

A Quantitative Method to Detect Fucoidan in Human Plasma Using a Novel Antibody

M.R. Irhimeh¹, J.H. Fitton^{1,2}, R.M. Lowenthal¹ and P. Kongtawelert³

¹Haematology/Oncology Unit, Royal Hobart Hospital, and Discipline of Medicine, University of Tasmania, Hobart, Tasmania, Australia; ²Marinova Pty. Ltd., Hobart, Tasmania, Australia; ³Thailand Excellence Centre for Tissue Engineering, Department of Biochemistry, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand

SUMMARY

We have developed an antibody-based method to assess plasma uptake of a proprietary *Undaria*-derived fucoidan galactofucan sulfate (GFSTM) after oral ingestion by human volunteers. Fucoidans have high-molecular-weights but exert biological effects in experimental animals after oral intake. By using a novel antibody raised against sulfated polysaccharides, we carried out a competitive ELISA to quantitate GFS in plasma samples from healthy volunteers who ingested 3 g/day of whole *Undaria* containing 10% GFS fucoidan, purified 75% GFS fucoidan, or 3 g of a nonsulfated placebo polysaccharide over 12 days. Increased reactivity to the novel antibody, as measured against preingestion levels, was detected at all time points. Assuming the measured material to be intact GFS, the concentration detected (median) was 4.002 and 12.989 mg/l when 3 g of 10% or 75% pure fucoidan was ingested orally over a period of 12 days, respectively. High-molecular-weight fucoidan can be detected in plasma using an ELISA competitive assay based on a novel antibody to sulfated polysaccharides. © 2005 Prous Science. All rights reserved.

Key words: Competitive ELISA - Fucoidan - Galactofucan sulfate - Heparin - 1B1-mAb

INTRODUCTION

Sulfated fucans constitute a class of polysaccharides first isolated in 1913 from marine brown algae. The sulfated fucans are often called fucoidans, which are fucose-rich sulfated carbohydrate polymers found in brown seaweeds, such as *Undaria* sp. and *Laminaria* sp., common in the Japanese diet as Wakame and Kombu (1, 2). Fucoidan extracts may have therapeutic benefits and are relatively nontoxic compared with other sea-derived substances. All research done on fucoidan showed that it is nontoxic, nonallergenic, has no harmful effects on any bodily function or organ, and is considered completely safe (2). The type of fucoidan, its sulfation, molecular weight, and conformation of sugar residues vary with the species of seaweed (3). Fucoidans are considered to have similarities with the much smaller mammalian molecule heparin, and modulate biological processes such as inflammation and resistance to viral infection (4) and coagulation (1).

It has recently been shown that intravenous fucoidan administration produces rapid mobilization of murine hematopoietic progenitor cells with long-term bone marrow repopulating potential. Fucoidan administration similarly increased circulating mature white blood cells and hematopoietic progenitor/stem cells in mice and nonhuman primates (5-7). Orally administered fucoidans have biological effects (8, 9).

Recently the search for new drugs has raised increased interest in fucoidans. In the past few years, several structures of algal and invertebrate fucoidans have been resolved, and many aspects of their biological activity have been elucidated. New methods have been developed to detect different polysulfated polysaccharides, including chondroitin sulfate (10, 11), dermatan sulfate, and hyaluronan (12), from urine and blood plasma samples after oral and intravenous routes of administration. Thus, the development of a rapid, accurate, and sensitive method to determine fucoidan found in biological samples, such as blood plasma and urine, is a key requirement for the use of these sulfated sugars in the production of new drug therapies.

The uptake of fucoidan from oral ingestion has not been assessed to date. High-molecular-weight materials are not considered absorbable. However, Structum[®], a high-molecular-weight chondroitin sulfate preparation (17 kDa), is accepted as a treatment for arthritis. The uptake of nondegraded chondroitin sulfate occurs across the small intestine, probably *via* endocytosis (13). Similarly, heparin can be delivered orally, and has a low but detectable uptake (14).

