Journal of Nutritional & Environmental Medicine (1999) 9, 199–207

ORIGINAL RESEARCH

Citrate: a Component of Bile and Calcium Chelator in Gallbladder Disease

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Citrate, a calcium chelator, was found to be a regular component of bile in the majority of patients with cholelithiasis. The elevation of citric acid levels was investigated in patients with indwelling T-tubes that were indicative of bile duct exploration for retained bile duct stones following cholecystectomy. The concentration of citrate in gallbladder bile in patients at cholecystectomy was variable. The mean (SEM) 95% confidence interval (CI) concentration in gallbladder bile was 57.8 (4.9) 48.2–67.3 with a range of 0–406 μmoles l^{-1}, and in bile duct bile was 39.6 (11.2) 17.5–61.6 with a range of 0–200 μmoles l^{-1}. Gallbladder or common duct bile infected with bacteria with at least one species with a CFU of $\geq 10^5$ recorded significantly lower citrate concentrations. It was not possible in this study significantly to infer that gallbladder bile associated with a particular stone type had an increased or decreased citric acid content. In 7 of 12 patients with indwelling T-tubes owing to choledocholithiasis, an oral citric acid load significantly increased T-tube bile citrate concentrations. This significant outcome prompted the possibility of manipulating citrate levels in bile and the further study of citrate use as an adjuvant in bile acid dissolution of calcified gallstones. Moreover, it is hypothesized that citrate may constitute a naturally occurring anionic chelator of ionized calcium in bile which, when increased in bile and used concurrently with diet management in selected gallstone patients, may augment bile salt dissolution and prevent stone recurrence.

Keywords: bile, calcium, chelator, citrate.

INTRODUCTION

It has been over half a century since citrate was shown to form soluble complexes with calcium [1]. It is a potent complex-forming anion of calcium cations [2]. Citrate is a known inhibitor of the crystallization process of stone-forming calcium salts (oxalate, phosphate) in urine. Moreover, it has also been shown that citrate can inhibit the precipitation of calcium oxalate and calcium phosphate [3–5]. It does so in urine by reducing the urinary saturation of calcium by chelation, thus inhibiting calcium salt nucleation, deposition and crystal growth [6]. Citrate excretion has also been investigated in pancreatic juice. Citrate has been shown to dissolve calcified pancreatic stones both *in vitro* and *in vivo* [7, 8].

We have documented the presence of citrate in bile [9, 10], with a view to it having a role in augmenting the prevention of gallstone formation, as well as being a useful adjuvant in the medical dissolution of calcified gallstones which are resistant to bile acid therapy, as

ISSN 1359-0847 print/ISSN 1364-6907 online/99/030199-09 ©1999 Taylor & Francis Ltd

TABLE 1. Site of origin of bile samples

Site of origin	Bile designations	Number (%)	Total
Gallbladder	Functional[a]	131 (42.6)	
	Infected[b]	22 (7.1)	166
	Non Functional[c]	13 (4.2)	
Common bile duct	Normal[a]	25 (8.1)	
	Infected	4 (1.3)	34
	Dilute[c]	5 (1.6)	
Duodenum	Normal	62 (20.1)	86
	Dilute	24 (6.5)	
T-tube	Norma	19 (6.2)	22
	Infected	3 (0.9)	
Totals		100%	308

[a]Bile salt concentrations >20 mmol l^{-1}. [b]Bile salt concentrations >20 mmol l^{-1} and infected $\geq 10^5$ CFU/ml of bile. [c]Bile salt concentrations <20 mmol l^{-1}.

well as their recurrence. This study presents comprehensive data on the levels of biliary citrate observed *in vivo* in patients with different types of gallstone, and on the effect of an oral dose of citrate on the plasma, urinary and biliary loads normally encountered.

PATIENTS AND METHODS

Patients

Two groups of patients participated in this study. The first group consisted of 166 patients with varying types of gallstone disease (Table 1), admitted to the Repatriation General Hospital, Heidelberg, Australia for elective cholecystectomy and operative cholangiogram. Of these, 22 patients had indwelling T-tubes inserted into their bile ducts because their ducts had been explored. The second group consisted of 86 patients undergoing medical bile salt (CDCA) dissolution of gallstones at the University Department of Surgery at the Repatriation General Hospital, Heidelberg, Australia.

