is the most critical step for in vitro methods aiming at prediction of the bioavailability of minerals and trace elements. Variation in the pH conditions in the course of intestinal digestion is a major cause of variability of results of dialyzability [13,19–21]. Therefore, this study has given particular attention to the process resulting in pH change and considered it important to provide a pH profile during dialysis corresponding to the dialysis profile. The dynamic in vitro methods developed by Miller [5], Minihane [12] and Shen et al. [13], Wolters et al. [9] and this study have slight differences in the pH change during dialysis and duration of dialysis as summarized in Table 1.

Optimization of the flow rate and concentration of dialyzing solution has been performed to achieve the following requirements for a close simulation of intestinal digestion in human:

1. Change of pH from 2.0 to about 5.0–6.0 in 30 min and to approximately 7.0–7.5 in 60 min and being constant thereafter.
2. Addition of digestive enzymes at required time via a three-way valve.
3. Continuous removal of dialyzed minerals from the dialysis system for determination.

Aiming at the above requirements, firstly a flow rate was selected. Then, the concentration of sodium bicarbonate was optimized.

Although the proposed dialysis system can be connected to the FAAS instrument for online detection, this study chose to collect the dialysate fractionwise (every 5 or 10 min) for subsequent calcium determination. By this way, dialysate samples are not totally consumed and can be kept for pH measurements and for repeated confirmation.

3.2. Selection of dialyzing solution flow rate

The peptic digestion was performed in pepsin solution at pH 2 in a batch system. For intestinal digestion, in contrast to

Table 1
A diagram comparing in vitro gastrointestinal digestion procedures

Item		Time (min) 0 30 60 90 120 150
1. pH adjustment and dialysis process		
(Miller) [5]	Place dialysis bag containing equivalent amount of $NaHCO_3$ in the digestion vessel; dialysis occurs through this dialysis bag	
(Minihane & Shen) [13]	Place a small dialysis bag containing equivalent amount of $NaHCO_3$ in an Amicon stirred cell; dialysis occurs through dialysis membrane at the bottom of the Amicon cell	
(This work)	Flow suitable concentration of $NaHCO_3$ to adjust pH and to continuously remove dialyzable minerals from peptic digest inside a dialysis tube	
2. Addition of PBE (same for all methods)		↓ PBE addition
3. pH change		
(Miller)		2.0 5.0 7.0
(Minihane & Shen)		2.0 7.0 7.0
(This work)		2.0 7.0 7.0
4. Dialysis process and duration of dialysis		
(Miller)	passive diffusion + equilibrium	← →
(Minihane)	diffusion under pressure of 50 psi + continuous removal of dialyzed minerals	← →
(Shen)	diffusion under pressure of 50 psi + continuous removal of dialyzed minerals	← →
(This work)	dialyzing solution flow accelerated diffusion + continuous removal of dialyzed minerals	← →
5. Dialysis membrane		
(Miller)	MMCO 6,000-8,000 Da	
(Minihane)	MMCO 1,000 Da	
(Shen)	MMCO 1,000 Da	
(This work)	MMCO 12,000-14,000 Da	

the equilibrium dialysis method where samples are incubated in sodium bicarbonate solution of sufficient concentration, optimal concentration of sodium carbonate solution was continuously flowed around the dialysis tubing containing peptic digest and the dialysate was collected fractionwise. Thus, the optimal flow rate was selected and the effect of concentration of sodium bicarbonate on pH change during the course of dialysis was studied. In theory, a faster flow rate can facilitate removal of the dialyzed minerals from the system and can speed up the dialysis. However, a fast flow can result in dilution of the dialyzed minerals in the dialyzing solution. The optimal flow rate should assist fast transfer of dialyzable mineral through the membrane and should not cause too much dilution of the dialyzed minerals. The dialysis profiles were obtained at different flow rates as shown in Fig. 2. Dialysis profiles show the kinetics of dialyzable calcium penetrating through the semipermeable membrane for 0.5, 1.0 and 2.5 ml min^{-1} flow rates.

It can be seen that faster flow rate at 2.5 ml min^{-1} could remove dialyzable calcium faster and quantitative dialysis was obtained in about 50 min while slow flow rate at 0.5 ml min^{-1} took a longer time (ca. 100 min) to complete the removal. However, the concentration of calcium in dialysate was lower for the faster flow as a result of dilution effect. Therefore, 1.0 ml min^{-1} flow rate was considered as a compromised flow rate for this application because the dialysis could be completed within 1 h and the dilution effect was acceptable.

