Glycyrrhizin treatment for Chronic Hepatitis C

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Aan Jan

Voor mams en pagie

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List of abbreviations

ABTS	2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate)
ALT	alanine aminotransferase
ANOVA	analysis of variance
AST	aspartate aminotransferase
AUC	area under the curve
BT	before treatment
CBG	cortisol binding globulin
CCl_4	carbon tetrachloride
Cl_{tot}	total clearance
C_{max}	maximum concentration
cpm	counts per minute
DHEA-S	dehydroepiandrosterone sulphate
DMSO	dimethyl sulphoxide
DNA	desoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EOT	end of treatment
EOF	end of follow up
FSH	follicle stimulating hormone
GA	glycyrrhetinic acid
Gen. Eq.	genome equivalent
GL	glycyrrhizin
GOT	glutamic oxaloacetic transaminase
γ-GTP	γ-glutamyltranspeptidase
HBeAg	hepatitis B envelop antigen
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HCV-RNA	Hepatitis C Virus - ribo nucleic acid
HIV	human immunodeficiency virus
11βHSD	11β-hydroxysteroid dehydrogenase
HPLC	high-performance liquid chromatography

IFN	interferon
IU	international units
IS	internal standard
i.v.	intravenous
LDH	lactic dehydrogenase
LH	luteinizing hormone
MANOVA	multiple analysis of variance
3MGA	3 mono-glucuronide-glycyrrhetinic acid
NFκB	nuclear factor kappa B
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffer saline
PHA	phytohemagglutinin
PPB	propylparaben
PRA	plasma renin activity
RAAS	renin-angiotensin-aldosterone system
RPMI	Roswell Park Memorial Institute
SD	standard deviation
SEM	standard error of the mean
SNMC	Stronger Neo-Minophagen C
SSAO	semicarbazide-sensitive amine oxidase
s-VAP-1	soluble vascular adhesion protein-1
TEAC	trolox equivalent antioxidant capacity
$t_{½}$	half-life
UDCA	ursodeoxycholic acid
ULN	upper limit of normal
V_{ss}	volume of distribution at steady state

Chapter 1

Introduction

Review article: glycyrrhizin as a potential treatment for chronic hepatitis C

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Summary

Chronic hepatitis C is a slowly progressive liver disease that may evolve into cirrhosis with its potential complications of liver failure or hepatocellular carcinoma. Current therapy with alpha-interferon is directed at viral clearance, but sustained response is only achieved in 20-40% of patients without cirrhosis, and less than 20% in patients with cirrhosis who need therapy most. Treatment for those who do not respond to antiviral therapy is highly desirable.

In Japan, glycyrrhizin has been used for more than 20 years as treatment for chronic hepatitis. In randomized controlled trials, glycyrrhizin induced a significant reduction of serum aminotransferases and improvement in liver histology compared to placebo. Recently, these short-term effects have been amplified by a well-conducted retrospective study suggesting that long-term usage of glycyrrhizin prevents development of hepatocellular carcinoma in chronic hepatitis C.

The mechanism by which glycyrrhizin improves liver biochemistry and histology are undefined. Metabolism, pharmacokinetics, side-effects, antiviral and hepatoprotective effects of glycyrrhizin are discussed.

Introduction

Chronic hepatitis C infection, usually a sub-clinical disease in its initial phase, can be associated with progressive liver disease that may evolve insidiously into cirrhosis and carries an increased risk of hepatocellular carcinoma (HCC).[1, 2] Spontaneous remission of viral replication is rare; therefore effective treatment is highly desirable. Treatment with alpha-interferon leads to serum alanine aminotransferase (ALT) normalization and HCV-RNA clearance in 20-40% of patients but relapses after treatment withdrawal are frequent.[3] So different treatment strategies have to be sought for those who don't respond. These approaches might include viral activity reducing or hepatoprotective medication.

In Japan glycyrrhizin, a natural compound extracted from the roots of *Glycyrrhiza glabra*, has been used for more than 20 years as a treatment for chronic hepatitis.[4] Glycyrrhizin is a conjugate of one molecule of glycyrrhetinic acid with two molecules of glucuronic acid (Figure 1). It has been used for many centuries in the traditional Chinese medicine as an anti-allergic agent. Because of its sweet taste it is also used as a food additive, for example in beverages and licorice.[5] In 1946 Revers reported the anti-ulcer effect of licorice;[6] since then, glycyrrhizin was used for many years as anti-ulcer drug in Europe. In Japan, intravenous glycyrrhizin has been used for allergic diseases, mainly in the dermatological field. In an attempt to use glycyrrhizin as a treatment for "allergic" hepatitis it lowered the transaminases. In 1977 Suzuki *et al.* performed a double blind randomized controlled trial in 133 cases with histologically documented chronic active liver disease.[7] The plasma transaminase activity improved significantly in the group treated with glycyrrhizin as compared to the placebo treated group. Hino *et al.* also found an improvement of the liver histology after treating chronic active hepatitis with glycyrrhizin.[8] The mechanism by which glycyrrhizin improves the biochemistry and histology is unknown. This review will deal with the metabolism and pharmacokinetics, the side-effects, the anti-viral and hepatoprotective action of glycyrrhizin. It will also discuss the long-term effects of glycyrrhizin in the treatment of chronic viral hepatitis C.

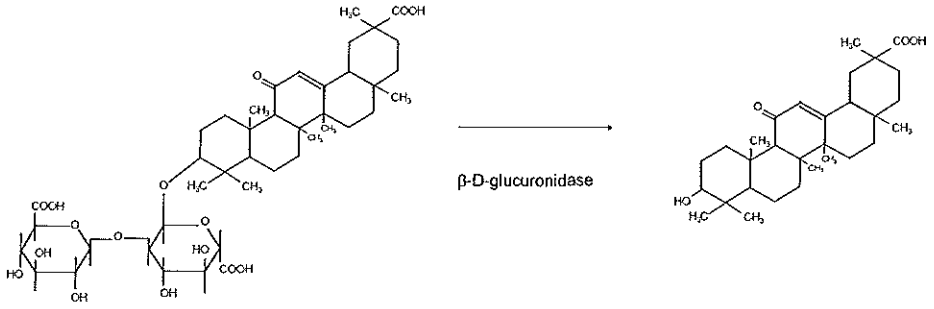
Methods

Medline (1966 - May 1997) was used as a reference source with the key words: glycyrrhizin(e), glycyrrhizic acid, licorice and liquorice. The retrieved publications were supplemented with relevant articles found in the respective reference lists.

Metabolism & Pharmacokinetics

After oral administration glycyrrhizin is metabolized to glycyrrhetinic acid by intestinal bacteria which contain β-D-glucuronidase (Figure 1).[9] After intravenous administration of

glycyrrhizin, both glycyrrhizin and glycyrrhetinic acid appear in the plasma. Intravenously administered glycyrrhizin is metabolized in the liver by lysosomal β-D-glucuronidase to 3-mono-glucuronide-glycyrrhetinic acid. The human liver is not able to metabolize to 3-mono-glucuronide-glycyrrhetinic acid to glycyrrhetinic acid; 3-mono-glucuronide-glycyrrhetinic acid is excreted with bile into the intestine where bacteria metabolize it to glycyrrhetinic acid, which is re-absorbed.[10]



Metabolism of glycyrrhizin to glycyrrhetinic acid by β-D-glucuronidase

Figure 1

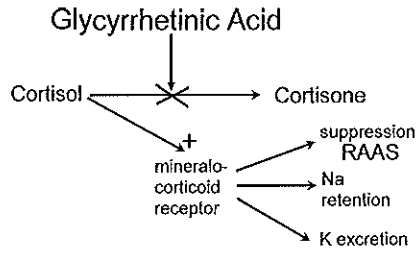
After intravenous injection of glycyrrhizin in healthy volunteers the terminal half life ($t_{½}$) and the total body clearance (Cl_{tot}) were 2.7-4.8 h and 16-25 mL/kg/h, respectively.[11] After an 80 mg dose the maximum glycyrrhizin plasma concentration was 29.3 µg/mL; in this study glycyrrhetinic acid was not detected in the plasma, probably because the detection limit of the assay for glycyrrhetinic acid (0.050 µg/mL) was too high. After oral administration of 100 mg glycyrrhizin in the same healthy volunteers no glycyrrhizin was found in the plasma; glycyrrhetinic acid was found at < 0.2 µg /mL. In the 24-hours period after oral administration, glycyrrhizin was excreted in urine, suggesting that glycyrrhizin is partly absorbed as intact drug, probably the detection limit of the assay for glycyrrhizin (0.5 µg/mL) was too high to determine the very small amounts of plasma glycyrrhizin.[11]

The terminal half life and the total body clearance of glycyrrhizin after 120 mg intravenous glycyrrhizin in eight chronic hepatitis patients were 6.0 h (range 4.3-10.7 h) and 7.9 mL/kg/h (4.5 -12.7 mL/kg/h), respectively. Compared to healthy subjects the $t_{½}$ and the Cl_{tot} were 2 and 0.7 times that of healthy subjects. The total body clearance

was inversely correlated with the transaminases (AST r=-0.739 and ALT r=-0.783). This inverse correlation between Cl_{tot} and ALT and AST was also found in six cirrhosis patients. After improvement of the liver functions the $t_{½}$ shortened from 7.6 to 3.4 h. and the Cl_{tot} increased from 2.8 to 11.4 mL/kg/h. Thus, pharmacokinetic profiles after intravenous administration of glycyrrhizin are correlated with the functional capacity of the liver.[12, 13]

Side Effects

Pseudo-aldosteronism, with sodium retention, hypokalemia and hypertension, is a well known side-effect of glycyrrhizin.[14] Stewart (1987) *et al.* concluded that glycyrrhizin's metabolite glycyrrhetinic acid causes this phenomenon by inhibiting the enzyme 11β-hydroxy-steroid-dehydrogenase (11β-HSD) in the kidney.[15] 11β-HSD exists as two isoforms. The renal isoform 11β-hydroxy-steroid-dehydrogenase converts cortisol to cortisone and the liver isoform catalyzes the reverse reaction.[16] Glycyrrhetinic acid does not inhibit the liver enzyme, but inhibits the renal 11-β-hydroxy-steroid-dehydrogenase.[17] This leads to increased cortisol levels in the kidney. Because cortisol and aldosterone bind with the same affinity to the mineralocorticoid receptor, a raise of renal cortisol will result in a hypermineralocorticoid effect.[15] Stimulation of the mineralocorticoid receptor leads to sodium retention, suppression of renin production, and subsequently to diminished formation of angiotensin I and aldosterone (Figure 2).[15]



Mechanism of side effects. Glycyrrhetinic acid inhibits conversion of cortisol to cortisone. The surplus of cortisol stimulates the mineralocorticoid-receptor in the kidney, which causes suppression of the renin-angiotensin-aldosterone system (RAAS), sodium retention and kaliuresis.

Figure 2

Table 1 shows the changes of plasma renin activity, aldosterone, sodium, potassium, cortisol and cortisone in plasma and urine during glycyrrhizin administration (225 mg/day

during 7 days) in eighteen volunteers.[18] Plasma renin activity and aldosterone were suppressed during glycyrrhizin administration and also their urinary excretion diminished significantly. Sodium was retained with no decrease in urinary excretion. There was a kaliuresis with a significant reduction of plasma potassium. Although the urinary excretion of cortisol increased, plasma cortisol levels remained normal. Both plasma and urinary concentration of cortisone decreased. In this study no alteration of body weight and mean blood pressure were observed, although gain of body weight and hypertension are common side-effects of glycyrrhizin.[14] After glycyrrhizin withdrawal the effects on electrolyte metabolism recovered after one week, plasma renin activity and aldosterone remained low for two to four months.[19, 20]

Table 1.

Changes of plasma renin activity (PRA), aldosterone, sodium, potassium, cortisol and cortisone in plasma and urine during oral glycyrrhizin administration (225 mg / day for 7 days)

Compound	plasma	urine
PRA	↓ *	n.d.†
aldosterone	↓	↓
sodium	↑ ‡	= §
potassium	↓	↑
cortisol	=	↑
cortisone	↓	↓

* significant decrease † not done

‡ significant increase § no significant difference

The incidence of side-effects is dose-dependent and differs among subjects: side effects occur more often in conditions favoring sodium retention, such as during the premenstrual period or oral contraceptive use and are facilitated by subclinical diseases as borderline hypertension and high plasma renin activity.[20, 21]

Three case reports are mentioned in which severe hypokalemia (< 2 mmol/L) caused by excess licorice consumption induced cardiac arrhythmia; in one case it led to cardiac arrest.[22, 23] Hypokalemia can also induce acute rhabdomyolysis with a clinical presentation of muscle weakness, paraparesis or quadriplegia. These symptoms totally

disappeared after cessation of licorice consumption and normalization of the potassium concentration.[24-26] Patients using thiazide diuretics, which enhance potassium loss, are at risk of developing severe hypokalemia and should be monitored carefully during glycyrrhizin treatment.

