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Relative contribution of oxalic acid, phytate and tannic acid on the bioavailability of calcium from various calcium salts - an *in vitro* study

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Abstract

Calcium is important for bone and teeth health. Due to its vital role in human health, people are aware about calcium intake through diet and from other supplements. But, it is not only the amount of intake but actual dose absorbed by the body plays important role. The objective of this study was to determine and compare the amount of bioavailable calcium from various calcium salts like calcium carbonate, calcium citrate, calcium formate, calcium gluconate and calcium lactate by *in vitro* method using equilibrium dialysis after simulating gastric digestion. Selected calcium salt solutions were used as the source of calcium, to which a series of inhibitors like oxalic acid, phytate and tannic acid of calcium absorption were added separately at various concentrations, and the extent of bioavailable calcium was determined. Among the salts studied, calcium citrate registered higher percentage of bioavailable calcium and among the inhibitors, oxalic acid decreased maximum bioavailable calcium in all selected calcium salts.

Introduction

Adequate calcium intake is essential for normal growth and development of the skeleton and teeth, and for adequate bone mineralization. In childhood and adulthood, low calcium intake has been associated with increased risk of osteoporosis, fluorosis (Harrison *et al.*, 1984) and bone fractures (Bailey *et al*., 2010; Kressel *et al*., 2010). The severity of fluorosis seems to be minimized by oral administration of certain nutrients. In support of this suggestion, skeletal effects of fluoride were prevented in experimental animals when calcium was administered along with fluoride. Intake of calcium greater than normal daily requirement may be required to combat the toxic effect of fluoride. Calcium prevents not only hypocalcemia but also the behavioral and dental effects of fluoride in animals exposed chronically to sodium fluoride (Harrison *et al*., 1984). The recommended dietary allowance (RDA) in adults for calcium varies between 800-1300 mg/d for adolescents, depending on the country, and 1000 mg/d for adults rising to 1200 mg/d for the elderly (Reginster *et al*., 1993; Meacham *et al*., 2008; Kressel *et al.*, 2010). Because many modern diets do not provide the recommended levels of calcium, dietary calcium supplements have been recommended for prevention of fluorosis and osteoporosis (Harrison *et al*., 1984; Hanzlik *et al*., 2005; Kressel *et al*., 2010).

Many forms of dietary calcium supplements are widely available, but products containing calcium carbonate and calcium citrate are the most common (Hanzlik *et al*., 2005). Supplements and fortified foods offer alternative sources of calcium to traditional food and in many cases show an improved absorbability (Rafferty *et al*., 2007). However, such alternative sources of calcium need to be evaluated in respect of bioavailability, convenience, taste, and compatibility with foods (Martin *et al*., 2002). Calcium carbonate is generally the least expensive and the most widely- used calcium salt. Data on the bioavailability of calcium salts like calcium citrate, calcium formate, calcium gluconate and calcium lactate are scarce. Bioavailability should be determined by *in vivo* measurements (Van Dyck *et al*., 1996). Ideally, this kind of research should have been performed in humans; however, such studies are difficult, expensive, and provide limited data with each experiment (Hansen *et al*., 1996). While animal assays are less expensive, they are somewhat limited by uncertainties with regard to differences in metabolism between animals and humans. As an alternative to human and animal *in vivo* studies, the availability of minerals or trace elements has also been estimated, based on simple, rapid and inexpensive *in vitro* methods. *In vitro* estimation of the bioavailability of minerals involves the simulation of gastrointestinal digestion and measurement of

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the mineral soluble fraction or the mineral fraction that dialyses across a semi-permeable membrane of a certain pore size. These methods are widely used because of their good correlation with *in vivo* studies. *In vitro* methods have been used to estimate the bioavailability of minerals from different foods and also from dishes and composite diets (Miller *et al.*, 1981; Luten *et al.*, 1996; Camara *et al.*, 2005). Food matrix may contain inhibitors which render some of the nutrients unavailable for human nutrition (Mosha *et al*., 1995). The potent inhibitors like oxalic acid, phytic acid and tannic acid could affect both the calcium naturally present in the food and added calcium (Cashman *et al*., 1996; Gupta *et al.*, 2006).