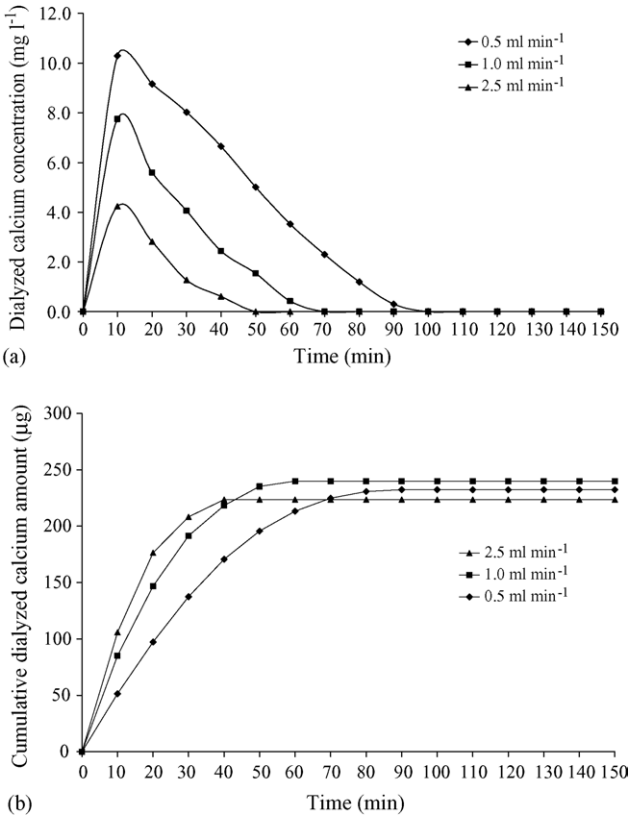

Fig. 2. Profiles of dialyzed amount (a) and cumulative dialyzed amount (b) of calcium at varying flow rate of dialyzing solution. Standard calcium (100 mg l^{-1}) 2.5 ml was used.

3.3. Optimization of concentration of sodium bicarbonate dialyzing solution

The concentration of dialyzing solution has to be optimized to obtain the required pH increase during the course of dialysis. It was found that $NaHCO_3$ concentration of 0.002 M was optimal for peptic digest of calcium carbonate tablets having titratable acidity of 0.05 M (Fig. 3b). The dialysis pH profiles for peptic digest of 0.01 and 0.1 M titratable acidities at various concentrations of dialyzing solution are also

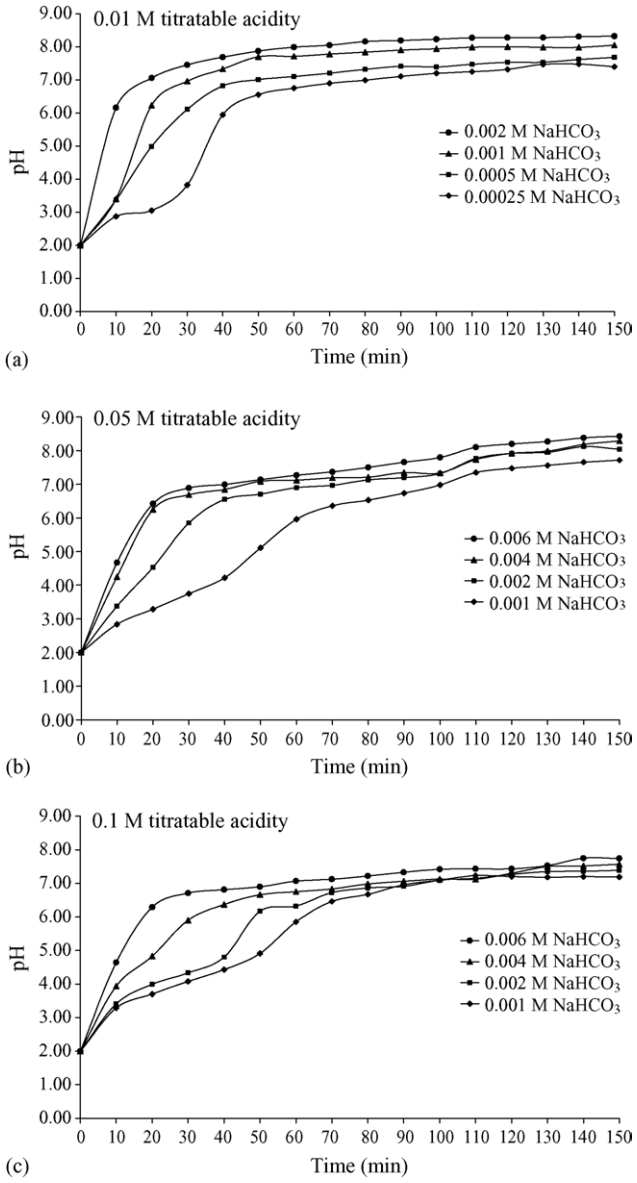

Fig. 3. Effect of concentration of dialyzing solution ($NaHCO_3$) on pH change during dialysis for peptic digests of different titratable acidities. Flow rate 1.0 ml min^{-1}.