Pharmacological Effects

Antiviral effect

In 1977, Pompei *et al.* found that glycyrrhizin inhibited growth and cytopathology of herpes simplex virus type 1 in human aneuploid HEp2 cells. This antiviral effect was not mediated by damaging the cells. Glycyrrhizin also induced irreversible loss of infectivity of herpes simplex virus type 1.[27] In 1990, Crance *et al.* investigated forty antiviral compounds for their effect on human hepatoma cell line PLC/PRF/5 infected with hepatitis A virus. They found a dose dependent inhibition of hepatitis A virus antigen expression and reduction of infectivity by glycyrrhizin. At the nontoxic concentrations of 1,000 and 2,000 µg/ml, glycyrrhizin completely suppressed viral antigen expression.[28] Further investigation showed that glycyrrhizin did not inactivate the virus nor influenced virus adsorption, but it inhibited the penetration of hepatitis A virus. Hepatitis A virus enters cells by receptor mediated endocytosis. This process is possibly inhibited by glycyrrhizin by causing a decrease of the negative charge on the cell surface and/or by decreasing the membrane fluidity. These changes of the cell could prevent penetration of the virus.[29]

Cytoprotective effect

Shiki *et al.* found that isolated rat hepatocytes incubated with anti-liver cell membrane antibody plus complement release aspartate aminotransferase (AST). This AST release was likely to be caused by lysis of the cell (membrane) after activation of the membrane phospholipase A_2 by the antigen-antibody reaction. After adding glycyrrhizin to this system the AST release by the hepatocytes decreased significantly. Further investigation suggested that glycyrrhizin inhibited the activation of phospholipase A_2.[30]

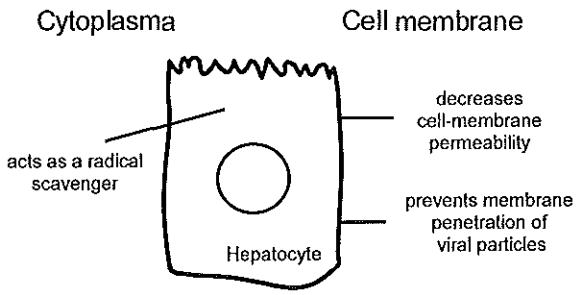
Nakamura *et al.* induced leakage of lactic dehydrogenase (LDH) and glutamic oxaloacetic transaminase (GOT) in a monolayer culture of rat hepatocytes by exposing these cells to carbon tetrachloride (CCl_4). This enzyme leakage was caused by a change in membrane permeability. The addition of glycyrrhizin caused a dose-dependent reduction of enzyme release; it was slightly effective at 25 µg/ml and maximally effective at 200 µg/ml. Glycyrrhizin probably acted by a direct protective effect on the hepatocyte membranes by preventing a change in cell permeability.[31]

In a comparable *in vitro* study Nose *et al.* compared the hepatoprotective action of glycyrrhizin and glycyrrhetinic acid. They found that glycyrrhetinic acid was effective in decreasing the transaminases in a concentration of 5 µg/ml and glycyrrhizin was only effective at a dosage of 1000 µg/ml. This study suggests that glycyrrhetinic acid is a better hepatoprotective drug than glycyrrhizin.[32]

After ischemia-reperfusion damage of the liver in rat models the serum levels of AST, ALT, LDH and lipid peroxides in liver tissue increased, while the liver gluthatione concentration decreased significantly. Pretreatment of the rats with subcutaneous glycyrrhizin for 10 days suppressed the elevation of lipid peroxides and serum AST, ALT and LDH, while the glutathione concentration in liver tissue remained at control level. Glycyrrhizin suppressed liver damage by acting as a hydroxyl-radical scavenger. Pretreatment with glycyrrhizin also reduced the morphological damage as assessed by electron microscopy.[33, 34]

Immunomodulation

Takahara *et al.* found that glycyrrhizin suppressed the secretion of HBsAg and enhanced its accumulation dose-dependently in the HBsAg expressing human hepatocellular carcinoma cell line PLC/PRF/5, without cytotoxicity. The mechanism of action was investigated in cells infected with recombinant varicella virus expressing HBsAg. It was found that glycyrrhizin inhibited the sialylation of HBsAg. As desialylated HBsAg has an improved immunogenicity in humoral antibody and cell mediated response, glycyrrhizin may improve the immunological status of HBV infected patients by enhancing the immunogenicity of HBsAg.[35]



Possible mechanism of action of glycyrrhizin in the hepatocyte

Figure 3

Abe *et al.* found that intravenous injection of glycyrrhizin in mice induced two peaks of γ-interferon. For this IFN induction T-cells and macrophages were required. Intravenous injection of glycyrrhetinic acid induced one interferon peak, so in this model glycyrrhizin is superior to glycyrrhetinic acid in inducing IFN.[36]

In mice, subcutaneously administered glycyrrhizin selectively activated extrathymic T cells in the liver, without affecting T-cells in the thymus. These activated T-cells were T cells with intermediate T-cell receptors, thus double negative CD_4^- CD_8^- as well as single positive CD_4^+ or CD_8^+; and may have cytotoxic activity against virally infected cells.[37]

Clinical investigations

The first double blind placebo controlled randomized study in chronic hepatitis patients was published in 1977 by Suzuki *et al.* in Japanese,[7] and in 1983 in English[38] In this study glycyrrhizin was given as Stronger Neo-Minophagen C (SNMC). This is a solution for intravenous use consisting of 2 mg glycyrrhizin, 1 mg cysteine and 20 mg glycine per ml in physiological saline solution. Glycine was added because it is supposed to prevent pseudoaldosteronism and cysteine should be detoxificative through cystine-conjugation in the liver. During 4 weeks 40 ml SNMC (= 80 mg glycyrrhizin) was given daily to 67 patients with histologically documented chronic hepatitis; 66 comparable patients received a placebo SNMC containing 0.1 mg/ml glycyrrhizin (= 4 mg glycyrrhizin daily). During treatment, a significant improvement in serum transaminases occurred in the SNMC treated patients compared to the placebo group. No marked side effects were observed; after discontinuation of the SNMC, the transaminase rebounced. Step-wise withdrawal therapy after eight weeks treatment with a daily dose of 100 ml SNMC resulted in less rebound of transaminases.[39]

Hino *et al.* found that 100 ml SNMC daily for 8 weeks not only improved the serum transaminases but also the pathological features of a liver biopsy as compared to the biopsy before treatment. Thirty-nine patients with chronic hepatitis were treated with SNMC, the control group consisted of 54 comparable patients. During and until 8 weeks after SNMC treatment the transaminases decreased significantly; in the control group no change occurred. The pathological diagnosis in the SNMC treated group improved in 44%, remained unchanged in 53% and worsened in 3%. No information is given about the histological changes in the control group.[8]

Xianshi *et al.* showed that oral administration of glycyrrhizin can significantly improve liver function. In this study 7.5 mg glycyrrhizin in a capsule had been given orally twice a day for 30 days for acute hepatitis and for 90 days for chronic hepatitis. Seroconversion

from HBeAg positive to HBeAg negative occurred in 15% and from HBsAg positive to HBsAg negative in 25% of the glycyrrhizin treated patients while no seroconversion occurred in the control group. No side-effects are mentioned.[40]

In Europe, experience with SNMC for the treatment of chronic hepatitis has been restricted to Germany. After 12 years experience Wildhirt concluded that the biochemical and histological improvements after SNMC were larger than those obtained after interferon, which is more expensive and has more side effects. The antiviral effect of SNMC was comparable to that of interferon. These conclusions came from open uncontrolled studies with patients that did not respond to previous medication.[41]

A retrospective study, performed by Arase *et al.*, suggests that long-term usage of SNMC is effective in preventing HCC development in patients with chronic hepatitis C. Eighty-four patients without cirrhosis treated with 100 ml SNMC daily for 8 weeks and 2 - 7 times a week for 2 - 16 years (median 10.1 years) were compared to 109 patients not treated with SNMC because there was no home health care professional available to give the intravenous injections. HCV was diagnosed in stored serum by second generation anti-HCV tests. The diagnosis of HCC was made by the typical hypervascular characteristics observed on angiography. The cumulative HCC appearance rate in the SNMC and control group were 7% and 12% respectively after 10 years, and 12% and 25%, respectively after 15 years. In the SNMC group ALT levels became normal in 30 of 84 patients (35.7%) compared to 7 of 109 patients (6.4%)in the control. In patients with ALT normalization the appearance of hepatocellular carcinoma was rare. Discontinuation because of side effects did not occur.[42]

Discussion

In Japan glycyrrhizin has been accepted as a treatment for chronic hepatitis for more than 20 years. Nowadays slightly less than 100 million ampoules a year with 40 mg of glycyrrhizin are produced in Japan for the treatment of chronic hepatitis.

Suzuki showed in a double blind randomized controlled trial that in patients with chronic hepatitis (not specified) the serum transaminases decreased during the treatment with intravenous glycyrrhizin given as SNMC. After discontinuation of the medication the serum transaminases rebounced, but this could be prevented by low dose maintenance therapy. In this respect glycyrrhizin resembles ribavirin.[43] As found by Arase *et al.*,[42] long-term usage of intravenous glycyrrhizin, or to be precise SNMC, appears effective in reducing the complications (hepatocellular carcinoma) of chronic hepatitis C. Contrary to IFN therapy, SNMC-treatment showed hardly any side-effects. These two studies suggest

that the long-term use of SNMC is safe and effective for non-responders after interferon therapy, in reducing the rate of HCV progression.

There are many gaps in our knowledge with regard to glycyrrhizin as a treatment for chronic viral hepatitis C. *In vitro* glycyrrhizin has antiviral activity against herpes simplex virus and hepatitis A virus. However, it is unlikely that glycyrrhizin induces viral clearance in chronic hepatitis C, because directly after cessation of the treatment, transaminases rise, indicating that the cause of the inflammation (HCV) is still present. *In vitro* glycyrrhizin is hepatoprotective, probably by preventing changes in cell membrane permeability. The observation that long-term glycyrrhizin treatment reduces HCV complications in patients suggests that glycyrrhizin also acts hepatoprotective in humans, although the precise mechanism is not known.

The optimal treatment schedule is not known. In Japan, SNMC is given according to the level of transaminases; if the transaminases rise, the dosage and frequency of SNMC-treatment will be increased.

The studies of Suzuki *et al.*[38] and Arase *et al.*[42] are performed with intravenous glycyrrhizin given as SNMC. Is the effect caused by glycyrrhizin or is it the combination of glycyrrhizin with glycine and cysteine that causes the effect? And is the intravenous way of administration essential to reach effectivity or is it possible to give an oral glycyrrhizin preparation? Orally administered glycyrrhizin will show up as glycyrrhetinic acid after absorption. Therefore it is important to find out whether the hepatoprotective effect is caused by glycyrrhizin or glycyrrhetinic acid or both.

To fill in these gaps it seems logical -on basis of the Japanese experience- to do a systematic evaluation of intravenous and oral glycyrrhizin therapy in controlled clinical studies. It will also be very important to elucidate unanswered questions regarding the underlying mechanism of action.

Although the optimal treatment strategy still has to be found, glycyrrhizin is a potentially effective drug in reducing long-term complications in chronic viral hepatitis C in patients who do not respond with viral clearance to IFN therapy.

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Chapter 2.1

Clinical trials

Intravenous glycyrrhizin for the treatment of chronic hepatitis C: a double-blind, randomized, placebo-controlled phase I/II trial

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Abstract

Background: In Japan, glycyrrhizin therapy is widely used for chronic hepatitis C and reportedly reduces progression of liver disease to hepatocellular carcinoma. The aims of this study were to evaluate the effect of glycyrrhizin on serum alanine aminotransferase (ALT), hepatitis C virus (HCV)-RNA and its safety in European patients

Methods: Fifty-seven patients with chronic hepatitis C, non-responders or unlikely to respond (genotype 1 / cirrhosis) to interferon therapy, were randomized to one of the four dose groups: 240, 160 or 80 mg glycyrrhizin or placebo (0 mg glycyrrhizin). Medication was administered intravenously thrice weekly for 4 weeks; follow-up also lasted 4 weeks.

Results: Within 2 days of start of therapy, serum ALT had dropped 15% below baseline in the three dosage groups ($p<0.02$). The mean ALT decrease at the end of active treatment was 26%, significantly higher than the placebo-group (6%). A clear dose-response effect was not observed (29, 26, 23% ALT decrease for 240, 160 and 80 mg, respectively). Normalization of ALT at the end of treatment occurred in 10% (four of 41). The effect on ALT disappeared after cessation of therapy. During treatment, viral clearance was not observed: the mean decrease in plasma HCV-RNA after active treatment was 4.1×10^6 genome equivalents/mL (95% confidence interval: 0 - 8.2×10^6, $p>0.1$). No major side-effects were noted. None of the patients withdrew from the study because of intolerance.