Methods

In order to obtain basal *in vivo* levels of biliary citrate, the first estimates of citrate levels were carried out on a number of bile aspirates as outlined in Table 1. To test *in vivo* citrate levels following an oral dose of citrate, post-cholecystectomy patients with inserted T-tubes were used. All patients were fasted overnight (for at least 10 h) prior to the commencement of the experiment. T-tubes had to be clamped and the integrity of the enterohepatic circulation restored for at least 24 h before a patient could participate. Patients were tested over two consecutive days. Oral citrate loads were administered on a given set day (day 1 or 2), with the control day the other day, where a glass of water was given orally as an alternate liquid. The oral citrate loads investigated were doses of 17.5, 35 and 50 mmol l^{-1}. In addition to estimating citrate levels in bile samples, plasma and urinary citrate levels were also concurrently estimated. All patients participating in the oral citrate load experiment were monitored for biochemical and haematological side effects of citrate, before and after the citrate load, namely, full blood examination, liver function tests, urea and electrolytes, calcium, chloride gap, alpha amylase and alanine transaminase. The enzymatic method for the determination of citrate in biological fluids was that adapted from Toftegaard–Nielsen [11]. The principle of the method is that based on the catalytic action of the enzyme citrate lyase (Fig. 1). In the presence of the enzymes malate dehydrogenase (MDH) and lactate dehydrogenase (LDH) reduced nicotinamide adenine dinucleotide

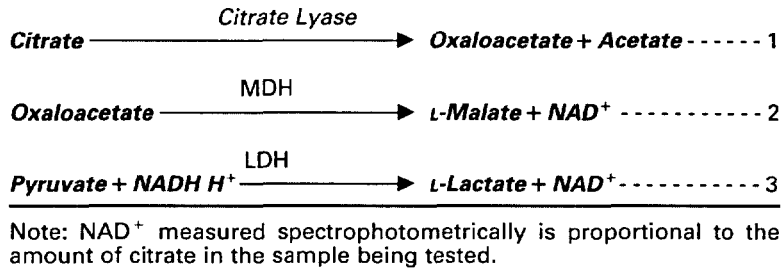

FIG. 1. Enzymatic reactions associated with the determination of citrate in biological fluids.

(NADH) is oxidized to NAD^+. The amounts of NADH oxidized in reactions 2 and 3 in Fig. 1 are stoichiometric with the amount of citrate. Thus NAD^+ was measured spectrophotometrically at a wavelength of 365 μm. The quantity of citrate was determined from a standard curve. Citrate was used as standard and 0.05, 0.1 and 0.2 mmol l^{-1} concentrations prepared. Bile with a known concentration was used as a control. Bile samples were stored at $-20°C$ for no longer than 5 days prior to assay.

Reagents

All reagents for the chemical determination of citrate were of analytical grade. The citric acid solution given to the patients was prepared by the hospital pharmacy and consisted of 240 g citric acid, 56 g of trisodium citrate, 53 g tripotassium citrate, and water added to 1000 ml. The patient's dose was 10.5, 21 and 30 ml of mixture in a glass of fruit juice, producing a citrate equivalent of 17.5, 35 and 50 mmol l^{-1} dose.

Bile Culture

Initially, bile samples were examined for bacteria by Gram's stain. Bile samples were then directly plated on to sheep blood agar, McConkey's agar, horse blood agar, lysed horse blood with vancomycin and vitamin K, Nagler's agar, phenyl-ethyl alcohol agar and cooked meat medium, these last five being incubated anaerobically in jars flushed with a commercial gas mixture of nitrogen, oxygen and carbon dioxide in a 8.5:0.5:1 proportion respectively. All plates were incubated at 35°C for 5 days and examined daily for growth.

Total Bile Salts

Total bile salts on bile samples were enzymatically determined by the method adopted by Admirand and Small (1968) [12,13]. Gallbladders with a total bile salt concentration of less than 20 mmol at cholecystectomy were considered to be poorly functioning or non-functional. Similarly, bile samples from duodenal aspirates were considered dilute, when the total bile salt concentrations were estimated to be less than 20 mmol (Table 6).

Statistical Analysis

Comparisons between groups of data were analyzed for significance using a student's t-test. As used in the test, p values of less than 0.05 were significant. The data where relevant were

TABLE 2. Mean (μmol l^{-1}) citrate levels from gallbladder, bile duct and duodenal bile samples

Bile Type	Number	Mean	SE	95% CI
Gallbladder Bile	131	57.8	4.9	48.2–67.3
Infected	22	12.6	3.4	6.0–19.3
Dilute	13	37.9	15.8	6.8–69.0
Common Bile Duct	25	39.6	11.2	17.5–61.6
Infected	5	26.0	8.2	9.8–42.2
Dilute	4	59.0	42.7	−25.0–143.0
Duodenal	62	38.9	7.4	24.4–53.4
Dilute	20	60.3	17.5	25.8–94.8

expressed as a mean (SEM). The statistical package for the social sciences (SPSS) was used to analyze the data.