Nowadays calcium salts are widely used as nutrient supplement, but they must be more bioavailable and compatible. Food industries are considering fortification of foods and beverages with calcium salts for protection against calcium deficiency disorders like osteoporosis, fluorosis etc. This study was undertaken to explore whether calcium supplementation may play a beneficial role against calcium deficiency disorder through enhancement of bioavailable calcium. The aim of this study was to determine and compare the level of bioavailable calcium from various cost effective calcium salts like calcium carbonate, calcium citrate, calcium formate, calcium gluconate and calcium lactate, and to determine the dose response relationships of oxalic acid, phytic acid and tannin acid with calcium bioavailability at different ratios by *in vitro* method.

Materials and Methods

Materials

All chemicals used for the study were AR grade procured from Sigma India (Mumbai, India). They were pepsin, pancreatin and bile salts as digestive enzymes. Calcium carbonate, calcium citrate, calcium formate, calcium gluconate and calcium lactate were used as the calcium source, and other chemicals include oxalic acid, sodium phytate and tannic acid. Dialysis tube was purchased from Hi-Media, Mumbai, India. Millipore - MilliQ distilled water was employed during the complete study.

Evaluation of calcium bioavailability by in vitro *simulated gastrointestinal method*

Bioavailability of calcium in selected calcium salts was determined by an *in vitro* method described by Camara *et al*. (2005). The procedure was followed as gastric stage and intestinal stage.

Gastric stage

Various concentrations of calcium salt solutions were taken, and the pH was adjusted to 2.0 with 6 N HCl. To carry out pepsin–HCl digestion, 0.5 g of pepsin solution per 100 g of sample was added. The mixture was then incubated for 2 h at 37°C in a shaking water bath.

Intestinal stage

A dialysis tube (molecular mass cut-off value 10000 Da) containing 25 ml of water and an amount of $NaHCO_3$ equivalent to the titrable acidity was placed in the flasks, together with 20 g aliquots of the pepsin digest. Incubation was continued for 45 min, the pancreatic-bile salt mixture (5 ml) was added, and incubation was continued up to 2 h. After incubation, the segments of dialysis tubes were removed from the flasks, washed and weighed. The titratable acidity was defined as the number of equivalents of NaOH required to titrate the combined pepsin digest pancreatin–bile salts mixture to pH 7.5. The calcium content of the dialysis tubes were analyzed by atomic absorption spectrophotometry (AAS) (Perkin–Elmer Analyst 100). Instrumental conditions: wavelength = 422.7 nm, slit = 0.7 nm, recommended flame = air-acetylene, oxidizing (lean, blue) and nebulizer = spoiler. Calibration of the instrument was performed using commercial standards. All measurements were carried out using standard flame operating conditions, as recommended by the manufacturer. Bioavailability (%) was calculated as follows: bioavailability (%) = 100 x D/T, where, D is the calcium content in the dialyzable portion for the bioavailable fraction (mg calcium), and T is the total calcium content (mg calcium).

Quality control

To eliminate the risk of contamination, all glasswares used were immersed in 10% (v/v) solution of nitric acid for 24 h, washed with Millipore - MilliQ distilled water before use Accuracy and reproducibility of the method for calcium was checked by adding two levels of known concentration of calcium (10 and 20 mg) to selected calcium salts.

Statistical analysis

All determinations were done in five replicates, and the average values, mean and standard deviation are reported. The data were also analyzed statistically by multiple regression analysis, to determine the extent of variation of calcium bioavailability by oxalic acid, phytic acid and tannic acid using the statistical software SPSS 16 and origin 6.