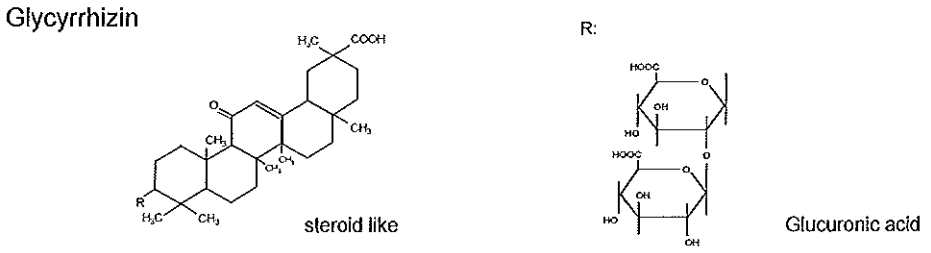
Conclusions: Glycyrrhizin up to 240 mg, thrice weekly, lowers serum ALT during treatment, but has no effect on HCV-RNA levels. The drug appears to be safe and is well tolerated. In view of the reported long-term effect, further controlled investigation of the Japanese mode of administration (six times weekly) for induction appears of interest.

Introduction

Chronic hepatitis C infection can be associated with progressive liver disease that may evolve insidiously to cirrhosis and carries an increased risk of hepatocellular carcinoma.[1]

Alpha-interferon monotherapy leads to hepatitis C virus (HCV)-RNA clearance in a minority of patients. Although combination therapy with Ribavirin increases efficacy, the sustained response rate is still below 50%.[2]

For patients who do not respond with viral clearance, different treatment strategies have to be sought. These approaches include virus suppressive or hepatoprotective medication. In long-term studies on chronic hepatitis B and C, persistent normalization of alanine aminotransferase (ALT) appears to be a key prognostic factor for reduction of complications, regardless of the presence of viral markers in the serum.[3-6]



Molecular structure of glycyrrhizin

Figure 1

In Japan, glycyrrhizin extracted from the roots of the plant *Glycyrrhiza glabra* (liquorice) has been used for the treatment of chronic hepatitis for more than 20 years.[7] Glycyrrhizin is a conjugate of a molecule of glycyrrhetinic acid and two molecules of glucuronic acid (Figure 1). Suzuki *et al.* found that treatment with glycyrrhizin, given as Stronger Neo-Minophagen C (SNMC, an aqueous solution for intravenous administration) lowered the serum transaminases significantly.[8] However, after discontinuation of the medication, the serum transaminases rebounded. Glycyrrhizin has no influence on the viral load.[9] Despite this finding, Arase *et al.* recently reported that long-term treatment with glycyrrhizin of Japanese patients with chronic hepatitis C, prevented the development of hepatocellular carcinoma when ALT normalized.[4]

At present millions of ampoules of SNMC, each containing 40 mg glycyrrhizin, are used each year in Japan for the treatment of chronic hepatitis. It is remarkable that a

drug which is used in Japan and other Asian countries on such a large scale is virtually unknown in Europe and the USA. Can this discrepancy be explained by a difference in efficacy of glycyrrhizin between Asian people and Caucasians, for instance due to genetic polymorphisms in drug metabolism? [10]

We performed a randomized, double-blind, placebo-controlled trial to evaluate whether the effect of glycyrrhizin on serum ALT is also demonstrable in European patients.

Methods

Patients

Patients were recruited from the University Hospital Rotterdam between February 1997 and March 1998. Patients between 18 and 65 years of age were eligible if they met all inclusion criteria: serum antibodies against HCV; HCV-RNA-positive; serum ALT at least 1.5-fold the upper limit of normal in the 8 weeks prior to treatment and at entry; liver biopsy consistent with mild to moderate fibrosis or cirrhosis with mild to moderately active hepatitis; relapse after, non-response to or unlikely to respond to interferon (cirrhosis, genotype 1); or contra-indication for or refusal to take interferon.

Patients were not eligible if they met one or more exclusion criteria: other causes of liver disease: chronic hepatitis B, alcohol abuse, auto-immune hepatitis, hemochromatosis, Wilson's disease, α-1-antitrypsin deficiency; decompensated cirrhosis, hepatocellular carcinoma; significant cardiovascular or pulmonary dysfunction in the past 6 months; malignancy in the previous 5 years; human immunodeficiency virus 1/2 infection; immunosuppressive therapy; antiviral treatment in the preceding 3 months; pregnancy, breast-feeding; hypokalemia and liquorice addiction.

The study was conducted according to the Declaration of Helsinki and Good Clinical Practice. The Protocol was approved by the Medical Ethical Committee of the Erasmus Medical Center Rotterdam and all patients gave their written informed consent.

Study Design

Patients were stratified for the presence or absence of cirrhosis and were randomized to receive a high (240 mg), a standard (160 mg), a low (80 mg) dose glycyrrhizin or placebo (0 mg glycyrrhizin). Randomization was performed by means of a computer-generated randomization schedule made by the statistician. The randomization code was known to the statistician and the Department of Pharmacy where the medication was packaged and labeled with consecutive randomization numbers. During the study neither the patients nor the physicians were aware of the dose of the trial drug. After completion of the clinical

part of the study and closure of data collection, the randomization code was broken and the physicians and patients were notified of the actual treatment.

Glycyrrhizin was given as Stronger Neo-Minophagen C (SNMC), a clear solution for intravenous use, consisting of 2 mg glycyrrhizin, 1 mg cysteine and 20 mg glycine per mL physiological saline. The placebo consisted of 1 mg cysteine and 20 mg glycine per mL physiological saline.

Both SNMC and placebo ampoules were supplied by Minophagen Pharmaceutical Co., Ltd., Tokyo, Japan.

Medication per visit consisted of 6 ampoules of 20 mL (placebo or verum). The 6 ampoules (120 mL) were added to a 250 mL infusion bag containing 100 mL of a 5% glucose solution. The time between preparation and administration was less than 4 hours. The 220 mL solution was administered intravenously within 15 minutes via an indwelling venous catheter. The system was flushed with 25 mL NaCl to ensure administration of all of the medication.

Because glycyrrhizin has a saponin-like structure it may foam, so that placebo and verum ampoules were not fully indistinguishable. After mixing with 100 mL 5% glucose both verum and placebo show foaming, so that they became indistinguishable. To keep administration double-blind, the infusion bag was prepared by an independent third person.

Patients were treated as out-patients. Medication was administered intravenously thrice weekly for 4 weeks (12 infusions). The duration of follow-up after the end of treatment was also 4 weeks. Routine hematological and biochemical assessments were performed weekly during the treatment period and every 2 weeks during follow-up. Virological assessments were performed on day 0 and day 28 (pretreatment and post-treatment).

Side effects were checked every visit by asking: "Have you had any (other) medical problem since your last visit?". Blood pressure and weight were checked every visit; a complete physical examination was carried out before treatment, at the end of treatment and at the end of follow-up. Electrolytes (Na and K) were assessed weekly during treatment and every two weeks during follow-up period.

Because administration took place in a hospital, patient compliance could be noted accurately by checking scheduled visits.

Virological assessments

Antibodies to hepatitis C virus were determined by screening for antibodies by third generation ELISA (IMX, Abbott, Chicago, IL, USA.).

EDTA-samples taken for HCV-RNA determination were processed into plasma within 2 h and stored at -70° C. Hepatitis C virus-RNA was determined qualitatively by a

modified version of the Amplicor HCV assay (Roche Molecular Systems, Alameda, CA, USA); the extracted RNA was resuspended in 150 µl instead of 1 mL. The level of HCV-RNA was assessed quantitatively by means of the branched DNA assay (Quantiplex 2.0, Chiron Corporation, Emeryville, CA, U.S.A.), which has a lower detection limit of 200,000 HCV genome equivalents per mL.

For genotyping, the polymerase chain reaction product generated with the Amplicor HCV assay was used. Sequence analysis was performed on an automated sequencer (Perkin Elmer, Nieuwerkerk, The Netherlands). The genotype was determined according to the rules described by the International HCV Collaborative Study Group.[11]

Assessments of outcome

The primary (biochemical) response parameters were the percentage decrease in ALT from baseline and the number of patients with ALT normalization at the end of treatment and at the end of follow-up. The secondary response parameter was the virological response, defined as the decrease in plasma HCV-RNA. Tolerability and occurrence of side-effects were the third outcome measures.

Statistical analysis

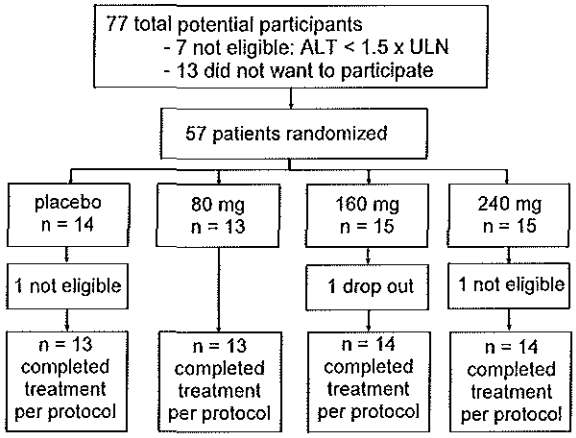
Before the beginning of the study, a power analysis was performed to determine the size of the study population. A linear relationship between the decrease in ALT and dose was assumed. The analysis was based on a two-tailed comparison with an α of 0.05 and a power of 0.80 ($\beta = 0.20$). It was calculated that to detect a correlation of $r = 0.40$ or more at least 12 patients were required per dosage group.

The baseline ALT was calculated as the mean ALT level at screening (in the 8 weeks before start of the study) and the ALT level on day 0. Statistical analysis was performed by using Stata 5.0 software (Stata Corporation, College Station, TX, USA). Comparisons between the four groups were evaluated using the Kruskal-Wallis test; if $p<0.05$ the Mann-Whitney U-test was used to perform further comparisons between the groups. Changes within groups were assessed with the signed-rank test. Fisher's exact test was applied to compare percentages.

Results

Characteristics of patients

Figure 2 shows the trial-profile. There were 77 potential participants, 13 patients were not willing to participate and 7 were not eligible. Therefore 57 patients were randomized to receive treatment. One patient dropped-out because of social problems. Two patients



Trial profile

Figure 2

were mistakenly randomized: one patient had a normal ALT value on day 0; the other had several protocol violations: ALT on day 0 was 1.3 × upper limit of normal, liver biopsy showed no fibrosis and the time between antiviral (interferon) treatment and start of the glycyrrhizin study was less than 3 months. These two patients were included in the safety and tolerability analyses. Statistical analysis of all randomized patients led to the same conclusion as analysis of the 54 patients who completed treatment according to protocol; the efficacy data on 54 patients and the safety data on 56 patients are presented.

The baseline characteristics of the 54 patients are shown in Table I. The groups appear well-balanced. Each group consisted of approximately 40% patients with liver cirrhosis. Most patients were Caucasian. About 80% of the patients per group were male. Although the average baseline level of ALT in the 240 mg group was lower than in the other groups, there was no significant difference between the four groups (Kruskal-Wallis p=0.13).

Median HCV-RNA levels were around 10^7 genome equivalents per mL.

Biochemical Response

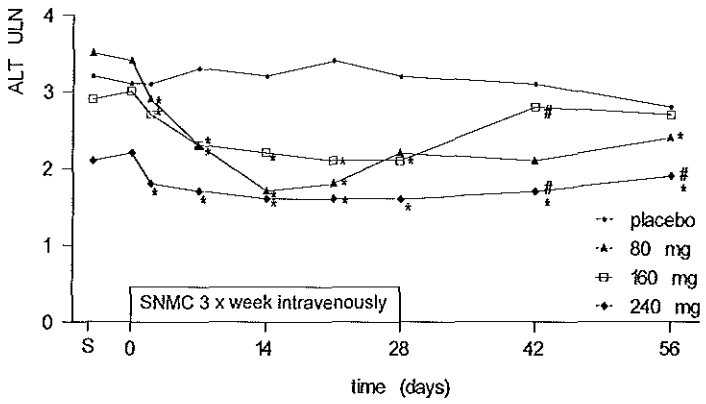
Figure 3 shows the effects of the placebo and the three doses of glycyrrhizin on the ALT level during the study. Within none of the four groups there was a significant difference between ALT at screening (in the 8 weeks before start) and ALT on day 0 (p>0.09).

Table I

General characteristics of the 54 patients analyzed for efficacy at baseline per group.

Group	0 mg	80 mg	160 mg	240 mg
number	13	13	14	14
caucasian/black/other	8/0/5	8/0/5	5/2/6	10/0/4
male/female	13/0	11/2	10/4	11/3
age median (range)	47 (37-60)	45 (32-66)	52 (35-69)	44 (34-61)
non cirrhosis/cirrhosis	7/6	8/5	8/6	8/6
previous interferon yes/no	12/1	10/3	11/3	11/3
ALT baseline (× ULN) median (range)	3.1(1.5-6.8)	3.4(1.4-11.8)	3.0(1.8-6.9)	2.2(1.6-5.2)
γ-GTP (× ULN) baseline median (range)	1.9(0.5-10.0)	1.4(0.4-10.4)	2.1(0.7-8.1)	1.2(0.4-3.7)
HCV-RNA $\times 10^5$ genome eq/ml, median (range)	4.5(1.4-39.2)	21.7(0.2-104)	11.1(1.4-66.9)	14.4(1.1-51.4)
genotype 1a/1b/2/3/4a	1/6/1/3/2	3/3/2/5/0	2/6/4/1/1	2/4/2/6/0

ALT= alanine aminotransferase; γ-GTP= gamma glutamyltranspeptidase. ALT and γ-GTP baselines were calculated as the mean value at screening (in the 8 weeks before start) and at day 0. ULN= upper limit of normal



Median ALT expressed as times the upper limit of normal (ULN) according to dosage group.