In an effort to understand clinically observed effects of fucoidan in humans, we wanted to assess the uptake of *Undaria* fucoidan after oral administration. We have developed a new monoclonal antibody (1B1) that

possesses reactivity against polysulfated polysaccharides. The antibody reacted with heparin and semisynthetic heparinlike substances, including dextran sulfate, pentosan polysulfate, and glucoseaminoglycan polysulfate (15). The aim of this current study was to develop a simple technique (microassay) to determine the concentration of fucoidan in plasma using this antibody.

MATERIALS AND METHODS

Materials

We used protamine sulfate (Salmon) from Aventis Pharmaceuticals Inc., New Jersey; heparin from Leo Pharmaceuticals, Ballerup, Denmark; microtiter nunc-immuno plate maxisorp surface from NalgeNunc International, Denmark; bovine serum albumin (BSA), pronase E, sigma fast *o*-phenylenediamine dihydrochloride tablet sets, and heparin from Sigma-Aldrich Co., St. Louis, Missouri; phosphate buffer saline Dulbecco-A (PBS) from Oxoid Ltd., Hampshire, England; tween-20 from BDH, Poole, England; peroxidase-mouse monoclonal antibiotin antibody from Zymed Laboratories Inc., South San Francisco, California; CM Affi-Gel Blue Gel and poly-prep chromatography columns from Bio-Rad Laboratories, Hercules, California; and guar gum fibers (benefiber) from Novartis Pty. Ltd., Victoria, Australia. All other chemicals were of analytical grade.

Volunteers

Healthy human volunteers of either sex, aged between 20–46 years (average 29) were chosen after advertising. All volunteers were nonsmokers with no history of thyroid abnormalities or sulfur allergy and had not received medication (including heparin) in the 30 days prior to commencing the study and had not taken food supplements or consumed seafood in the 7 days prior to commencing the study. Informed consent was obtained from all volunteers after human ethics approval was obtained from the Southern Tasmania Health & Medical Human Research Ethics Committee.

Volunteers were divided into three groups. The first group of volunteers ($n = 6$) took 3 g of guar gum as placebo. The second group ($n = 6$) took 3 g of Galactofucan sulfate (GFSTM) containing 10% fucoidan. The third group ($n = 40$) took 3 g of GFS containing 75% fucoidan daily for 12 days. All volunteers took three capsules thrice daily at the same time each day. During

the study time, volunteers were asked not to eat any sort of seafood or food containing seaweed-derived products, and they did not take any drugs or food supplements. Blood samples from the three groups were collected as described later.

Preparation of capsules

Three different types of capsules were prepared. The first contained dietary fibers derived from guar gum. The other two types contained seaweed extract GFS 10% and 75% pure fucoidan. The GFS is a high-molecular-weight fucoidan derived from Tasmanian *Undaria pinnatifida* by Marinova Pty. Ltd. (Hobart, Tasmania, Australia). The structures of these compounds were described previously (16-19). The guar gum and GFS were filled in sterile gelatinous capsules under aseptic conditions. A special encapsulator was used in the filling process. Each capsule contained 0.33 g. The capsules were carefully weighed to ensure they conformed to the 0.33-g standard. The therapeutic characteristics of the polysaccharides used in the study are shown in Table 1 (16, 17).

Blood sample collection

Citrated venous blood was collected from the three groups of volunteers and centrifuged at 1500 g for 5 min. Plasma fractions were collected and stored in aliquots at -80°C within 30 min of collection for later analysis. Some of these samples were pretreated using pronase enzyme or purified using affinity chromatography, as described later, in an attempt to increase the antibody 1B1 sensitivity in the fucoidan assay. Other samples were tested directly for fucoidan.

Blood parameters

Complete blood count was obtained using an automated cell counter (CELL-DYN 4000 System, Abbott Laboratories, Abbot Park, Illinois). Venous blood samples were collected after an overnight fasting for monitoring blood glucose level during the study, using glucose oxidase colorimetric method (Vitros-950 Chemistry System, Johnson & Johnson Clinical Diagnostics, Inc., New York).