Table 1. Bioavailability of calcium from various calcium salt solutions at different concentrations

Concentration of calcium (mg)	Calcium carbonate		Calcium citrate		Calcium formate		Calcium gluconate		Calcium lactate	
	Bioavailablity (mg)	%	Bioavailablity (mg)	%	Bioavailablity (mg)	%	Bioavailablity (mg)	%	Bioavailablity (mg)	%
500	138.9 ± 1.5	27.8	201.1 ± 2.2	40.2	102.3 ± 1.7	20.5	181.9 ± 2.1	36.4	134.3 ± 1.3	26.9
600	143.1 ± 1.2	23.9	223.4 ± 2.4	37.2	118.6 ± 1.4	19.8	204.8 ± 2.6	34.1	152.8 ± 1.6	25.5
700	158.8 ± 1.9	22.7	245.8 ± 1.4	35.1	125.8 ± 2.8	18.0	227.4 ± 2.2	32.5	171.3 ± 2.4	24.5
1000	198.8 ± 2.0	19.9	295.4 ± 1.2	29.5	149.4 ± 1.6	14.9	268.7 ± 2.6	26.9	207.8 ± 1.5	20.8
1300	218.6 ± 2.3	16.8	312.5 ± 2.9	24.0	177.8 ± 2.1	13.7	295.7 ± 3.2	22.7	231.8 ± 2.0	17.8

Values are means ± SD of five independent determinations.
Significant correlation (P<0.05) between concentration of calcium and bioavailable calcium
Significant negative correlation (P<0.05) between concentration of calcium and percentage of bioavailable calcium

Results and Discussion

Bioavailable calcium from selected calcium salts

Calcium supplements are used to prevent osteoporosis and fluorosis and to treat calcium deficiencies. In this study, various known concentrations of calcium salt solutions were used as the model system for the determination of bioavailable calcium. From the results bioavailable calcium was found to be the highest in calcium citrate (201.2 mg/L) with an initial concentration of 500 mg/L and followed by calcium gluconate, calcium carbonate and calcium lactate which were 181.9, 138.9, and 134.3 mg/L respectively. Calcium formate had the least bioavailable calcium of 102.3 mg/L (Table 1). Significant correlation (P<0.05) was observed between total calcium intake and the bioavailability of calcium content in the selected calcium salts. Certain studies have demonstrated differences in the bioavailability of various calcium salts, whereby primarily the solubility and ionization of the respective salts were deemed to be the decisive factors for calcium absorption (Pak and Avioli, 1998). However, it has been pointed out that only a weak correlation exists between the water solubility of calcium salts and their intestinal absorption, and that not only ionic calcium but also certain calcium complexes can be absorbed (Hansen and Werner *et al.*, 1996). Calcium solubility does not seem to be the major responsible factor for calcium bioavailability. Heaney *et al*. (1990) found no difference in calcium absorption from seven salts over a range of solubilities in humans. Similarly, Hansen and Werner *et al.* (1996) reported that calcium absorption was not determined only by the solubility of the calcium salt administered in therapeutical doses to healthy volunteers. The bioavailability of calcium from calcium salts appears to be influenced by gastric acid secretion and by simultaneous ingestion of food

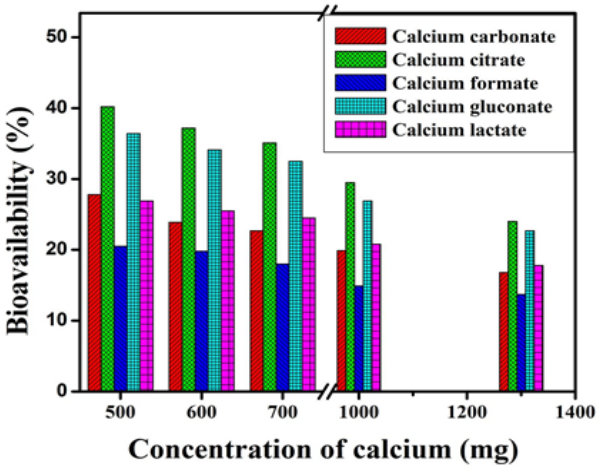

Figure 1. Bioavailability of calcium from various calcium salt solutions at different concentrations

(Kohls, 1991), rather than primarily by its solubility.