Table 2
Analytical recovery of calcium for powder milk sample (three individual replicates are shown)

Sample	Dialyzed		Non-dialyzed		%Recovery
	Amount (mg kg^{-1})	%Dialyzability	Amount (mg kg^{-1})	%Remaining	
Powder milk-based formula	4680	68.1	1800	26.2	94.3
	4500	65.4	2270	33.1	98.4
	4780	69.5	2080	30.3	99.8
Average	4650 ± 140	67.6 ± 2.1	2060 ± 240	29.9 ± 3.4	97.5 ± 2.9

Total calcium 6890 ± 120 mg kg^{-1}.

shown in Fig. 3a and c. From the results of Fig. 3, optimum concentration of dialyzing solution was found to show an approximate linear relationship with the titratable acidity and the following equation can be drawn:

$$\text{Optimum } NaHCO_3 \text{ concentration} = \frac{\text{titratable acidity in M}}{25}$$

The optimal $NaHCO_3$ concentration for more than 10 peptic digests of calcium carbonate tablet samples with different titratable acidities were calculated using the above equation and the pH profiles were found to demonstrate satisfactory pH change during dialysis. So this equation will be used to obtain appropriate concentration of dialyzing solution for calcium carbonate peptic digests. It should be noted that the above equation can be applied for calcium carbonate and other tablets, and may not be applied to other types of food digest. For peptic digests of powder cow milk, the optimum dialyzing solution was found to be one fiftieth of the titratable acidity in M. This difference is probably due to the higher concentration of suspended matter in peptic digest of powder cow milk which resulted in slow rate of mass transfer in the dialysis tube and across the dialysis membrane. Therefore, this should be determined when different new types of sample are to be studied.

3.4. Method validation by analytical recovery study

Since there is no reference materials providing bioavailability data available, validation of the proposed method can only be done by studying of analytical recoveries of the mineral of interest. A milk sample was subjected to the proposed analytical procedure to determine the dialyzable calcium in the dialysate and the non-dialyzable calcium in the retentate. The results are given in Table 2. It can be seen that percent dialyzability of calcium is reproducible and the percent recoveries are acceptable.

3.5. Application of the proposed method to estimate calcium dialyzability of calcium carbonate tablets and milk samples

As examples to show the applicability of the dialysis system developed, calcium dialyzability for calcium carbonate tablets and milk samples was studied. Table 3 shows the results of dialyzable calcium determined by the developed continuous-flow method and the equilibrium method together with some previously reported values.

Since there has not been an accepted standard procedure for in vitro method for estimation of bioavailability, different authors used different procedures or conditions in their work. Furthermore, components in the samples (especially for formulated milk, which may contain different additives)

Table 3
Comparison of percent bioavailability (or dialyzability) of calcium for milk samples and calcium carbonate by different authors and procedures

Sample	In vitro		In vivo	Ref.
	Continuous flow	Equilibrium		
Powder cow milk	42.7 ± 2.5[a]	16.3 ± 1.1[a] (32.6 ± 2.2)	–	This work
Powder milk-based formula	67.7 ± 2.1	–	–	This work
Cow milk	–	20.2 ± 1.4	–	[22]
Cow milk	–	17.0 ± 0.8	–	[18]
Milk-based formula	13.9 ± 2.6[b]	10.2 ± 0.7	–	[13]
Milk-based formula	4.3 ± 0.6[c]	–	–	[13]
Cow milk	–	–	46.3 ± 9.5	[23]
Whole milk	–	–	31 ± 3	[24]
Powder cow milk	–	–	37.4 ± 8.7	[25]
Calcium carbonate	72.8 ± 2.2[a]	32.4 ± 1.5[a] (64.8 ± 3.0)	–	This work
Calcium carbonate	–	–	43.0 ± 5.9	[25]
Calcium carbonate	–	–	39 ± 3	[24]

[a] $n = 3$, in brackets are corrected values as indicated in Section 2.9.
[b] By Shen's method.
[c] By Minihane's method.