RESULTS

Citrate in Gallbladder Bile

The concentration of citrate in gallbladder bile duct biles and ranged from a low value of 0 mmol l^{-1} to a high value of 406 mmol l^{-1}. The bile samples from 131 functioning gallbladders at cholecystectomy provided estimates of biliary citrate concentrations that could be observed *in vivo* in the gallbladder, at any one time. The calculated mean total bile salt concentrations from gallbladders containing large cholesterol solitaire or black pigment gallstones were higher than for any other type of gallstone. However, the respective mean citrate concentrations did not follow a proportional relationship to the concentration of total bile salts measured (Table 2). Gallbladder bile citrate concentrations, though, were consistently higher from functional gallbladders harboring cholesterol solitaire or black pigment gallstones. Hence gallbladder bile citrate levels were variable among different gallstone types as well as different total bile salt concentrations. However, there was no apparent relationship between gallstone type, total bile salts in gallbladder bile and the bile citrate concentration.

Citrate and Duodenal Aspirated Bile

Citrate concentrations estimated in gallbladder bile obtained at cholecystectomy were comparable to the estimates obtained from duodenal aspirated bile, whose concentration range was from 0 to 380 mmol l^{-1}. The individual mean concentrations of citrate for gallbladder, bile duct and duodenal bile are presented for comparisons (Table 2). The citrate concentrations again were not constant, but were highly variable within a specific bile type, or between different groups of gallbladder bile. On comparing different types of bile sample obtained under different conditions, it was observed that bacterial infection of gallbladder bile was associated with significantly low citrate concentrations, when a comparison was made with other bile samples from different groups.

Citrate and Post-cholecystectomy T-tube Bile

Three different citrate concentrations were tested with the T-tube patients, namely, 17.5 (patients 1 and 2), 35 (patients 3 and 4), and 50 mmol l^{-1} solutions (patients 5–12) (Tables 3, 4 and 5). The level of citrate was first determined concurrently in the plasma and urine of the T-tube patients. All patients taking an oral citrate load recorded significant increases

TABLE 3. Plasma citrate concentrations in T-tube patients

Patient no.	Citrate load Given (mmol l^{-1})	Day	Sample time 0 h	Sample time 2 h
1	17.5	1*	0.068	0.130
		2	0.081	0.088
2	17.5	1	0.030	0.025
		2*	0.026	0.073
3	35.0	1*	0.040	0.149
		2	0.030	0.030
4	35.0	1	0.035	0.029
		2*	0.023	0.127
5	50.0	1*	0.020	0.030
		2	0.066	0.063
6	50.0	1	0.045	0.041
		2*	0.045	0.106
7	50.0	1*	0.049	0.096
		2	0.032	0.049
8	50.0	1	0.056	0.034
		2*	0.063	0.102
9	50.0	1*	0.036	0.058
		2	0.030	0.030
10	50.0	1*	0.018	0.031
		2	0.010	0.005
11	50.0	1	0.020	0.025
		2*	0.030	0.102
12	50.0	1*	0.060	0.128
		2	0.030	0.035

*An orally administered citrate load was given on day 1 or 2 post collection of 0 h sample. On the alternate day water was given as a substitute.

in their plasma and urine levels respectively (Tables 3 and 4). Seven out of 12 patients with T-tubes showed a significant positive response in their bile to the oral citrate load, recording significantly elevated levels of citrate (Table 5). No abnormal biochemical side effects were detected in any patient on any day of the test.

DISCUSSION

Citrate is an important component of biological fluids. Its presence is extensive in human tissues. Previous studies have confirmed that it is also present in human bile [9]. Clinically, citrate (as the alkaline potassium salt) has been extensively employed in patients with calcium nephrolithiasis, whereby its administration has effected successful long-term management [14–17].