The percentage values of bioavailable calcium measured after administration of 500 mg of calcium by *in vitro* method (Table 1) were 40.2% for calcium citrate, 36.4% for calcium gluconate, 27.8% for calcium carbonate 26.9% for calcium lactate and 20.5% for calcium formate. If the initial concentration of calcium in selected calcium salts was increased, the percentage of bioavailable calcium decreased. Calcium citrate, which had the highest percentage of bioavailable calcium decreased from 40.2% to 24.0% when initial concentration of calcium was increased. Significant negative correlations (P<0.05) were observed between total calcium intake and percentage of bioavailability of calcium.

Most experts recommend that supplements be taken with food and that no more than 600 mg should be taken at a time because the percent of calcium absorbed decreases as the amount of calcium in the supplement increases (Institute of Medicine, (IOM) 2010)). The percentage of calcium absorbed depends on the total amount of elemental calcium consumed at one time; as the amount increases, the percentage

Table 2. Effect of inhibitors on the bioavailability of calcium from different calcium salt solutions (500 mg)

Ratio of Ca : Inhibitors	Calcium carbonate		Calcium citrate		Calcium formate		Calcium gluconate		Calcium lactate	
	Bioavailability (mg) & (%)	% of decrease	Bioavailability (mg) & (%)	% of decrease	Bioavailability (mg) & (%)	% of decrease	Bioavailability (mg) & (%)	% of decrease	Bioavailability (mg) & (%)	% of decrease
Oxalic acid										
1 : 0.25	114.5 ± 1.8 (22.9)	17.6	172.6 ± 1.2 (34.5)	14.2	84.8 ± 1.4 (17.0)	17.5	158.4 ± 2.6 (31.7)	12.9	107.4 ± 1.1 (21.5)	20.0
1 : 0.50	107.8 ± 2.2 (21.6)	22.4	160.4 ± 1.8 (32.1)	20.2	78.4 ± 1.7 (15.7)	23.4	144.2 ± 1.9 (28.8)	20.7	101.8 ± 2.1 (20.4)	24.2
1 : 0.75	99.4 ± 1.2 (19.9)	28.4	151.9 ± 2.8 (30.4)	24.5	72.9 ± 1.4 (14.6)	28.8	137.6 ± 2.6 (27.5)	24.4	94.9 ± 1.5 (19.0)	29.3
1 : 1	92.8 ± 1.9 (18.6)	33.2	144.2 ± 1.5 (28.8)	28.3	67.7 ± 1.8 (13.5)	33.8	130.9 ± 1.3 (26.2)	28.0	92.4 ± 1.3 (18.5)	31.2
Phytic acid										
1 : 0.25	118.2 ± 1.3 (23.6)	14.9	178.4 ± 2.2 (35.7)	11.3	87.1 ± 1.8 (17.4)	14.9	162.4 ± 1.2 (32.5)	10.7	112.4 ± 2.1 (22.5)	16.3
1 : 0.50	111.6 ± 1.2 (22.3)	19.7	163.3 ± 1.8 (32.7)	18.8	82.8 ± 1.4 (16.6)	19.1	156.3 ± 2.6 (31.3)	14.1	104.7 ± 1.1 (20.9)	22.0
1 : 0.75	107.6 ± 1.9 (21.5)	22.5	151.7 ± 1.5(30.3)	24.6	76.2 ± 1.6 (15.2)	25.5	149.6 ± 2.2 (29.9)	17.8	97.2 ± 1.7 (19.4)	27.6
1 : 1	102.3 ± 1.8 (20.5)	26.3	148.1 ± 1.7 (29.6)	26.4	71.7 ± 2.4 (14.3)	29.9	142.8 ± 1.6 (28.6)	21.5	94.1 ± 1.3 (18.8)	29.9
Tannic acid										
1 : 0.25	121.4 ± 1.5 (24.3)	12.6	182.6 ± 2.1 (36.5)	9.2	90.1 ± 1.2 (18.0)	11.9	164.6 ± 2.4 (32.9)	9.5	118.8 ± 1.8 (23.8)	11.5
1 : 0.50	117.7 ± 1.9 (23.5)	15.3	177.6 ± 2.4 (35.5)	11.6	85.8 ± 1.8 (17.2)	16.1	159.7 ± 1.6 (31.9)	12.2	107.3 ± 2.2 (21.5)	20.1
1 : 0.75	113.2 ± 1.8 (22.6)	18.5	168.4 ± 1.8 (33.7)	16.3	79.3 ± 2.4 (15.9)	22.5	152.5 ± 2.0 (30.5)	16.2	102.8 ± 1.7 (20.6)	23.5
1 : 1	108.8 ± 2.4 (21.8)	21.7	155.5 ± 1.5 (31.0)	22.7	73.6 ± 1.4 (14.7)	28.1	146.2 ± 1.5 (29.2)	19.6	98.5 ± 1.3 (19.7)	26.7