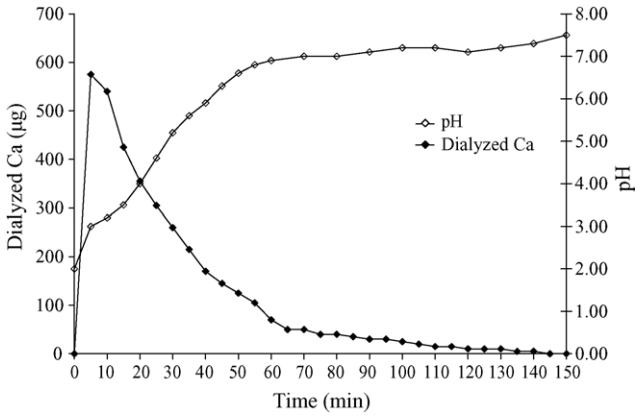

Fig. 4. Profile of dialyzed calcium and the pH change for peptic digest of calcium carbonate tablet by a continuous flow in vitro method.

may inhibit or promote dialyzability. This makes it rather impossible to compare results of different authors.

For milk samples, it was found in this work that dialyzability of calcium in powder cow milk was 32.6 and 42.7% for equilibrium and continuous-flow methods, respectively. The powder milk-based formula has a high dialyzability at 67.7%. Many authors have reported different values of bioavailability of calcium in milk ranging from 4.3 [13] to 46.3% [23]. This probably was attributed to the different sample compositions, procedures and conditions being used. For calcium carbonate tablet, our results of %dialyzability were very high at 64.8 and 72.8 for equilibrium and continuous-flow methods, respectively. Some authors indicated that incomplete disintegration and/or dissolution of the tablet could limit the degree of dialysis [26]. In this study, the peptic digest was seen to be clear after 2 h of peptic digestion at pH 2.0 indicating of solubilization very close to completion. This could possibly be the reason for high dialyzability. The dialyzability for equilibrium method gave slightly lower value probably due to loss during transfer of the dialysate from the dialysis chamber for AAS measurement, the step not required in the continuous-flow method. The in vivo result from a balance study [24,25] reported lower bioavailability values (43.0 and 39%).

Because in vitro dialysis method is a relative rather than an absolute estimation, the use of this method to estimate bioavailability has to be done with careful consideration of dialysis conditions [20,21] and report should serve as a relative evaluation under the same dialysis procedure and conditions. To confirm the similar conditions of dialysis being performed, this work considers monitoring of pH during dialysis a crucial necessity. Fig. 4 shows the dialysis profiles and the pH change during dialysis for calcium carbonate after peptic digestion. The pH profiles demonstrate pH change following the physiological conditions. The dialyzed calcium profiles show maximum value at the first dialysate fraction and gradually lower values similar to the profile of Fig. 2. The dialysis took about 60 min to complete. Similar observation was also evident for milk samples (not shown).

The continuous-flow dialysis profile and pH change are expected to be useful for comparative study of dialyzability of different foods and the study of the effects of food components on dialyzability. Such detailed investigation is not possible using the equilibrium dialysis system.

4. Conclusions

Simulated intestinal digestion has been developed for estimation of nutrient bioavailability. The continuous flow in vitro method is believed to be more representative of in vivo physiological conditions than that based on equilibrium dialysis because dialyzable components are continuously removed from the simulated intestinal digestion system during dialysis. In this study, a simple in vitro continuous-flow dialysis method was developed and used for estimation of calcium availability in comparison with the conventional in vitro equilibrium dialysis method. The most important part for successful simulation of the intestinal absorption was the pH adjustment during intestinal digestion. This was obtained by flowing dialyzing solution of appropriate concentration through a glass dialysis chamber containing the dialysis tubing with peptic digest inside. The optimum conditions for continuous flow in vitro method were a flow rate of 1.0 ml min^{-1} and varying concentration of dialyzing solution ($NaHCO_3$) depending on titratable acidity of the sample. In order that the PBE mixture would not drastically affect the pH change on its addition, PBE was prepared in 0.001 M sodium bicarbonate instead of 0.1 M as in the equilibrium dialysis. As a result, this proposed method achieved a gradual change of pH. The results from continuous-flow dialysis system not only can be used for estimating dialyzability of minerals but also provide dialysis profiles for detailed investigation. Since pH change during dialysis can greatly affect dialyzability due to precipitation for some elements, simultaneous monitoring of the pH change was also performed. The dialysis profiles of dialyzed mineral together with corresponding pH change can help understand the dialysis changes with time and the effect of food components on mineral dialyzability.