Pronase-treated plasma samples

Citrated plasma samples were mixed in equal volumes with pronase (1 g/l) and incubated at 37°C overnight. The digested samples were boiled for 3 min

TABLE 1. General characteristics of the therapeutic polysaccharides administered orally.

| Polysaccharide | Sugar type | Mean MW (kDa) | Type of linkage | % SO ₄ |
|--------------------------------|----------------------|---------------|---|-------------------|
| Guar gum (dietary fibers) | Galactomannan | 220 ± 20 | Backbone: (1→4)-β-D-mannopyranose; branches: 1→6-linked-α-D-galactopyranose | 0 |
| GFS TM 10% fucoidan | Galactofucan sulfate | 713 | L-fucose linked 1→3 and 1→4, and C-2 or C-4 is sulfated | 29.07 |
| GFS TM 75% fucoidan | Galactofucan sulfate | 713 | L-fucose linked 1→3 and 1→4, and C-2 or C-4 is sulfated | 29.07 |

in a boiling water bath and cooled in an ice bath, and then centrifuged at 2500 g for 10 min. The resulting supernatants were collected as pretreated samples for a further assay (11, 15).

Affinity chromatography

A bifunctional affinity/ion exchange chromatography matrix (CM Affi-Gel Blue Gel) that binds both albumin and serum proteases was used to remove these proteins and purify plasma samples rapidly. The gel was prepared and used according to the Bio-Rad instruction manual. Briefly, the gel was washed in a column with 5 bed volume of prewash buffer (0.1 mol/l acetic acid, pH 3, 1.4 mol/l NaCl, 40% isopropanol), followed by 7 bed volumes of deionized water. The gel was then washed with 2 bed volumes of 1.4 mol/l NaCl and then with running buffer (10 mmol/l K₂HPO₄, pH 7.25, 0.15 mol/l NaCl). It was then packed into a poly-prep chromatography column. The plasma sample was applied and the column was washed with 2 bed volumes of running buffer. The effluent from this step was then collected in fractions in Eppendorf tubes for further analysis.

To use the gel again, the albumin was eluted from the column with 2 bed volumes of 1.4 mol/l NaCl in running buffer, and the column was regenerated with 2 bed volumes of regeneration buffer (2 mol/l guanidine HCl), followed by 2 bed volumes of running buffer.

Preparation of 1B1 monoclonal antibody

The novel biotinylated monoclonal antibody IgM isotype was prepared as described previously (15). Briefly, this antibody was developed against protamine sulfate, heparin, and heparinlike substances by immunizing Balb/c mice with protamine sulfate and polyolpoly-sulfate. The monoclonal antibody was purified by thiophilic adsorption (T-gel, a gift from Dr Jan Carlsson, Pharmacia Diagnostics AB, Uppsala, Sweden) column chromatography, then biotinylated, aliquoted, lyophilized, and stored at -20 °C. On the day of the experiment, the antibody was reconstituted with 100 µl of distilled water, and diluted with PBS to the optimum concentration of 20 µg/ml.

Micro-titer plate preparation

A competitive ELISA technique was used to detect and measure the concentration of fucoidan in plasma samples. Briefly, a 96-welled, maxisorp surface, micro-titer plate was coated with 100 µl of 200 µg/ml protamine sulfate solution in PBS (pH 7.4) per well overnight at 4 °C. Some wells of the plate remained uncoated with protamine sulfate (blank wells). Distilled water (100 µl) was used in these blank wells. All wells in the plate were then blocked with 200 µl of PBS Tween-20 0.1% (v/v) (PBS-T) supplemented with 6% BSA and incubated at 37 °C for 1 h. The plate was then washed thrice

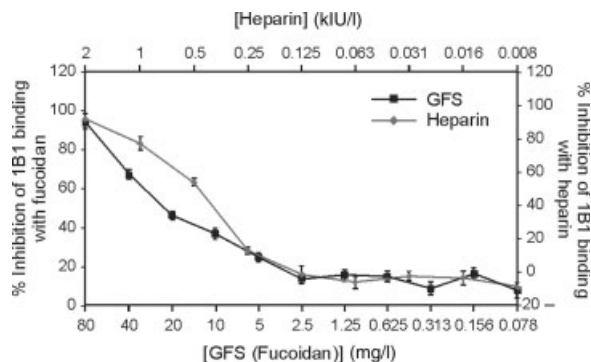


FIG. 1. Inhibition curves for heparin and fucoidan using 1B1 antibody: a comparison between the affinity of the monoclonal antibody 1B1 with both heparin sulfate and fucoidan at equivalent concentrations. Each point represents the mean of triplicate readings from three different experiments \pm standard deviation.

with PBS-T and air-dried. These protamine-sulfate-coated plates were prepared freshly each time.