Values are means ± SD of five independent determinations.
Significant between both bioavailable calcium and percentage of bioavailable calcium and the different ratios of studied inhibitors were at $P< 0.01$ level.

absorption decreases. Absorption is highest in doses ≤ 500 mg. So, for example, one who takes 1,000 mg/day of calcium from supplements might split the dose and take 500 mg at two separate times during the day (IOM, 2010).

Absorbability of calcium form the calcium carbonate and calcium citrate salts was compared at 300 mg and 1000 mg calcium loads ingested as part of a light breakfast meal. Absorption was measured both by tracer appearance in serum and by the absorptive increment in urinary calcium. When taken with food, calcium from the carbonate salts is fully as absorbable as from the citrate (Heaney *et al*., 1999). Singh *et al.* (2008) reported that calcium in fruit yogurt can be increased up to 50 mg calcium per 100 ml by addition of calcium lactate without any negative influence on the organoleptic properties. Addition of calcium caused an increase in colloidal calcium phosphate cross linking between casein micelles which resulted in comparatively high water holding capacity and final apparent viscosity and comparatively less shear thinning when compared with control fruit yogurt. Milk fortified with calcium lactate or gluconate when stabilized with disodium phosphate improved its sensory acceptance, calcium bioavailability and heat stability. Bioavailability of calcium was higher from fortified milk containing organic salts of calcium compared to the inorganic salts (Singh *et al*., 2007). Patwardhan *et al.* (2001) studied to evaluate the effect of extra calcium supplementation in the form of three different calcium salts like calcium carbonate, calcium lactate and calcium phosphate in normocalcemic and hypocalcemic status of the *in vivo* milieu. Among these salts calcium phosphate seems to be a better supplement for the *in vivo* system compared to calcium lactate and calcium carbonate. Hernandez-Munoz *et al.* (2008) studied the effect of chitosan coating combined with postharvest calcium treatment of strawberry fruits. Incorporation of calcium gluconate increased the nutritional value of fruit with altering visual appear. Van Der Hee *et al.* (2009) have designed a calcium fortified ice cream formulation that is lower in fat than regular ice cream and calcium absorption was determined in young adults and compared with milk. Calcium fortified ice cream provides a useful dietary source than can contribute to total daily intake of calcium. Selgas *et al.* (2009) studied calcium lactate, calcium gluconate and calcium citrate have been added to dry fermented sausages to determine whether they are useful for enriching these meat products with calcium. Twenty percentage of recommended dietary allowance (200 mg) calcium salt was the most sensorially adequate, with this amount can be considered a source of calcium. Kruger *et al.* (2003) studied and compared the bioavailability of calcium from skim milk fortified with calcium carbonate or milk calcium using young growing rats. They concluded that calcium fortified food products or milk is a convenient option to increase dietary calcium intake.