Although other elements, elemental detection systems and online measurement can be performed to demonstrate additional advantages of this proposed system, only calcium with a FAAS and off-line detection was attempted to prove the feasibility of the concept in this report. Future studies with online and other elemental detection systems such as inductively-coupled plasma spectrometry will be performed to cover more elements and to show the usefulness of the dialysis profiles obtained.

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References

[1] L.H. Allen, Am. J. Clin. Nutr. 35 (1982) 783.
[2] L. Shackleton, R.A. McCance, Biochem. J. 30 (1936) 583.
[3] B.S. Narasinga Rao, T. Prabhavathi, Am. J. Clin. Nutr. 31 (1978) 169.
[4] S. Lock, A.E. Bender, Br. J. Nutr. 43 (1980) 413.
[5] D.D. Miller, B.R. Schricker, R.R. Rasmussen, D. Van Campen, Am. J. Clin. Nutr. 34 (1981) 2248.
[6] B.R. Schricker, D.D. Miller, R.R. Rasmussen, D. Van Campen, Am. J. Clin. Nutr. 34 (1981) 2257.
[7] T. Hazell, I.T. Johnson, Br. J. Nutr. 57 (1987) 223.
[8] R.F. Hurrell, S.R. Lynch, T.P. Trinidad, S.A. Dassenko, J.D. Cook, Am. J. Clin. Nutr. 47 (1988) 102.
[9] M.G.E. Wolters, H.A.W. Schreuder, G. Van Den Heuvel, H.J. Van Lonkhuijsen, R.J.J. Hermus, A.G.J. Voragen, Br. J. Nutr. 69 (1993) 849.
[10] P. Hocquellet, M.D. L'Hotellier, J. AOAC Int. 80 (1997) 920.
[11] J. Veenstra, M. Minekus, P. Marteau, R. Havenaar, Int. Food Ingredients 3 (1993) 1.
[12] A.M. Minihane, T.E. Fox, S.J. Fairweather-Tait, Proceedings of Bioavailability'93 Nutritional, Chemical and Food Processing Implications of Nutrient Availability, Part II, FRG, 1993, p. 175.
[13] L.H. Shen, I. Luten, H. Robberecht, J. Bindels, H. Deelstra, Lebensm Unters Forsch 199 (1994) 442.
[14] M. Minekus, P. Marteau, R. Havenaar, J.H.J. Huis in't Veld, Altern. Lab. Anim. (ATLA) 23 (1995) 197.
[15] M. Larsson, M. Minekus, R. Havenaar, J. Sci. Food Agric. 74 (1997) 99.
[16] C. Krul, A. Luiten-Schuite, R. Baan, H. Verhagan, G. Hohn, V. Feron, R. Havenaar, Food Chem. Toxicol. 38 (2000) 783.
[17] M. Chu, D. Beauchemin, J. Anal. At. Spectrom. 19 (2004) 1213.
[18] P. Chaiwanon, P. Puwastien, A. Nitithamyong, P.P. Sirichakwal, J. Food Comp. Anal. 13 (2000) 319–327.
[19] D.R. Van Campen, R.P. Glahn, Field Crop. Res. 60 (1999) 93.
[20] C. Ekmekcioglu, Food Chem. 76 (2002) 225.
[21] D. Bosscher, Z.L. Lu, R. Van Cauwenbergh, M. Van Callie-Bertrand, H. Robberecht, H. Deelstra, Int. J. Food Sci. Nutr. 52 (2001) 173.
[22] M.J. Roig, A. Alegria, R. Barbera, R. Farre, M.J. Lagarda, Food Chem. 65 (1999) 353.
[23] R.P. Heaney, C.M. Weaver, S.M. Hinders, B. Martin, P.T. Packard, J. Food Sci. 58 (1993) 1378.
[24] M.S. Sheikh, C.A. Santa Ana, M.J. Niocar, L.R. Schiller, J.S. Fordtran, N. Engl. J. Med. 317 (1987) 532.
[25] M.C. Kruger, B.W. Gallaher, L.M. Schollum, Nutr. Res. 23 (2003) 1229.
[26] M.J. Brennan, W.E. Duncan, L. Wartofsky, V.M. Butler, H.L. Wray, Calcif. Tissue Int. 49 (1991) 308.