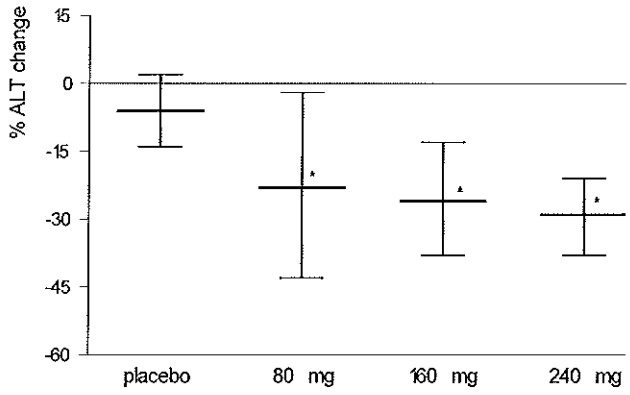
* significantly lower than baseline ($p<0.05$, signed rank test) # significantly higher than ALT at the end of treatment (day 28) ($p<0.05$, signed rank test)

Figure 3

During the study period there was no significant change in ALT in the placebo group.

Within 2 days of start of therapy serum ALT dropped 15% below baseline in the three dosage groups ($p<0.02$) and remained significantly lower, except in the 80 mg group on day 28.

During follow-up after the end of treatment ALT levels tended to increase again; only in the 240 mg group were ALT levels at the end of follow-up significantly higher than at the end of treatment ($p=0.02$). At the end of follow-up the ALT values in the 80 and 240 mg groups were significantly lower than the baseline value ($p<0.01$ and 0.05, respectively).



Mean percentage change in ALT at the end of treatment with respect to baseline ± 95% confidence interval per dosage group.

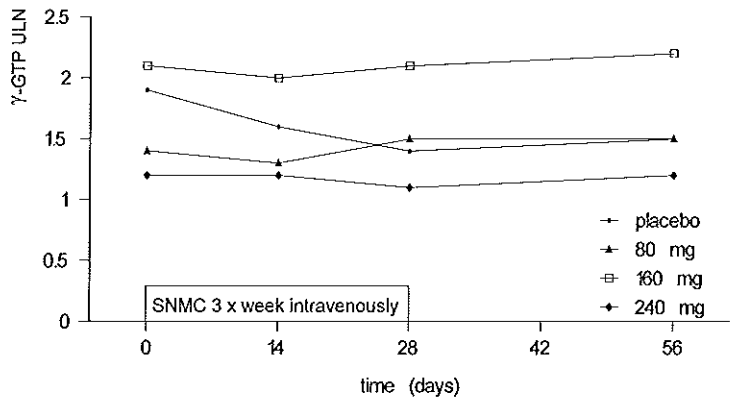
* significantly greater than placebo group ($p<0.03$, ranksum test)

Figure 4

During treatment it was found that on all assessment days the percentage decrease in ALT was significantly higher in the treatment groups compared to the placebo group (all $p<0.03$), while there was no difference between the three active groups. Figure 4 shows the mean percentage ALT decrease from baseline at the end of treatment per group. The mean percentage decrease for the three glycyrrhizin groups at that time was 23, 26 and 29 percent, respectively, all being significantly larger than the 6% decrease found for the placebo group.

The percentage patients exhibiting ALT normalization at the end of treatment was 0 (0/13), 15 (2/13), 0 (0/14) and 14 (2/14) in the 0, 80, 160 and 240 mg glycyrrhizin groups, respectively. Only 1 patient in the 240 mg group had a normal ALT at the end of follow-up. There was no significant difference in ALT normalization between the 4 groups

at the end of treatment and at the end of follow-up. There was no significant difference in γ-glutamyltranspeptidase (γ-GTP) at baseline and γ-GTP at the end of treatment (all p>0.08) within any of the four groups (Figure 5).



Median γ-GTP expressed as times upper limit of normal (ULN) according to dosage group.

Figure 5

Virological Response

The HCV-RNA levels at baseline were high (range of 0.2 - 104×10^6 genome equivalents per mL) as a result of selection of the patients (the majority were non-responders to interferon). After treatment HCV-RNA was still detectable in all patients. In each of the four groups the HCV-RNA levels at the end of treatment did not differ significantly from those at baseline (all p>0.11; Table II). The mean change in plasma HCV-RNA after active treatment was -3.1×10^6 genome equivalents/mL (95% confidence interval: -6.2, -0.1×10^6).

Safety and Tolerability

The expected side-effects of glycyrrhizin are: hypokalemia, sodium retention, increase in body weight, elevated blood pressure and retention of body fluids (i.e. edema).[12] None of these expected side-effects were observed during the study.

Table III shows the adverse events mentioned by more than 2 patients in the course of the study spontaneously or after the question: "Have you had any (other) medical problem since your last visit?".

Table II

Median HCV-RNA levels before and at the end of treatment and mean HCV-RNA change per group. Figures in parentheses represent the range of data.

	Glycyrrhizin treatment			
HCV-RNA (10^6 geq/mL)	**0 mg**	**80 mg**	**160 mg**	**240 mg**
Before treatment	4.5 (1.4-39.2)	21.7 (0.2-104)	11.1 (1.4-66.9)	14.4 (1.1-51.4)
At the end of treatment	6.9 (1.4-38.3)	17.8 (0.5-73.2)	11.5 (1.9-61.6)	8.9 (4.4-43.8)
Mean change	-0.3 (-4.3, 5.0)	-3.6 (-30.8, 13.3)	-1.6 (-27.7, 17)	-7.3 (-42.3, 12.1)

HCV=hepatitis C virus; geq=genome equivalents.

Table III

Number of patients, who mentioned an adverse event spontaneously or after the question: "Have you had any (other) medical problem since your last visit?" Fifty-six patients were analysed.

	Glycyrrhizin treatment (mg)					
Adverse Event \ Group	**0 (n=14)**	**80 (n=13)**	**160 (n=14)**	**240 (n=15)**	**Total**	**p-value [a]**
Headache	5	2	1	5	13	0.21
Having a cold / "flu like" symptoms	0	1	6[b]	3	10	0.01
Increased fatigue since treatment	2	2	3	1	8	0.73
Pain/haematoma at puncture site	1	1	3	1	6	0.67
Strange feeling during administration	2	2	0	1	5	0.48
Diarrhea	0	1	1	3	5	0.35
Stitches in region of liver	1	0	1	1	3	1.00
Rash/itching	0	0	1	2	3	0.61
Dizziness	1	1	0	1	3	0.89
Nausea	0	1	1	1	3	0.89
Total with at least one side effect	10	10	13	10	43	0.63

[a] over-all p-value determined by using Fisher's exact test. [b] significantly higher than placebo (p= 0.016 Fisher's exact test).

Headache, increased fatigue since treatment, pain or hematoma at the puncture site, strange feeling during administration, stabs of pain in the region of the liver and dizziness were mentioned by patients of the placebo as well as the 3 dosage groups. No significant differences between the four groups were observed. Cold or "flu-like" symptoms, diarrhea, rash or itching and nausea were not mentioned by patients of the placebo group, only by patients of one of the three dosage groups. The only statistically significant difference was six patients of the 160 mg group with cold or "flu-like" symptoms; this was significantly higher than the 0 patients of the placebo group (Fisher's exact test, p=0.016). The number of patients reporting an adverse event did not differ significantly between the four groups (Fisher's exact test, p=0.63).

Treatment was generally well tolerated, no patients were lost due to intolerance.

Discussion

This European phase I/II study confirms the effect of glycyrrhizin in lowering serum ALT, as already observed in Japan. The efficacy of glycyrrhizin in normalizing serum ALT in our study was less than that reported in Japan. In our study 10% (4/41) of the patients treated with glycyrrhizin achieved ALT levels within the normal range at the end of treatment, while in Japan 36% of the patients exhibited this important outcome measure.[4] It is possible that Asian people benefit more from glycyrrhizin treatment due to genetic polymorphisms in drug metabolism. Another possible explanation might be the difference in treatment schedules. In Japan, daily administration of glycyrrhizin is standard practice. In Europe, however, it is unusual to treat patients daily with intravenous medication, for which they have to visit the hospital. Therefore in our study glycyrrhizin was administered three times a week. We expected at least a 15% drop-out rate because of intolerance. However, we did not have any dropouts due to intravenous drug administration. In view of our observation that the thrice weekly schedule was well tolerated, it seems logical to conduct an additional study in which European patients are treated according to the Japanese schedule to find out whether the different treatment schedule was the cause of the difference in efficacy.

Treatment with SNMC rapidly induces a significant decrease in ALT. Within the four groups there was no significant difference between ALT at screening and ALT on day 0, but within 2 days of the first dose of glycyrrhizin ALT became significantly lower than baseline ($p<0.02$ signed rank test), while no such effect was seen in the placebo group. We may therefore conclude that the observed ALT decrease during active treatment is not caused by a natural fluctuation of ALT in chronic hepatitis C patients. After cessation of therapy the ALT-decreasing effect of glycyrrhizin disappeared.

The mechanism by which glycyrrhizin decreases the transaminases is not known. One of the proposed mechanisms is that glycyrrhizin induces ALT decrease by stabilizing the cell membrane of the hepatocyte.[13]

In the present study glycyrrhizin was given as SNMC, which contains glycyrrhizin and a ten-fold higher concentration of glycine. Both molecules are said to be hepatoprotective *in vitro*.[13, 14] The placebo used in this trial consisted of all the ingredients of SNMC except for glycyrrhizin. Because the serum ALT did not drop in the placebo group, whereas it was decreased significantly in the three SNMC groups, we can conclude that the ALT-decreasing effect of SNMC in this study is caused by glycyrrhizin and not by glycine.

None of the 57 randomized patients left the study because of drug intolerance. Pseudo-aldosteronism, with sodium retention, hypokalemia and hypertension, is a well known side-effect of glycyrrhizin.[12] This phenomenon is caused by the metabolite glycyrrhetinic acid, which inhibits the conversion of cortisol to cortisone by the enzyme 11β-hydroxy-steroid-dehydrogenase in the kidney. This inhibition leads to increased cortisol levels in the kidney. Because cortisol and aldosterone bind with the same affinity to the mineralocorticoid receptor, a rise in renal cortisol will result in a hypermineralocorticoid effect.[15] In our study we did not observe sodium retention, hypokalemia, hypertension or an increase in bodyweight. Possibly the addition of glycine prevented the occurrence of these side-effects, as reported by Suzuki *et al.*[8] Alternatively, the administered dose of glycyrrhizin -a maximum of 720 mg glycyrrhizin per week (103 mg/day)- is too low to cause side-effects. Side-effects are more likely to occur when the daily dose of glycyrrhizin is several times more than 103 mg.[16]

Unexpected side-effects as headache, increased fatigue since treatment, a strange feeling during administration and pain or hematoma at the puncture site were encountered as often in the placebo group as in the glycyrrhizin groups; so these symptoms are -in all likelihood- not caused by glycyrrhizin itself but by the total treatment (e.g., visiting the hospital three times a week; receiving intravenous medication). Therefore treatment with SNMC up to 240 mg, thrice weekly for 4 weeks, appears to be safe and well tolerated.

In conclusion, we confirmed that a minority of the European patients normalized ALT during glycyrrhizin therapy.

Acknowledgments

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Chapter 2.2

Clinical trials

Glycyrrhizin induced reduction of ALT in European patients with chronic hepatitis C

a double–blind randomized placebo-controlled trial combined with an open trial

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submitted

Abstract

Background: In Japan, ALT-normalization induced by long-term intravenous glycyrrhizin treatment reportedly reduces the progression of liver disease to hepatocellular carcinoma in chronic hepatitis C patients.

Aims: To evaluate the short-term (4 weeks) feasibility and efficacy on serum ALT of 3 or 6 times per week intravenous glycyrrhizin therapy in European patients.

Methods: Patients with chronic hepatitis C, non-responders or unlikely to respond (genotype 1 / cirrhosis) to interferon therapy, were included in this study. Medication was administered intravenously 3 or 6 times per week for 4 weeks; follow-up also lasted 4 weeks.

Results: Sixty-nine patients completed the treatment according to protocol. There were no significant changes in ALT-levels within the placebo group (n=13). The mean percentage ALT decrease from baseline at the end of treatment was 26% and 47% for the three times per week and six times per week treatment group, respectively (both $P<0.001$ versus placebo). At the end of active treatment 10% (4/41) and 20% (3/15) of the patients reached normal ALT levels for the three times per week and six times per week treatment group, respectively. The ALT lowering effect disappeared after cessation of treatment. No major side effects were observed.