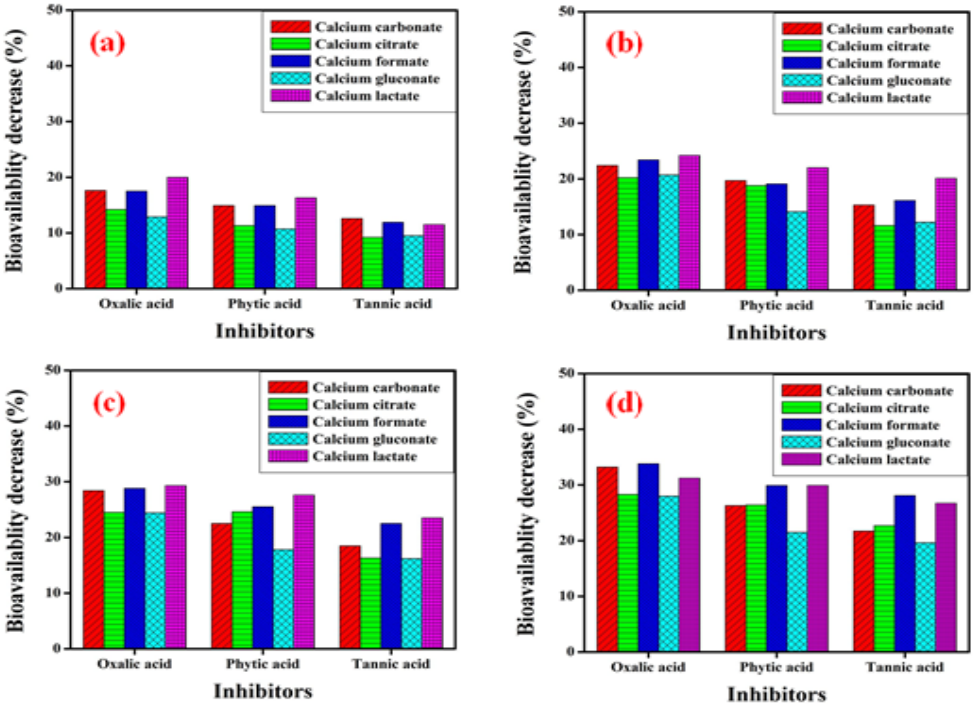

Figure 2. Effect of various ratios between calcium and inhibitors (a) 1:0.25; (b) 1:0.50; (c) 1:0.75 and (d) 1:1 on the bioavailability of different calcium salts

Effect of oxalic acid

Oxalic acid is one of the anti-nutritional factors, which is widely distributed in commonly consumed foods. Oxalic acid is known to interfere with calcium absorption by forming insoluble salts of calcium (Gupta *et al*., 2005). High oxalic acid levels in foods may reduce the bioavailability of such metals particularly calcium. In all selected calcium salts, decrease in percentage of bioavailable calcium was noted when the ratio of oxalic acid was increased (Table 2). Addition of oxalic acid at various doses showed a remarkable reduction in bioavailable calcium. With a further increase in oxalic acid content the inhibition was drastic. The level of percentage of bioavailability reduced greatly from 34.5% to 28.8% in calcium citrate, 31.7% to 26.2% in calcium gluconate, 22.9% to 18.6% in calcium carbonate, 21.5% to 18.5% in calcium lactate and 17.0% to 13.5% in calcium formate. Among the selected calcium salts maximum percentage of decrease in bioavailability was found in calcium formate and followed by calcium carbonate and the minimum percentage of decrease was found in calcium gluconate.

Effect of phytate

Phytate is the major storage form of phosphorus in commonly consumed foods. It is negatively charged under physiological conditions and thus strongly chelates metal ions, especially calcium, forming insoluble complexes in the gastrointestinal tract that cannot be digested or absorbed in humans because of the absence of intestinal phytase enzymes (Iqbal *et al.*, 1994). It also complexes endogenously secreted minerals such as calcium making them unavailable for re-absorption into the body (Abebe *et al*., 2007). With the addition of sodium phytate in all selected calcium salts, a remarkable decrease was observed even at the lowest concentration itself (Table 2). Among the selected calcium salts, percentage of decrease on the bioavailability was found maximum in both calcium formate and calcium lactate (29.9%) and minimum in calcium gluconate (21.5%) and followed by calcium carbonate (26.3%) and calcium citrate (26.4%) with the addition of sodium phytate along calcium salt solutions in the ratio of 1:1 level. Comparison of reduction in percentage of bioavailability showed that it between 35.7% to 29.6% in calcium citrate, 32.5% to 28.6% in calcium gluconate, 23.6% to 20.5% in calcium carbonate, 22.5% to 18.8% in calcium lactate and 17.4% to 14.3% in calcium formate.