Calcium salts of bilirubin, carbonate, phosphate and palmitate have all been detected in varying amounts in gallstones [18–21]. Calcium precipitation is a crucial event in the nucleation and growth of gallbladder and bile duct stones, as exemplified by the variable distribution of calcium salts from the nuclear areas of cholesterol gallstones to the more dispersed distributions in most brown and black pigment gallstones [18]. Calcium in bile has been reported to be present partly bound and partly in a soluble and ultra-filterable form [22]. Further, it has been postulated that calcium ions may be in equilibrium complexed to a number of bile components that include bile salts, mixed micelles of bile salts and phospholipids, glycoproteins and albumin [23–25]. Ionized calcium levels in bile are directly related to the pH and the 'anaerobicity' and carbon dioxide content of the bile fluid. A useful addition to this study would have been to measure bound and unbound calcium

TABLE 4. Urine citrate concentrations (mmol l^{-1}) in T-tube patients

Patient no.	Citrate load Given (mmol l^{-1})	Day	Sample time 0 h	1 h	2 h	4 h
1	17.5	1*	0.94	1.92	1.88	2.22
		2	1.50	0.82	0.80	0.74
2	17.5	1	1.24	1.60	1.40	1.40
		2*	1.36	1.58	1.42	2.42
3	35.0	1*	1.30	1.50	2.10	2.60
		2	1.25	1.30	1.31	1.34
4	35.0	1	1.40	1.60	3.70	2.60
		2*	1.30	1.59	1.78	2.45
5	50.0	1*	0.80	0.90	1.10	1.00
		2	2.10	2.40	2.50	2.30
6	50.0	1	3.30	3.30	3.10	2.90
		2*	3.60	3.80	4.80	5.30
7	50.00	1*	–	7.80	7.80	7.30
		2	6.90	–	5.40	5.60
8	50.0	1	3.00	–	3.60	2.90
		2*	–	3.20	6.42	–
9	50.0	1*	2.80	4.20	4.30	4.30
		2	2.20	3.50	3.30	2.50
10	50.0	1	0.40	0.07	1.00	0.09
		2*	1.50	1.80	2.10	2.30
11	50.0	1*	5.70	5.70	6.50	7.40
		2	3.50	3.80	–	–
12	50.0	1	1.80	1.90	2.50	3.50
		2*	3.20	3.70	4.50	4.40

*An orally administered citrate load was given on day 1 or 2 post collection of 0 h sample. On the alternate day water was given as a substitute.

in our bile samples, supplemented with pH bile values. However, insufficient bile sample collections from the patients' T-tubes as well as the inappropriate facility for measuring the pH by the patient's bedside were limiting factors that precluded a detailed analysis of calcium in bile in this study. We have, however, previously examined citrate levels in model bile solutions and have positively measured their concentration effects [10]. This study endeavored, then, to show changes in citrate levels as measured by a pre- and post-oral citrate load analysis on bile directly emanating from a T-tube inserted into patients' bile duct systems for suspected bile duct calculi.

To assess citrate levels *in vivo* further, bile with gallstones that represented all the major types as previously documented were first investigated in this study [20]. Corresponding gallbladder bile–citrate concentrations showed that usually cholesterol solitaire stones and black pigment gallstones had biliary citrate concentrations that were regularly at the higher end of the concentration range, when compared to bile harboring multiple cholesterol or brown pigment gallstones. However, it was not possible in this study significantly to infer that gallbladder bile associated with a particular stone type had increased or decreased citric acid content.

We observed that biliary citrate had a complex distribution *in vivo*; we then designed an intervention that would investigate orally administered citric acid output in bile. The intention was to elevate biliary citrate levels with an acute (fasting) citric acid load, orally administered to post-cholecystectomy patients with indwelling T-tubes. Seven out of 12 patients registered a significantly ($p < 0.05$) increased elevation of T-tube bile citrate concentration at some time during the study period, following the oral dose. Failure to elevate the biliary citrate levels in the other 5 patients was not known. However, the reported intolerability to the citrate beverage led to reports of copious consumption of

TABLE 5. T-tube bile citrate concentrations (mmol l^{-1}) in T-tube patients

Patient no.	Citrate load given (mmol l^{-1})	Day	Sample time 0 h	1 h	2 h	4 h
1	17.5	1*	0.0	0.015	0.010	0.073
		2	0.0	0.0	0.012	0.0
2	17.5	1	0.0	0.0	–	0.0
		2*	0.0	0.036	0.008	0.020
3	35.0	1*	0.0	0.010	0.025	0.0
		2	0.0	0.0	0.0	–
4	35.0	1	0.0	0.001	–	–
		2*	0.004	0.040	0.050	0.01
5	50.0	1*	0.019	0.029	0.014	0.016
		2	0.0	0.0	–	–
6	50.0	1	0.044	0.058	0.044	0.037
		2*	0.055	0.039	0.092	0.078
7	50.00	1*	0.015	0.036	0.018	0.009
		2	0.017	0.0	0.017	0.011
8	50.0	1	0.0	0.002	0.001	–
		2*	0.0	0.034	0.048	0.001
9	50.0	1*	0.0	0.003	0.038	0.013
		2	0.001	0.01	–	0.0
10	50.0	1	0.0	0.0	0.013	0.001
		2*	0.0	0.001	0.025	–
11	50.0	1*	0.015	0.015	0.051	0.077
		2	0.001	0.002	–	–
12	50.0	1	0.005	0.005	–	–
		2*	0.014	0.104	0.095	0.215