Conclusions: It appeared feasible to treat European outpatients with chronic hepatitis C 3 or 6 times per week with intravenous glycyrrhizin. Glycyrrhizin treatment induces a significant ALT decrease in patients with chronic hepatitis C. Six times a week treatment appears more effective than 3 times a week.

Introduction

Treatment of chronic hepatitis C infection with alpha interferon and ribavirin combination therapy results in a sustained virological response in less than 50% of the treated patients.[1-3] For those who do not respond with viral clearance, different treatment strategies have to be sought. In long-term studies on chronic hepatitis B and C, persistent normalization of alanine aminotransferase (ALT) appears to be a key prognostic factor for reduction of long-term complications, regardless of the presence of viral markers in the serum.[4-8]

In Japan, glycyrrhizin - extracted from the roots of the plant *Glycyrrhiza glabra* (liquorice) - has been used for the treatment of chronic hepatitis for more than 20 years.[9] Glycyrrhizin is a conjugate of a molecule of glycyrrhetinic acid and two molecules of glucuronic acid.

In 1997 Arase *et al.* reported that in Japanese patients with chronic hepatitis C, long-term treatment (median 10.1 years) with 2-7 times per week intravenous glycyrrhizin, prevented the development of hepatocellular carcinoma when ALT normalized.[5] Ito reported that glycyrrhizin treatment does not influence the viral load.[10]

At present nearly 100 million of ampoules of SNMC - each containing 40 mg glycyrrhizin - are used each year in Japan for the treatment of chronic hepatitis. It is remarkable that a drug, which is used in Japan and other Asian countries on such a large scale, is virtually unknown in Europe. In Europe it is unusual to treat patients with daily intravenous medication in the outpatient clinic. Therefore the feasibility and the efficacy of glycyrrhizin treatment in European patients should be evaluated. We performed a double-blind, randomized, placebo-controlled study in which medication was administered 3 times a week.[11] In this study only ten percent of our patients treated with glycyrrhizin reached a normal ALT-value at the end of treatment, while in Japan 36% of the patients reached ALT normalization albeit with daily administration.[5] To evaluate whether we could increase the efficacy and whether it is feasible in Europe to administer 6 x week i.v. medication we extended the study with a second part in which glycyrrhizin was given 6 times a week.

Here we report the combined results of the two treatment modalities for intravenous glycyrrhizin in a European setting.

Material & methods

Patients

Patients between 18 and 70 years of age were eligible if they met all inclusion criteria: serum antibodies against HCV, HCV-RNA-positive; serum alanine aminotransferase at least 1.5 times the upper limit of normal (ULN) in the 8 weeks prior to treatment and at start of treatment; liver biopsy consistent with mild to moderate fibrosis or cirrhosis with mild to moderately

active hepatitis; relapse after, non-response to or unlikely to respond to interferon based therapy (cirrhosis, genotype 1); or contra-indication for or refusal to take interferon based therapy. Patients were not eligible if they met one or more exclusion criteria: other causes of liver disease: chronic hepatitis B, alcohol abuse, auto-immune hepatitis, hemochromatosis, Wilson's disease, alpha-1-antitrypsin deficiency; decompensated cirrhosis, hepatocellular carcinoma; significant cardiovascular or pulmonary dysfunction in the past 6 months; malignancy in the previous 5 years; HIV infection; immunosuppressive therapy; antiviral treatment in the preceding 3 months; pregnancy, breast-feeding; hypokalemia and liquorice addiction.

The studies were conducted according to the Declaration of Helsinki and Good Clinical Practice. The Medical Ethical Committee of the Erasmus Medical Center Rotterdam approved both protocols and all patients gave their written informed consent.

Study Design

Glycyrrhizin was given as Stronger Neo-Minophagen C (SNMC, Minophagen Pharmaceutical Co., Ltd., Tokyo, Japan.), a clear solution for intravenous use, containing 2 mg glycyrrhizin, 1 mg cysteine and 20 mg glycine per ml in saline.

Part I (double-blind, randomized, placebo-controlled trial): placebo (SNMC without glycyrrhizin) or glycyrrhizin (80, 160 or 240 mg) was given intravenously 3 times a week. Medication was diluted with 100 ml glucose 5% and administered by drip infusion in 15-20 minutes.

Part II (open study): 200 mg glycyrrhizin (100 ml SNMC) was administered six times per week. Undiluted medication was directly injected into a peripheral vein via a butterfly needle (Neofly® 21G Ohmeda, Japan) in 3-5 minutes. Eight of the 15 patients in this study received placebo and 5 of the 15 received active treatment in part I; the time between the two treatments was at least 6 months.

Treatment duration for both parts was 4 weeks; follow-up thereafter was also 4 weeks. Routine hematological and biochemical assessments were performed weekly during the treatment period and every 2 weeks during follow-up. Virological assessments were performed on day 0 and day 28 (pre-treatment and at the end of treatment). Side effects were checked every visit by asking: "Have you had any (other) medical problem since your last visit?". Patients were treated as outpatients. Because administration took place in a hospital, patient compliance was directly linked with scheduled visits.

Virological assessments

Anti-HCV was determined by third generation ELISA (IMX, Abbott, Chicago, IL, USA.).

EDTA-blood samples were centrifuged within two hours, plasma was stored at -70° C. HCV-RNA was determined qualitatively by a modified version of the Amplicor HCV assay (Roche Molecular Systems, Alameda, CA, USA); the extracted RNA was re-suspended in 150 µl instead of 1 ml. HCV-RNA was quantified by means of the branched DNA assay (Quantiplex 2.0, Chiron Corporation, Emeryville, CA, U.S.A.), which has a lower detection limit of 200,000 HCV genome equivalents per ml.

For genotyping, the PCR product generated with the Amplicor HCV assay was used. Sequence analysis was performed on an automated sequencer (Perkin Elmer, Nieuwerkerk, The Netherlands). The genotype was determined according to the rules described by the International HCV Collaborative Study Group.[12]

Assessments of outcome

The primary (biochemical) response parameters were ALT-normalization at the end of treatment and the mean percentage decrease of ALT from baseline at the end treatment.

The secondary response parameter was the virological response, defined as more than one ^{10}log decrease in plasma HCV-RNA.

Tolerability and side effects were also monitored.

Statistical analysis

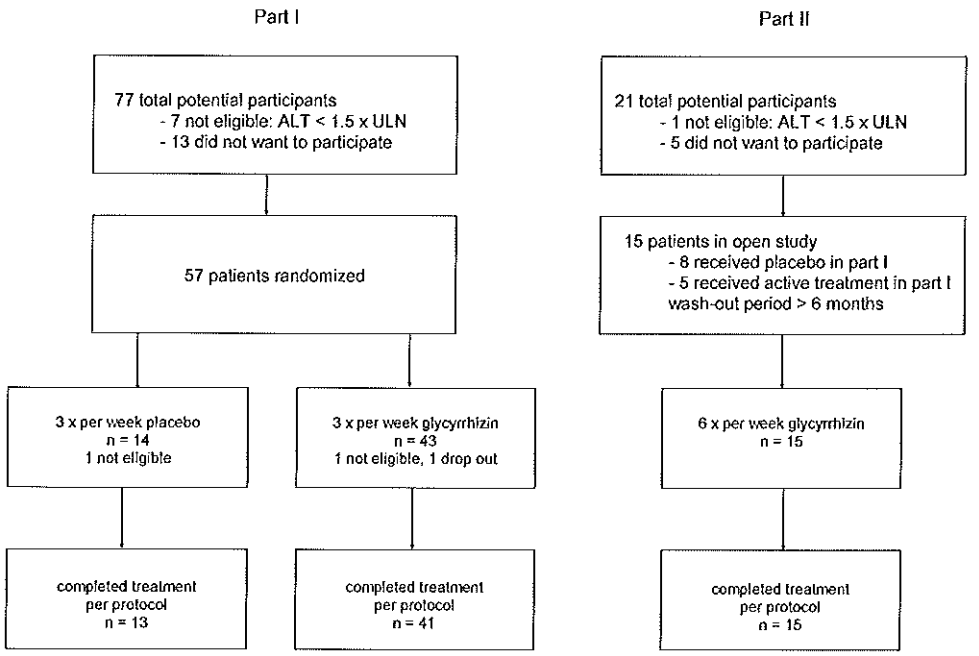
Statistical analysis was performed by using Stata 5.0 software (Stata Corporation, College Station, Texas, USA). The baseline ALT was calculated as the mean of two values: ALT level at screening (in the 8 weeks before start of the study) and ALT level on day 0. A linear relationship between the mean decrease in ALT and treatment dose was assumed in the power analysis of the randomized part of the study. This analysis, based on a two-tailed comparison with an alpha of 0.05 and a power of 0.80, showed that to detect a correlation of r=0.40 or more at least 12 patients per dosage group were required.

Changes within the group were assessed with the Wilcoxon signed-rank test. Comparisons between groups were evaluated with Mann-Whitney's test. Fisher's exact test was applied to compare percentages. p=0.05 (two-sided) was considered the limit of significance.

Results

Characteristics of patients

In total fifty-nine patients were treated in our studies. Thirteen of the 15 patients included in part II participated also in part I: 8 received placebo and 5 received active treatment. Two patients were found not to have met the inclusion criteria; these two patients



Patient flow chart

Figure 1

were included in the safety and tolerability analysis. One patient discontinued after one administration due to social circumstance, this patient was excluded from analysis. Figure 1 shows the flow of patients.

As no significant differences regarding ALT normalization and ALT decrease were found between the three groups receiving active treatment three times per week (80 mg (n=13), 160 mg (n=14), or 240 mg (n=14)), these 41 actively treated patients were combined into one group (the three times per week frequency group).

The baseline characteristics of the patients are shown in table I. About 80% of the patients were male; more than 80% of the patients were interferon or interferon/ribavirin non-responders. ALT levels were 2.5-3 times the upper limit of normal (ULN: 31 IU/l for female and 41 IU/l for male). The median HCV-RNA levels were around 10^7 genome equivalents per ml.

Table I

Baseline characteristics of the 69 patients evaluable for efficacy per group.

Treatment group	Placebo	3 x week	6 x week
number of patients	13	41	15
male/female	13/0	32/9	12/3
caucasian/other	8/5	23/18	11/4
median age* in years (range)	47 (37 - 60)	46 (32 - 69)	49 (39 - 70)
noncirrhosis/cirrhosis	7/6	24/17	7/8
previous interferon (ribavirin) yes/no	12/1	32/9	13/2
median ALT ULN** (range)	3.1 (1.5 - 6.8)	2.6 (1.4 - 11.8)	3.0 (1.6 - 12.5)
median HCV-RNA Mgeneq#./ml (range)	4.5 (1.4 - 39.2)	14.9 (0.2 - 104)	14.1 (0.7 - 76.3)
genotype-1/genotype non-1	7/6	20/21	7/8

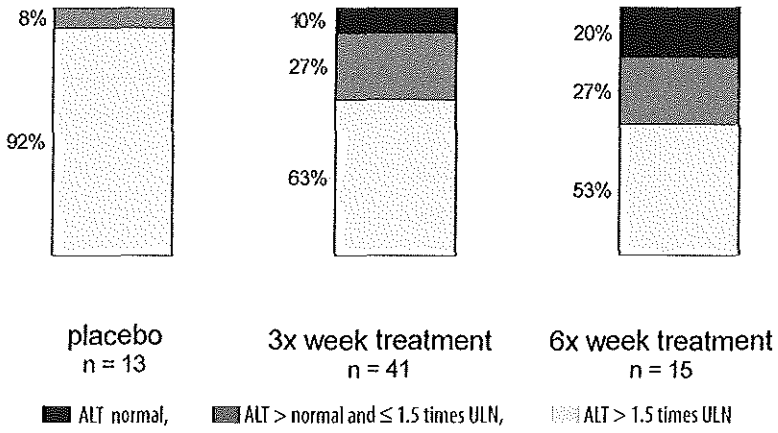
*at start of treatment ** upper limit of normal # Mega genome equivalent

Biochemical Response

For none of the groups there was a significant difference between ALT at screening and ALT at day 0. No significant changes were observed during study period in the placebo group.

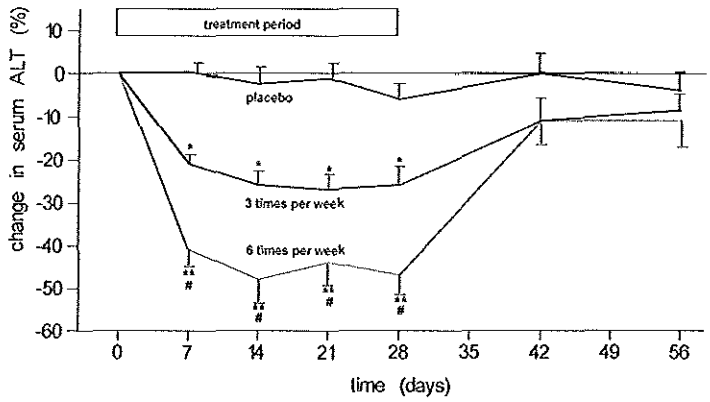
None of the patients receiving placebo showed ALT normalization at the end of treatment, 10% of the patients (4/41) after three times per week and 20% (3/15) of the patients after six times per week glycyrrhizin treatment reached normal ALT levels at the end of treatment; ALT normalization at the end of treatment was not significantly different between the three studied groups (p_{trend}=0.12). Figure 2 shows the distribution of ALT at the end of treatment.