Construction of standard curves

A stock solution of heparin (4 kIU/l) and another stock solution of fucoidan (160 mg/l) were used to produce a twofold dilution series, as shown in Figure 1. Concentrations shown in Figure 1 are the final working concentrations after diluting fucoidan or heparin with the antibody to prepare "inhibition mixtures." All dilutions were performed using a plasma mixture prepared from baseline-plasma samples for those samples tested in the same plate as an intraassay control. Each plate contained a set of serial standard concentrations to construct its own standard curve. The standard curve was created using four-parameter logistic fitting algorithm, using a computer software Genesis Lite, version 3.03, Life Sciences, UK. The inhibition percentage for both heparin and fucoidan was calculated using the following formula:

$$\% \text{ Inhibition} = 100 - \left[\frac{\text{Unknown}}{\text{Positive Control}} \times 100 \right]$$

Blank readings were subtracted from all other readings. The positive control was prepared by mixing equal volumes of 1B1 antibody and plasma to prepare the inhibition mixture. About 100 µl of inhibition mixture was then added to the protamine-sulfate-coated wells in triplicates. The 50% inhibition concentration (IC₅₀) for both heparin and fucoidan standard curves was calculated.

Detection of fucoidan in plasma

A set of serial standard concentrations to generate a standard curve was included in each microtiter plate. Each standard, blank, control, or sample was mixed in 1:1 ratio with the preprepared monoclonal antibody 1B1

TABLE 2. Average inhibition concentration 50% (IC₅₀) for heparin and fucoidan standard curves using 1B1 antibody and the competitive ELISA.

| | IC ₅₀ | Range |
|-----------------|---------------------------|-------------|
| Heparin (kIU/l) | 0.52 ± 0.05 ^a | 0.47–0.57 |
| Fucoidan (mg/l) | 21.95 ± 3.66 ^b | 18.29–25.61 |

^aMean ± SD; n = 3.^bMean ± SD; n = 6.

and incubated for 1 h at 37 °C to prepare inhibition mixtures. One hundred microliters of each inhibition mixture was added to the designated wells in triplicate and the plate was incubated for 1 h at 37 °C. Then, the plate was washed thrice with PBS-T and air-dried, followed by the addition of 100 µl of 1:1000 of peroxidase-conjugated monoclonal antibiotin antibody to each well (prepared in PBS-T supplemented with 0.5% BSA) and left for 1 h at 37 °C. Then, the plate was washed thrice with PBS-T and air-dried. Next, 100 µl/well of *o*-phenylenediamine dihydrochloride substrate (0.4 mg/ml) was added. The plate was then incubated in the dark for 10 min, and the reaction was stopped by adding 50 µl of 4 M H₂SO₄ to each well. The absorbance was measured immediately at 490/630 nm using a microtiter plate reader (MR 5000 Micro-plate Reader, Dynatech Laboratories Inc., Virginia). The amount of fucoidan in the plasma samples was calculated after creating the standard curve.

Statistical analysis

The standard curve readings were calculated using mean and standard deviation. All other statistic values are based on nonparametric statistics using the median and 95% confidence interval. Triplicate readings for each standard, control, and sample were averaged and subtracted from the average blank readings. Statistical parameters were calculated using Microsoft Office, Excel, and SPSS, Version 12.1.

RESULTS

The reactivity of the antibody toward GFS was assessed prior to its use in the clinical assay by comparing its affinity for heparin and fucoidan. After this, clinical

samples from the study were assessed using the developed competitive ELISA assay.

The reactivity of the antibody toward GFS *Undaria* fucoidan was similar to that of heparin (Fig. 1). The calculated IC₅₀ for each fucoidan standard curve ranged from 18.29 to 25.61 mg/l, with an average of 21.95 mg/l, whereas the IC₅₀ for each heparin standard curve ranged from 0.47 to 0.57 kIU/l, with an average of 0.52 kIU/l (Table 2).