*An orally administered citrate load was given on day 1 or 2 post collection of zero hour sample. On the alternate day water was given as a substitute.

water, which may have resulted in dilution of the initial citric acid concentration, as well as possibly increasing its rate of excretion in the urine. An examination of the citrate concentration of the respective plasma and urine samples showed that citrate was being absorbed into the bloodstream; however, high concentrations of citrate were observed as being excreted in the urine. It was noted that no gross biochemical side effects were observed in any patient following citrate ingestion over a short period of time.

The successful elevation of the levels of biliary citrate in T-tube bile invoked the possibility of its use as an adjuvant in bile acid dissolution therapy of gallstones in some patients, given that medical dissolution with chenodeoxycholate (CDCA) and

TABLE 6. Gallstones total bile salts and biliary citrate from functional gallbladders

Gallstone type	Total bile salts mean (SEM) mmol l^{-1} (range)	Gallbladder citrate mean (SEM) mmol l^{-1} (range)
Cholesterol single	125.6 (13.7)	44.4 (59.0)
$n = 10$	(22.5–237.5)	(0–262.0)
Cholesterol multiple	72.4 (4.3)	31.1 (55.0)
$n = 86$	(20.0–224.3)	(0–406.0)
Brown pigment	100.6 (11.2)	19.6 (23.4)
$n = 13$	(30.7–170.2)	(0–69.0)
Black pigment	150.8 (12.0)	33.5–(68.9)
$n = 24$	(74.0–261.9)	(0–326.0)

ursodeoycholate (UDCA) have proved to be deficient [22–24]. CDCA has been reported to be ineffective with calcified cholesterol gallstones and UDCA effective in calcifying previously radiolucent cholesterol gallstones, thus rendering further dissolution ineffective [26–28]. A preliminary study which has been completed has shown that a combined CDCA and citrate regime was successful in partial or complete dissolution of gallstones in 12 out of 20 patients tested [29].

Although we have shown that citrate is a normally occurring component of bile in the majority of patients with gallstones, significant ethical issues precluded obtaining bile from patients with no gallstone disease. Thus the citrate concentrations in 'normal' bile remained elusive. Notwithstanding this outcome, we strongly believe that citrate is also a normally occurring component of alithogenic bile. Citric acid is known as a chelating anion in solution that allows it to form a soluble complex with ionized calcium. Moreover, the bond between calcium ions and citric acid is a very stable one with a reported [2] formation constant of 1.9×10^3 with a Pk_3 for citric acid of 5.48. Thus, citrate and calcium ion complexes become relevant at a pH above 4.5, which is in accordance with known estimates of gallbladder bile pH [10]. The consequence of this could be that citrate may afford bile with the capacity to buffer ionized calcium in solution. Bile already contains a number of calcium buffers that reduce the potential of calcium precipitation, such as bile salts, conjugated bilirubin, bicarbonate ions and proteins such as mucin [30–33]. Although citrate was present in micromolar concentrations in bile, successfully elevating its concentration in bile via an oral dose may constitute a way of easily increasing its potential hypocalcibiliary effect. Acting in conjunction with other biliary calcium buffers, citrate may reduce gallstone dissolution resistance and recurrence.

Over a decade ago it was proposed that vegetarianism was protective against gallstones [34]. This was consistent with studies which confirmed that obesity and high calorie intake constituted a risk for gallstone disease [35]. The consumption of vegetables and fruit may favor calcium solubility in bile, by neutralizing, at least in part, the potential nucleating capacity of ionized calcium [36, 37]. More recently, nephrologic studies have reported that decreases in vegetable fiber intake may lead to low citric acid levels in the urine, thereby further increasing the risk of formation of calcium nephrolithiasis [37, 38]. Dietary manipulation with a lemonade beverage consisting of reconstituted lemon juice (from lemon pulp) in 2 l of water was used to treat hypocitraturic acid nephrolithiasis [38]. Whether citric acid, as a regular component of certain fruits and vegetables [39], contributes with other unknown compounds as an aid to blocking gallstone nidation through an additional solubilization effect on calcium ions in bile remains as yet inconclusive.

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