After one week of treatment the mean percentage ALT decrease from baseline was 21% and 41% and at the end of treatment it was 26% and 47%, for the three times per week and six times per week treatment group, respectively (Figure 3). The mean percentage ALT decrease during active treatment (three times and six times per week) was significantly greater compared to placebo at all time points ($p<0.001$). Also the differences between both active treatment groups were significant ($p<0.002$). After cessation of active treatment ALT levels increased again; there was no significant difference between ALT at the end of follow-up and ALT at baseline for any of the groups.



Distribution of ALT at the end of treatment. ALT is expressed as upper limit of normal (ULN: male 41 IU/L, female 31 IU/L). Patients were treated with 3 times per week intravenous placebo or 3 or 6 times per week with intravenous glycyrrhizin for 4 weeks. Before treatment all patients had ALT levels at least 1.5 x ULN.

Figure 2



The mean percentages ALT change from baseline. Vertical bars indicate the standard error of the means. After active treatment the mean percentage ALT decrease was significantly lower compared to placebo (*$p<0.001$, **$p<0.0001$). The mean percentage ALT decrease at the end of treatment from baseline was 26% and 47% for three times and six times per week treatment, respectively. Six times per week treatment caused a larger decrease than three times per week (#$p<0.002$).

Figure 3

Virological Response

The HCV-RNA levels at baseline were high: range 0.2 – 104x10^6 genome equivalents per ml. HCV-RNA levels at the end of treatment did not differ significantly from those at baseline. None of the patients cleared HCV-RNA.

Safety and Tolerability

Expected side effects of intravenous glycyrrhizin treatment are haematoma at the injection place and pseudo-aldosteronism with: hypokalemia, sodium retention, elevated blood pressure and retention of body fluids.[13, 14] Only within the six times a week treatment group minor reversible symptoms of pseudo-aldosteronism occurred; the mean serum potassium decreased from 4.2±0.3 before treatment to 4.0±0.3 at the end of treatment (p=0.05) and the mean systolic blood pressure rose from 132±15 to 142±14 (p=0.01). There was no significant difference between cirrhotic and non-cirrhotic patients with regard to decrease of potassium or rise of systolic blood pressure during glycyrrhizin treatment. Ten patients suffered haematoma at the injection place. Headache, common cold, more tired than before treatment and strange feeling during administration of the medication were unexpected adverse events mentioned spontaneously by more than 5 patients (Table II). There were no clinically significant differences between the three frequency groups.

Table II

Number of patients who mentioned an adverse event spontaneously or after the question:"Have you had any (other) medical problem since your last visit?"

Adverse event/frequency group	0 x week (n=14)	3 x week (n=42)	6 x week (n=15)	total	p*
Headache	5	8	0[a]	13	0.03
Common cold	0	10	3	13	0.1
Haematoma at injection place	1	5	4	10	0.4
Strange feeling during administration	2	3	5[b]	10	0.04
More tired than before treatment	2	6	0	8	0.3

* overall p value (Fisher's exact test)

[a] significantly lower than placebo (p=0.01)

[b] significantly higher than three times per week group (p=0.03)

Discussion

The problem with most herbal remedies is, that there are no well conducted clinical trials in which their efficacy has been evaluated. During the congress "Complementary and Alternative Medicine in Chronic Liver Disease (NIH, Bethesda, Maryland, USA, August 1999) the need for such well conducted trials was stressed. Therefore we performed this study, according to Good Clinical Practice, in order to evaluate the short-term efficacy of glycyrrhizin treatment on serum ALT levels and the feasibility of 3-6 times per week intravenous glycyrrhizin administration in a European setting.

Clinically significant adverse events did not occur more often in the six times per week group compared to 3 times per week or placebo; none of the patients left the study because of treatment intolerance. So short-term treatment with 3-6 times per week intravenous glycyrrhizin appeared feasible and safe for patients with chronic hepatitis C or compensated cirrhosis.

We did not objectivate the quality of life of patients in this short-term study. Most patients were interferon nonresponders, so they were well aware of the benefits of selfinjection and the side effects of interferon treatment. However, a number of patients requested for longer treatment with glycyrrhizin instead of maintenance therapy with interferon, because they felt better during glycyrrhizin therapy. Therefore the quality of life during long-term glycyrrhizin treatment should be evaluated.

Five patients participating in part II received active treatment in part I. It is likely that patients who respond well to the treatment are willing to receive another treatment, while patients who did not respond are not willing to receive another treatment. The occurrence of this bias appears unlikely in our study because none of these 5 reached normal ALT levels during part I, while the mean percentage ALT decrease of these 5 was only 13%, in this part of the study.

The proportion of patients with ALT normalization at the end of treatment was higher in actively treated patients than in placebo, and higher in six times per week than three times per week glycyrrhizin administration; but the differences failed to reach statistical significance. However, due to the small numbers studied, real differences cannot be excluded. Comparing our data with the results reported by Arase *et al.*,[5] outcomes differed significantly between our placebo versus Arase's data (0% (0/13) vs. 36% (30/84), p=0.008) and our three times per week versus Arase's data (10% (4/41) vs. 36% (30/84), p=0.002), while no significant difference was found between our six times per week

versus Arase's data (20% (3/15) vs. 36% (30/84) p=0.4). This confirms that six times per week glycyrrhizin administration is more effective than three times per week or placebo.

The mean percentage ALT decrease at the end of treatment after 6 times per week treatment was significantly greater than after 3 times per week treatment (47% (95% CI: 38-57) vs. 26% (95% CI: 18-34) p=0.002). Within the three times per week treatment group the mean percentage ALT decrease did not show a linear dose response relationship, the mean percentage ALT was 23% (95% CI: 2-43), 26% (23-38) and 29% (21-38) after 80, 160 and 240 mg glycyrrhizin respectively. This suggests that not only a total week dosage of 1200 mg glycyrrhizin but also six times per week administration are necessary to reach a mean percentage ALT decrease of 47%. This hypothesis is corroborated by the half-life of glycyrrhizin, which is 8 hours in patients with chronic hepatitis C; administration of the medication every 24 hours results in a continuously detectable level of glycyrrhizin, while with 3 times per week glycyrrhizin administration, glycyrrhizin is not detectable anymore after 48 hours.[15]

Is treatment with glycyrrhizin only beneficial when ALT normalization occurs? Tarao *et al.* recently reported that persistently high ALT levels (>80 IU/L) in patients with chronic hepatitis C were associated with a more rapid development of hepatocellular carcinoma (HCC) than persistently low ALT levels (<80 IU/L). They followed 69 consecutive HCV patients with cirrhosis for more than 5 years; the 5-year rate incidence of HCC was 53.6% versus 7.1%, for the high ALT and low ALT group, respectively.[16] Therefore there is some evidence that not only patients with ALT normalization but also patients with an ALT decrease to below 80 IU/L might benefit from long-term glycyrrhizin treatment.

To confirm the efficacy of glycyrrhizin treatment with regard to the retardation of the progression of liver disease, a prospective long-term treatment trial in Europe should be performed. If this long-term study shows histological improvement, glycyrrhizin can be an important treatment in order to reduce the progression of the disease for patients who do not clear the hepatitis C virus.

We observed that the decrease of ALT, induced by glycyrrhizin treatment, occurs within the first two weeks of treatment, thereafter ALT levels remain stable. This is in agreement with the finding of Tsubota *et al.* that ALT levels did not significantly change during 3 times per week 200 mg glycyrrhizin therapy from 4-24 weeks.[17] Based on our current results and experience we suggest to start with an induction period in which glycyrrhizin is given 6 times per week for at least two weeks followed by a maintenance regimen with 3 times a week glycyrrhizin administration. The development of an oral form of

glycyrrhizin for maintenance therapy would be a more convenient option for long-term treatment.

The mechanism by which glycyrrhizin reduces the progression of liver disease without clearing the virus is unknown. A few *in vitro* and animal (rat) studies suggest that glycyrrhizin or its metabolite glycyrrhetinic acid inhibits lipid peroxidation, thereby protecting the hepatocytes.[18-21] Shiota *et al.* recently reported the first animal model in which they described that treatment with glycyrrhizin significantly reduced the occurrence of hepatocellular carcinoma in diethylnitrosamine treated mice.[22] The mechanism in this mouse-model is still unclear, but this model might help to unravel a possible mechanism of action.

In conclusion, it is feasible to treat European patients with 3-6 times per week intravenous glycyrrhizin for 4 weeks. Glycyrrhizin treatment induces a significant ALT decrease in patients with chronic hepatitis C. Six times per week treatment is more effective than three times per week treatment. To evaluate the benefit of treatment on liver histology a prospective long-term treatment study with glycyrrhizin should be performed in Europe.

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Chapter 3

Pharmacokinetics

Pharmacokinetics of intravenous glycyrrhizin after single and multiple doses in patients with chronic hepatitis C

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Abstract

Intravenous glycyrrhizin has been used in Japan for the treatment of chronic hepatitis for more than 20 years. Nevertheless, only brief reports of its pharmacokinetic profiles after multiple intravenous doses in small numbers of Japanese patients have been published.

The present study compared these Japanese data with the pharmacokinetic characteristics of glycyrrhizin after single and multiple intravenous doses in 35 European patients with chronic hepatitis C infection.

We administered 80, 160, or 240 mg glycyrrhizin 3 times/week or 200 mg glycyrrhizin 6 times/week for 4 weeks. Twenty-four-hour pharmacokinetic assessments were performed on day 1 and on or around day 14. Glycyrrhizin levels were determined by high-performance liquid chromatography.

The mean (±SD) volume of distribution at steady state on day 1 in the 80, 160, 200 and 240 mg groups were 67±11; 62±13; 54±7 and 66±8 mL/kg, respectively. The respective terminal elimination half-lives on day 1 were 7.7±2.8, 10.1±1.4, 9.0±2.3, and 8.6±2.1 hours. The area under the curve (AUC) increased linearly with doses ≤ 200 mg ($r=0.67$, $p<0.001$). No significant differences between day 1 and day 14 were found in any dose group, with the exception of AUC in the 200 mg group, which was significantly higher on day 14 compared with day 1 ($p=0.03$). Comparing the European and Japanese data, the mean (±SD) AUC was 289±244 μg*h/ml for the former and 402±372 μg*h/ml for the latter; the half-life was 8.2±2.6 versus 8.8±9.0 hours; and the total clearance was 7.6±3.6 versus 8.5±5.7 ml/h/kg.

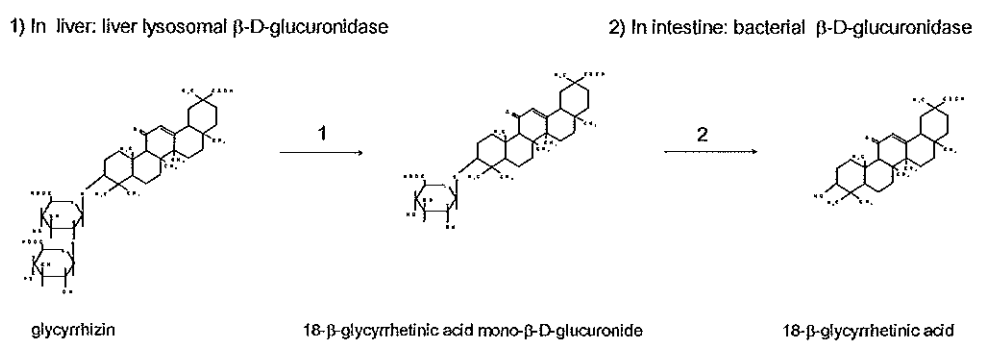
Thus our pharmacokinetic data are comparable to those from Japan. Glycyrrhizin's pharmacokinetics are linear up to 200 mg. Drug accumulation is seen after 2 weeks of treatment with 200 mg administered 6 times/week.

Introduction

Glycyrrhizin, extracted from the roots of the plant *Glycyrrhiza glabra* (liquorice), has been used as a treatment for chronic hepatitis in Japan for more than 20 years.[1] According to information from the manufacturer, Minophagen Pharmaceutical Co., Ltd. (Tokyo, Japan), tens of millions of ampoules Stronger Neo-Minophagen® C (SNMC) containing 2 mg of glycyrrhizin per ml are used annually for this indication in Japan.