Plasma samples collected from volunteers were quantified for the presence of fucoidan using the described competitive ELISA method. The median concentration of fucoidan for the three different groups of volunteers after taking the capsules orally is summarized in Table 3. The plasma fucoidan concentration (median) in samples from volunteers who received guar gum as placebo was –0.28 mg/l. The plasma fucoidan concentration (median) in samples from volunteers who received 10% or 75% GFSTM fucoidan was 4.00 and 12.99 mg/l, respectively (Figs. 2 and 3).

Another set of clinical plasma samples, and standard curve plasma, was protein-purified using the affinity/ion exchange chromatography matrix (CM Affi-Gel Blue Gel). This matrix binds to both serum albumin and proteases. The resulting fractions were then tested for the presence of fucoidan using the same ELISA technique. The fucoidan level was low in most of the samples (n = 10) and lower than the level of untreated samples.

Another 10 plasma samples from the same volunteers, and a standard curve, were treated with pronase enzyme and then tested for the presence of fucoidan. Fucoidan levels were too low for detection also using this method.

DISCUSSION

The monoclonal antibody 1B1 was raised to react with heparin and heparinlike substances. It also reacts with pentosan polysulfate and dextran sulfate and chondroitin sulfate E from cells. It reacts weakly with heparin sulfates, chondroitin sulfate, and dermatan sulfate. The antibody had good reactivity against GFS fucoidan in this assay. From our early experiments, we found that

TABLE 3. Concentration of fucoidan (median) in human plasma samples of the three groups of volunteers, measured by the competitive ELISA using the 1B1 antibody.

| Days | Fucoidan concentration (mg/l) | | |
|--|-------------------------------|---|---|
| | Guar gum ^a | GFS TM 10% fucoidan ^a | GFS TM 75% fucoidan ^b |
| 4 | –0.852 (2.334) [2.177] | 4.002 (0.689) [2.722] | 11.906 (1.573) [2.35] |
| 8 | –0.281 (2.873) [1.96] | 2.087 (1.045) [1.485] | 13.015 (1.433) [1.757] |
| 12 | 2.394 (3.327) [0.368] | 5.082 (2.961) [1.288] | 15.746 (4.008) [1.703] |
| Median for all point readings at 4, 8, and 12 days | –0.281 (2.905) [2.675] | 4.002 (1.915) [2.721] | 12.989 (1.165) [1.267] |

Values in parentheses indicate lower 95% confidence interval; values in square brackets indicate upper confidence interval.

^an = 6, median representing the mean of triplicate readings for each point ± 95% confidence intervals.

^bn = 40, median representing the mean of triplicate readings for each point ± 95% confidence intervals.

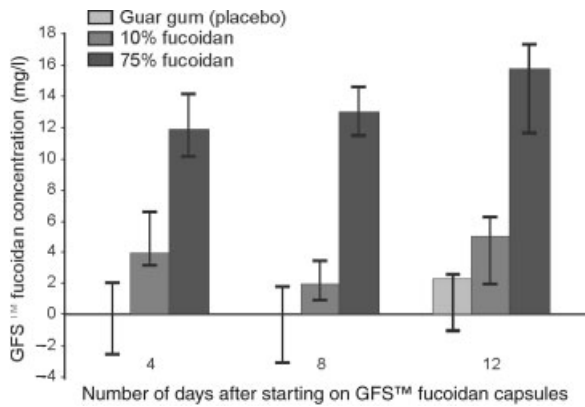


FIG. 2. Concentration of free fucoidan in plasma. The histogram shows three different groups at 4, 8, and 12 days after starting the ingestion of the designated seaweed capsules. The first group of volunteers ($n = 6$) ingested capsules containing guar gum (placebo). The second group ($n = 6$) and the third group ($n = 40$) ingested capsules containing GFS™ fucoidan at two different concentrations (10% and 75%). Each point represents the median of readings \pm 95% confidence intervals.

the 1B1 antibody lower detection limit for dextran sulfate was about 156 mg/l (15).

The amount of fucoidan detected in serum was small, correlating with 0.6% of the oral dose. In this assay, we assumed that all material detected by the antibody was any sulfated polysaccharide with affinity for the antibody or GFS fucoidan.