Effect of tannic acid

Tannic acid which is a phenolic compound (Khanbabaee *et al.*, 2001), inhibits the activities of digestive enzymes (Rehman and Shah, 2001) and chelate minerals (Lestienne *et al.*, 2005), mainly calcium, interfering in its absorption and, consequently, reducing its bioavailability (Marin *et al*., 2009). Addition of tannic acid at various levels

Table 3. Regression equation and correlation coefficients for the association of bioavailable calcium (X) and ratio of inhibitors (Y) in the selected calcium salts

Ratio of inhibitors (Y)	Regression equation	Correlation coefficient*
Oxalic acid		
1 : 0.25	Y = -12.703+0.9925X	0.997
1 : 0.50	Y = -8.826+0.839X	0.999
1 : 0.75	Y = -13.099+0.820X	0.998
1 : 1	Y = -14.044+0.789X	0.999
Phytic acid		
1 : 0.25	Y = -12.405+0.950X	0.998
1 : 0.50	Y = -8.265+0.870X	0.993
1 : 0.75	Y = -9.058+0.827X	0.986
1 : 1	Y = -12.770+0.821X	0.992
Tannic acid		
1 : 0.25	Y = -7.960+0.946X	0.999
1 : 0.50	Y = -15.413+0.957X	0.996
1 : 0.75	Y = -16.585+0.921X	0.997
1 : 1	Y = -13.252+0.855X	0.995

*Significant ($P < 0.01$)

showed a significant decrease in the dialyzable calcium in all selected calcium salts (Table 2). The percentage of bioavailability was reduced from 36.5% to 31.0% in calcium citrate, 32.9% to 29.2% in calcium gluconate, 24.3% to 21.8% in calcium carbonate, 23.8% to 19.7% in calcium lactate and 18.0% to 14.7% in calcium formate. Among the selected calcium salts maximum percentage of decrease of bioavailability was found in calcium formate (28.1%) and the minimum percentage of decrease was noted in calcium gluconate (19.6%) in 1:1 ratio between calcium and tannic acid.

Multiple regression analysis carried out to explain calcium bioavailability of selected calcium salts and the influence of different ratios of selected inhibitors on bioavailable calcium. Among the inhibitors used, oxalic acid exhibited the maximum inhibitory effect of all the selected salts with high correlation coefficient 0.99 at significant level of $P<0.05$. Other two inhibitors also showed a significant inhibitory effect ($P<0.05$) on various concentrations of selected salts used in this study. Significant correlation between both bioavailable calcium and percentage of bioavailable calcium and the different ratios of oxalic acid were observed at $P< 0.01$ level (Table 3). The results of the effect of inhibitors are in agreement earlier reports in the presence of naturally occurring inhibitors in commonly consumed foods (Cashman and Flynn, 1996; Gupta *et al.*, 2005; Ma *et al*., 2005; Ghavidel *et al*., 2007).

Conclusion

Fluorosis is a global health problem due to high fluoride contamination of water and food. The inhibition of fluoride absorption by calcium would take place through the formation of chemical complex as insoluble CaF_2 from within the intestinal lumen by consuming calcium as supplements. Calcium salts are effective and appropriate as supplements and must be bioavailable. From the results, bioavailable calcium was found to be the highest in calcium citrate and followed by calcium gluconate, calcium carbonate and calcium lactate. Calcium formate had the least bioavailable calcium. This study emphasizes the beneficial effect of enhanced calcium intake by people of fluorotic area to minimize the effects of fluorosis. By this study, it was demonstrated that various ratio of inhibiting factors like oxalic acid decreased maximum bioavailable calcium in all selected calcium salts. Calcium citrate, calcium gluconate and calcium carbonate might be seen as good calcium salts with satisfactory calcium content and calcium bioavailability and serve as good source of the daily calcium intake.

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