Glycyrrhizin is a conjugate of one molecule of glycyrrhetinic acid and two molecules of glucuronic acid (Figure 1). Suzuki *et al.* reported that intravenously administered glycyrrhizin lowered the serum transaminase levels significantly in patients with chronic hepatitis.[2,3] Arase *et al.* reported that in Japanese patients with chronic hepatitis C infection, long-term treatment with glycyrrhizin prevented the development of hepatocellular carcinoma when alanine aminotransferase (ALT) normalizes.[4] Intravenously administered glycyrrhizin is metabolized in the liver by lysosomal β-D-glucuronidase to 3-mono-glucuronide-glycyrrhetinic acid. The metabolite is excreted with bile into the intestine, where it is metabolized by bacteria into glycyrrhetinic acid, which can be reabsorbed (Figure 1).[5]

A Medline search of the literature from January 1966 to July 1999 using the key words glycyrrhizin(e) and pharmacokinetics yielded a small number of Japanese studies involving 10 or less patients. These studies described the pharmacokinetic profile of glycyrrhizin after multiple doses at a single dose strength in patients with acute and chronic hepatitis and cirrhosis of different causes.[6-9] In these patients, the total body



Metabolism after intravenous administration of glycyrrhizin. Glycyrrhizin is metabolized in the liver by lysosomal β-D-glucuronidase to 3-mono-glucuronide-glycyrrhetinic acid; 3-mono-glucuronide-glycyrrhetinic acid is excreted with bile into the intestine where it is metabolized by bacteria to glycyrrhetinic acid that can be re-absorbed (enterohepatic cycle).

Figure 1

clearance was inversely correlated with serum ALT level ($r=-0.7$, $p<0.05$), suggesting a correlation of pharmacokinetic variables with hepatic function.

We conducted a Phase I/II clinical trial in Europe to evaluate the dose-dependent pharmacokinetics, safety and efficacy of glycyrrhizin treatment. The safety and efficacy results have been reported elsewhere.[10] The study reported here was performed to compare the Japanese data with pharmacokinetics of increasing doses of glycyrrhizin in European patients.

Patients and Methods

Only patients with chronic hepatitis C infection, with a positive hepatitis C virus RNA titer, serum ALT at least 1.5 times the upper limit of normal (ULN), and findings on liver biopsy consistent with mild to moderate liver fibrosis or cirrhosis were included. Patients were not eligible for inclusion if they had other causes of liver disease, decompensated cirrhosis (Child-Pugh score > 7) or hepatocellular carcinoma.

The study was conducted according to the Declaration of Helsinki and Good Clinical Practice. The protocol was approved by the Medical Ethical Committee of the University Hospital Rotterdam and all patients gave their written informed consent.

Study treatment

Glycyrrhizin was given as SNMC, a clear solution for intravenous use, consisting of 2 mg glycyrrhizin, 1 mg cysteine and 20 mg glycine per ml in physiological saline solution. Patients received 80, 160 or 240 mg intravenous glycyrrhizin 3 times/week or 200 mg intravenous glycyrrhizin 6 times/week for 4 weeks. The medication administered 3 times/week was given by drip infusion over 15 to 20 minutes in a total volume of 220 ml. The infusion line was then flushed with 25 ml sodium chloride (0.9%). The medication given 6 times/week was administered undiluted into a peripheral vein over 3 to 5 minutes.

Pharmacokinetic measurements

Pharmacokinetic measurements were obtained on the first day of treatment and on or around day 14. Patients were not allowed to consume food or drink (except water) after 11.00 p.m. of the night before pharmacokinetic measurements were to be taken. Food was allowed 4 hours after administration of medication, and water was allowed as required. Patients remained semirecumbent from 0.5 hours before until 4 hours after receiving medication.

Blood sampling

An indwelling cannula was placed in one arm for blood sampling; medication was admini-

stered in the other arm. Before sampling, approximately 1 ml blood was discarded. Ethylene-diamine tetraacetic acid-blood samples (7 ml) were collected at the following times: before administration of medication and at 0, 5, 15, 45, 60, 90 minutes, 2, 4, 6, 8, 10, 12, 16, 18 and 24 hours after administration of medication (at time 0, all the medication was administered). Samples were stored on ice, and plasma was separated within one hour by centrifugation (4° C and 3000 g for 10 min). Plasma samples were stored at -20° C until analyzed.

High-Performance Liquid Chromatography

Plasma samples were analyzed by a validated high-performance liquid chromatographic (HPLC) method modified from Raggi *et al.*.[11] Fifty µl propylparaben (25 mg/l internal standard solution) and 2 ml methanol were added to 500 µl plasma. After mixing and centrifugation, the supernatant was decanted into another test tube and evaporated at 40° C with flushing nitrogen. The residue was dissolved in 500 µl acetonitrile/citrate (180:320) buffer (0.1 mol/l, pH 2.8). After vortexing and centrifugation, 20 µl of the supernatant was injected into the HPLC-system. The extract was separated on a ChromSpher-5 C8 column (200 x 3 mm; 5 µm particles) with a acetonitrile/citrate buffer at a flow of 0.6 ml/min at ambient temperature. Detection was by ultraviolet absorption at 250 nm with a diode array detector.

As validated in our laboratory, the assay was linear over a range of at least 0.5-150 mg/l. The limit of quantification was 0.5 mg/l. Day-to-day variations were less than 2% at concentrations of >30 mg/l and less then 5% in the lower range (around 1 mg/l).

Pharmacokinetic Analysis

Weighted least-squares regression analysis, with $1/y^2$ as a weighting factor for each data point, was performed using Topfit version 2.0 to analyze the plasma concentration-time data for each patient.[12] We calculated the maximum concentration (C_{max}), total clearance (Cl_{tot}), distribution volume of the steady state (V_{ss}), area under the curve (AUC) from time zero to infinity, and terminal elimination half-life ($t_{½}$).

Data analysis was based on a weighted 3-compartment disposition model, which was deemed the most appropriate model based on visual inspection and minimized residuals.

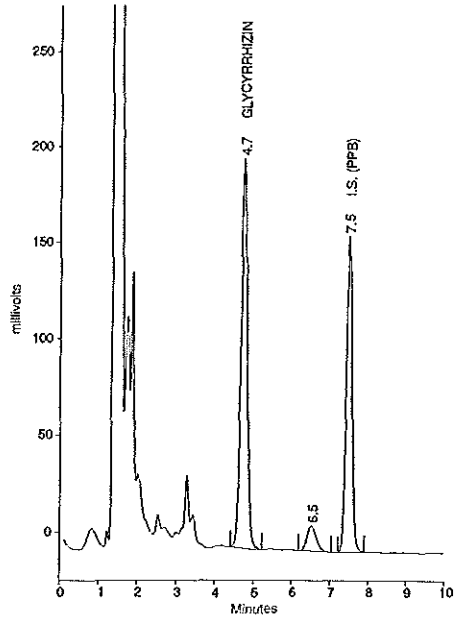
Statistical Analysis

Statistical analysis was performed using Stata 5.0 software (Stata Corporation, college Station, Texas, USA). Results are given as mean ± SD. Comparisons between groups were conducted using the Kruskal-Wallis test; if $p<0.05$ the Mann-Whitney test was used to perform further pairwise comparisons between groups. Differences within groups were

assessed with the Wilcoxon signed-rank test. Spearman correlation coefficients were used. Significance was set at p=0.05 (two-sided) for all tests.

Results

The baseline characteristics of patients are shown in Table I. The four groups were comparable in terms of all characteristics except baseline ALT value, which was significantly lower in group receiving 240 mg 3 times/week than in the group receiving 200 mg six times/week (p=0.02).



Chromatogram of a plasma sample obtained 6 hours after a single dose of 200 mg glycyrrhizin. The glycyrrhizin peak occurs after 4.7 minutes. I.S. = internal standard, PPB = propylparaben

Figure 2

Figure 2 shows a chromatogram 6 hours after a single dose of 200 mg glycyrrhizin. Figure 3 shows the mean course of the measured plasma concentration on day 1 and day 14 for the group receiving 200 mg glycyrrhizin 6 times/week. The plasma concentration of glycyrrhizin declined according to a 3-compartment model.

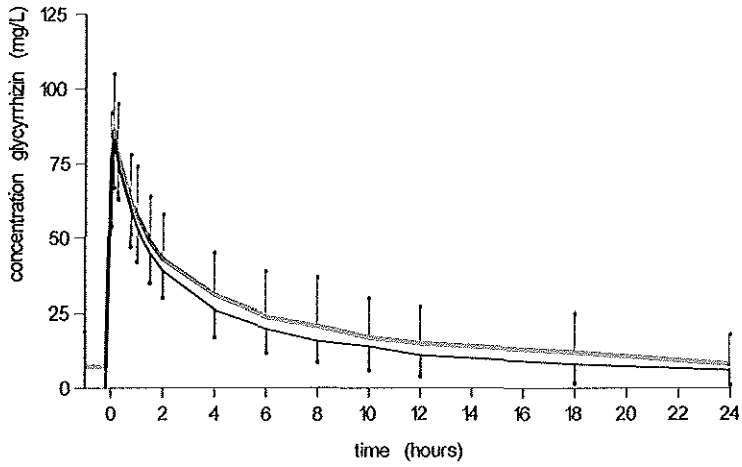
The second of the two pharmacokinetic measurements was performed on day 13 in 1 patient; day 14 in 27 patients; day 15 in 2 patients; and day 16 in 3 patients.

Table I

Baseline characteristics of the 35 patients per dosage group.

Dose (number of patients)	80 mg (8)	160 mg (7)	240 mg (7)	200 mg (13)
frequency of administration	3 times/wk	3 times/wk	3 times/wk	6 times/wk
male / female	6 / 2	5 / 2	7 / 0	11 / 2
age in years, mean (range)	47 (35-66)	54 (43-60)	43 (34-59)	50 (39-70)
weight in kg, mean (range)	71 (55-107)	78 (64-94)	79 (62-119)	79 (63-109)
cirrhosis / noncirrhosis	3 / 5	3 / 4	3 / 4	7 / 6
ALT (ULN), mean (95% CI)	3.8 (2.0-5.7)	3.6 (2.0-5.1)	2.1* (1.8-2.5)	3.9 (2.2-5.7)

* significantly lower than 200 mg 6 x week (p=0.02)



Mean measured plasma concentration-time profile of the 200 mg group on day 1 (black line) and day 14 (gray line), dots represent standard deviation. Decline of glycyrrhizin according to three-compartment model.

Figure 3

Results for the pharmacokinetic variables are shown in Table II. A dose-response relationship was found for both the C_{max} and the AUC. C_{max} increased in a log-log plot with increasing dosage (day 1, r=0.82, p<0.001; day 14, r=0.89, p<0.01); there was no significant deviation from linearity. A linear increase in AUC with dose was found only for the 80, 160 and 200 mg doses (day 1, r=0.67, p<0.001; day 14, r=0.57, p<0.002).

Table II

Pharmacokinetic results (expressed as mean ± standard deviation) by dosage group on days 1 and 14.

Dose frequency	80 mg 3 times/wk		160 mg 3 times/wk		240 mg 3 times/wk		200 mg 6 times/wk	
Day (number of patients)	day 1 (8)	day 14 (7)	day 1 (7)	day 14 (7)	day 1 (7)	day 14 (7)	day 1 (11)	day 14 (12)
C_{max} (mg/l)	42 ± 16	31 ± 4	70 ± 14	72 ± 11	102 ± 7	106 ± 16	112 ± 39	116 ± 40
V_{ss} (ml/kg)	67 ± 11	66 ± 10	62 ± 13	57 ± 15	66 ± 8	63 ± 10	54 ± 7	53 ± 14
AUC (µg*h/ml)	138 ± 76	112 ± 37	415 ± 156	466 ± 232	319 ± 66	345 ± 99	468 ± 210	574 ± 389
Cl_{tot} (ml/h/kg)	9.9 ± 3.3	10.8 ± 2.9	5.9 ± 2.5	5.7 ± 2.7	10.3 ± 3.1	9.8 ± 3.2	6.0 ± 2.6	5.5 ± 2.6
$t_{½}$ (h)	7.7 ± 2.8	6.2 ± 2.7	0.1 ± 1.4	10.2 ± 1.6	8.6 ± 2.1	6.6 ± 2.0	9.0 ± 2.3	9.1 ± 2.2

Table III

Total clearance (Cl_{tot}) and half-life ($t_{½}$) (expressed as mean ± standard deviation) for patients with chronic hepatitis and cirrhosis on days 1 and 14.

	Chronic hepatitis		Cirrhosis	
	day 1 (n=19)	day 14 (n=17)	day 1 (n=14)	day 14 (n=16)
Cl_{tot} (ml/h/kg)	8.3 ± 3.0	8.1 ± 3.2	7.2 ± 4.0	7.1 ± 4.0
$t_{½}$ (h)	8.2 ± 2.2	7.7 ± 2.5	9.7 ± 2.3	8.7 ± 2.7

The mean ± SD V_{ss} on day 1 was between 54±7 and 67±11 ml/kg; the mean Cl_{tot} was between 5.9±2.5 and 10.3±3.1 ml/h/kg; and the mean $t_{½}$ was between 7.7±2.8 and 10.1±1.4 hours. No significant difference was noted on day 1 and day 14 between dose groups, except for the significantly higher AUC in the 200 mg group on day 14 compared with day 1 (p=0.03). All variables showed a strong correlation between day 1 and day 14.