Figure 1 shows that both heparin and fucoidan have similar binding affinities with 1B1 antibody, using plasma as diluents, which is a clear indication that this antibody can be used to detect fucoidan in biological systems. However, at high concentrations of both heparin (0.25–2 kIU/l) and fucoidan (5–80 mg/l), the avidity of the antibody with heparin was higher than the avidity of the antibody with fucoidan. This may be because the antibody was originally produced against heparin, which has a sulfation pattern different to that of fucoidan. At lower concentrations of both heparin (0.0078–0.25 kIU/l) and fucoidan (0.078–5 mg/l), the reactivity of the antibody for both substances was low under the same experimental conditions. This implies limits on the use of the antibody at very low concentrations. The data on the inhibition concentration of heparin and fucoidan by 1B1 antibody, shown in Table 2, confirm that heparin and fucoidan have comparable IC_{50} values at the comparable concentrations used in the study.

The antibody used in this assay is not specific to fucoidan, but was raised against sulfated polysaccharides. It is possible that the increased readings in this assay were not due to fucoidan, but due to the elevation of another sulfated polysaccharide, such as heparin. We reported previously (15) on the measurement of heparin using this antibody. An important part of the method was digestion of plasma samples with broad-spectrum proteolytic enzymes such as pronase. In the previous

work, pretreatment with pronase enhanced the sensitivity of the assay toward heparin. In the work presented here, pretreatment of clinical samples with pronase decreased sensitivity, indicating that the detected molecule was not heparin.

In addition, the same samples were prepurified using affinity/ion exchange chromatography matrix (CM Affi-Gel Blue Gel) prior to the assay. This pretreatment reduced the amount of reacting material in the samples. This may be due to an association of the fucoidan with proteins that are stripped out by the affinity matrix. Fucoidans are charged molecules and may react in ion-exchange procedures.

Despite the fact that *Undaria* fucoidan, a large-molecular-weight material, was given orally, we were able to detect small amounts in the plasma. Although acidic conditions in the stomach may cause a limited hydrolysis of the fucoidan, humans do not produce enzymes capable of breaking down fucoidans, and the latter also appear to be unaffected by human fecal flora (20). We suggest that small quantities of orally administered fucoidan may cross the intestinal wall as whole molecules probably by the process of endocytosis. This theory complies with results reported by Barthe and coworkers for chondroitin sulfate (13). They suggested that chondroitin sulfate (a slow-acting drug taken orally in humans to treat osteoarthritis) may cross the upper intestine intact. Unlike fucoidan, chondroitin sulfate is effectively degraded in the distal gastrointestinal tract, presumably by the enzymes in the intestinal flora.

In conclusion, we have shown that the novel antibody 1B1 can be used to detect the bioavailable free fucoidan as whole or as fragments in human plasma samples using a quantitative competitive ELISA assay. It is generally safe to take purified fucoidan orally. The assay we have developed may be useful for monitoring fucoidans when used for therapeutic purposes. Further development of the assay may be needed to make it possible to measure the total fucoidan concentration.

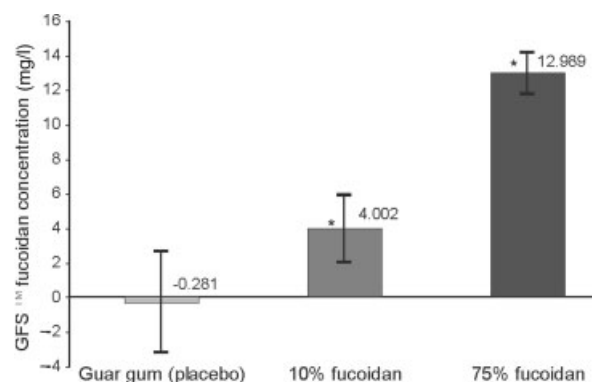


FIG. 3. Concentration of free fucoidan in plasma (median). The concentration observed on the 4th, 8th, and 12th day for each group (placebo, 10% fucoidan, and 75% fucoidan) is plotted (confidence intervals, 95%; * $p < 0.05$ [ANOVA]).

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Address for correspondence: Mohammad R. Irhimeh, Haematology/Oncology Unit, Clinical School, University of Tasmania, Hobart, TAS 7000, Australia.
E-mail: mirhimeh@utas.edu.au