Table III shows the Cl_{tot} and $t_{½}$ for cirrhotic and noncirrhotic patients on day 1 and day 14 after combining all dose groups. There were no significant differences between cirrhotic and noncirrhotic patients. No correlation could be found between ALT levels at baseline and Cl_{tot} or $t_{½}$.

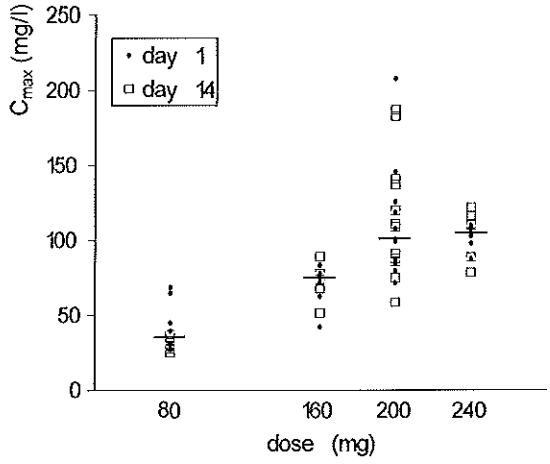
Discussion

This study reports the pharmacokinetic characteristics of increasing doses of intravenous glycyrrhizin after single and multiple doses in the largest cohort of patients (35 patients) studied to date.

The variation in C_{max} in the 200 mg group appeared to be substantially larger than in the other 3 groups (Figure 4). An explanation for this observation could be that the 200 mg dose was administered by manual direct intravenous injection in 3-5 minutes while the other 3 dosages were administered by drip infusion in 15-20 minutes; it is likely that the slower infusion rate caused a more equal administration of glycyrrhizin between patients than injection.

It is possible that different rates of infusion might affect the dose linearity and number of compartments. However, in our study, the AUC increased linearly with dose between 80 to 200 mg, although the 80 and 160 mg doses were administered over 15 to 20 minutes, and the 200 mg dose was given over 3 to 5 minutes. The pharmacokinetic data after both administration rates fitted best according to a 3-compartment model. Thus the difference between administration over 3 to 5 minutes or 15 to 20 minutes does not appear to affect dose linearity or compartmentalization.

With glycyrrhizin's half-life of approximately 9 hours, a dosing interval of 24 hours might lead to some accumulation. Indeed, the AUC for the group receiving 200 mg



Calculated maximum concentration (C_{max}) per patient per dose on day 1 and day 14. Line indicates the median per dose for day 1.

Figure 4

6 times/week was significantly higher at day 14 compared with day 1; after 14 days of treatment, the mean ± SD glycyrrhizin concentration before administration of medication was 7.8±11.0 mg/l.

Our pharmacokinetic data are based upon samples taken between 0 minutes and 24 hours after administration of medication. We observed a 3-compartment distribution over the entire period. Tanaka *et al.*[6] investigated the pharmacokinetic profile of multiple doses of intravenously administered glycyrrhizin (120 mg) in 8 patients with chronic hepatitis of unreported cause, and Yamamura *et al.*[7] investigated the same regimen in 4 patients with acute hepatitis and 6 patients with cirrhosis (5 of 6 cases were caused by chronic hepatitis C infection). In these studies, samples were taken between 2 and 10 hours after drug administration. Over this 8-hour period, the investigators observed a monoexponential decline in glycyrrhizin, as in our study.

Combining the pharmacokinetic data on the 8 patients from Tanaka *et al.*[6] and the 10 patients from Yamamura *et al.*[7] yields a mean ± SD $t_{½}$ of 8.8±9.0 hours, a Cl_{tot} of 8.5±5.7 ml/h/kg and an AUC of 402±372 µg*h/ml. These data obtained after multiple doses of glycyrrhizin are comparable to those obtained in our study (Table IV).

Table IV

Japanese and European pharmacokinetic data after multiple dosages (expressed as mean ± standard deviation)

	Japan (n=18)	Europe (n=33)
AUC for 120 mg (µg*h/ml)	402 ± 372	289 ± 244
Cl_{tot} (ml/h/kg)	8.5 ± 5.7	7.6 ± 3.6
$t_{½}$ (h)	8.8 ± 9.0	8.2 ± 2.6

The $t_{½}$ of glycyrrhizin in 3 healthy volunteers has been reported as 3.5 hours.[13, 14] Yamamura *et al.* observed an increase in $t_{½}$ and a decrease in Cl_{tot} among cirrhotic patients compared with noncirrhotic patients. We found no significant differences between cirrhotic and noncirrhotic patients (Table III). Cl_{tot} and $t_{½}$ are pharmacokinetic parameters that depend on physiological variables. Therefore it seems logical that a decrease in hepatic function would lead to a decrease in Cl_{tot} and an increase in $t_{½}$. We did not observe such a relationship in the present study, probably because patients with severe liver disease were excluded. Mean ALT levels before the initiation of treatment in the

Japanese patients were around 300 IU/l, much higher than our baseline ALT levels of approximately 3.5 times the ULN (or 150 IU/l).

Comparisons of pharmacokinetic data between centers should be interpreted cautiously. Variations in population, stage of disease, dose rate and regimen, sampling frequency and duration of observation are all capable of influencing study results. Given these limitations, we conclude that our data corroborate and strengthen those from the smaller studies.

The first phase of the 3-compartment model can be explained predominantly by distribution of glycyrrhizin. The V_{ss} was about 4.5 l per patient which means that glycyrrhizin was confined mainly to the vascular compartment. Glycyrrhizin is not taken up in blood cells.[13] The second phase can be explained by elimination, predominantly through the metabolism of glycyrrhizin to 3-mono-glucuronide-glycyrrhetinic acid in the liver by lysosomal β-D-glucuronidase.[5] The third phase can be explained by an enterohepatic cycle of glycyrrhizin, which would extend the elimination.[15, 16]

Conclusions

Glycyrrhizin exhibits linear pharmacokinetics up to 200 mg; steady state kinetics are attained after 2 weeks of 200 mg administered 6 times/week .

Our pharmacokinetic data are comparable to the Japanese findings, although we did not find a correlation between hepatic function and pharmacokinetics. This difference may be explained by our patients having milder liver disease.

Acknowledgments

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Chapter 4.1

Side effects

"Pseudo-aldosteronism" induced by intravenous glycyrrhizin treatment of chronic hepatitis C patients

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Summary

Background: Treatment with intravenous glycyrrhizin reduces the progression of liver disease caused by chronic hepatitis C (HCV) infection. Glycyrrhetinic acid, a metabolite of glycyrrhizin, inhibits the renal conversion of cortisol to cortisone by the enzyme 11β-hydroxysteroiddehydrogenase in the kidney. The resulting excess of cortisol subsequently stimulates the mineralocorticoid receptor, leading to pseudo-aldosteronism with hypertension, hypokalemia and eventually renin and aldosterone suppression.

Aim: To evaluate the occurrence of pseudo-aldosteronism after treatment of chronic hepatitis C patients with increasing doses of intravenous glycyrrhizin.

Design/patients: Forty-four HCV patients with chronic hepatitis or compensated cirrhosis were treated with intravenous glycyrrhizin 6 x 200 mg / week, or 3 x 240 mg / week or 3 x 0 mg / week (placebo) for 4 weeks.

Measurements: In all patients body weight, blood pressure, and plasma concentrations of sodium, potassium, cortisol, DHEA-S (dehydroepiandrosterone sulphate), renin and aldosterone were measured before and 0 and 4 weeks after treatment.

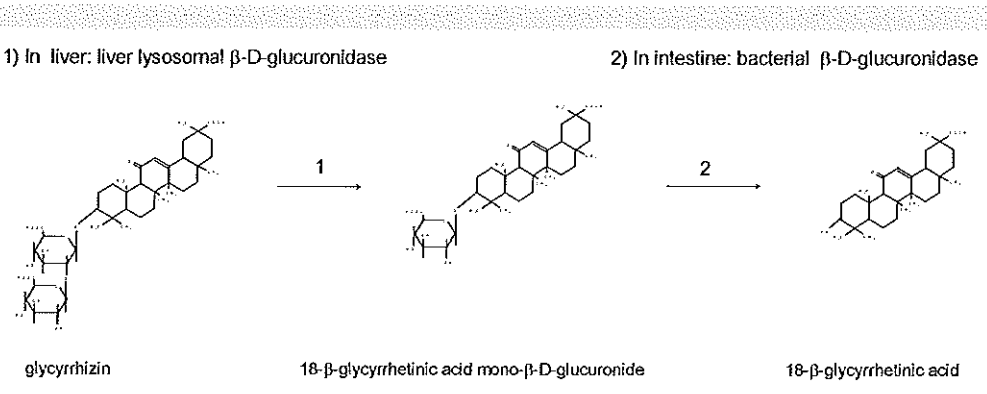
Results: Within the placebo group no significant changes were observed. Within the 1200 mg group systolic blood pressure was significantly higher at the end of treatment, while aldosterone was significantly lower; at the end of follow-up these values had returned to baseline. The changes from baseline in systolic and diastolic blood pressure at the end of treatment were significantly higher in the 1200 mg group compared to the placebo group. The changes in aldosterone and potassium concentrations at the end of treatment increased with increasing dosage although not significantly.

Conclusion: HCV patients with chronic hepatitis or compensated cirrhosis show minor reversible symptoms of pseudo-aldosteronism after treatment with 1200 mg glycyrrhizin weekly for 4 weeks.

Introduction

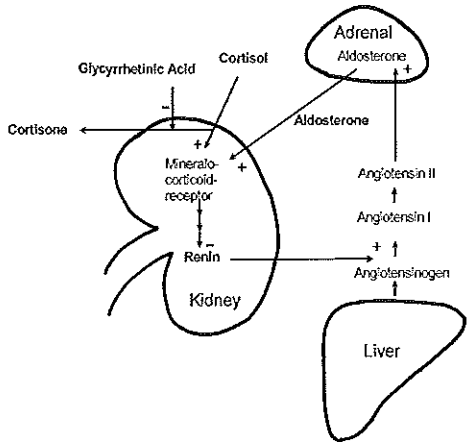
Glycyrrhizin, a natural compound extracted from the roots of Glycyrrhiza glabra, has been used for more than two decades to treat chronic hepatitis in Japan.[1] Glycyrrhizin is usually given as Stronger Neo-Minophagen C (SNMC), a clear solution for intravenous use containing 2 mg glycyrrhizin per ml. SNMC therapy significantly lowers the serum alanine aminotransferase (ALT) levels.[2,3] ALT normalization, induced by SNMC, reportedly reduces the progression of liver disease to hepatocellular carcinoma in patients with chronic hepatitis C infection.[4]

Glycyrrhizin (GL) is a conjugate of one molecule of glycyrrhetinic acid (GA) and two molecules of glucuronic acid. After intravenous administration GL is metabolized in the liver by lysosomal β-D-glucuronidase to 3-mono-glucuronide-glycyrrhetinic acid (3MGA). The human liver is not able to metabolize 3MGA to GA; 3MGA is excreted with bile into the intestine where bacteria metabolize it to GA, which is re-absorbed (Figure 1).[5] GA inhibits 11β-hydroxysteroiddehydrogenase (11β-HSD).[6] 11β-HSD is an enzyme of which two isoforms exist. The renal isoform 11β-HSD (type 2) has dehydrogenase activity and converts cortisol to cortisone; the liver isoform 11β-HSD (type 1) has oxo-reductase activity and catalyses the reverse reaction.[7] Inhibition of 11β-HSD type 2 leads to increased cortisol levels in the kidney. Because cortisol and aldosterone bind with the same affinity to the mineralocorticoid receptor, a rise in renal cortisol will result in stimulation of the mineralocorticoid receptor, leading to sodium retention, hypokalemia, hypertension and eventually to renin and aldosterone suppression (Figure 2).[8,9]



Metabolism of intravenously administered glycyrrhizin. Glycyrrhizin is metabolized in the human liver to 18-β-glycyrrhetinic-acid-mono-β-D-glucuronide. This is excreted with bile into the intestinal lumen where it is metabolized by bacteria to 18-β-glycyrrhetinic-acid. 18-β-glycyrrhetinic-acid is reabsorbed.

Figure 1



Glycyrrhetinic acid inhibits the enzymatic (11β-HSD type 2) conversion of cortisol to cortisone in the kidney. This results in an increased level of cortisol, which stimulates the mineralocorticoid receptor, eventually leading to inhibition of renin formation and, via the angiotensin system, inhibition of aldosterone formation.

Figure 2

Patients with liver disease have impaired cortisol metabolism;[10] there is a negative correlation between severity of the liver disease and cortisol levels after ACTH stimulation; baseline morning cortisol and dehydroepiandrosterone sulfate levels are comparable to those found for healthy subjects.[11]

The aim of this study was to evaluate the occurrence of pseudo-aldosteronism after treatment of chronic hepatitis C patients with different stages of liver disease with increasing doses of intravenous glycyrrhizin.

Methods

Subjects

Patients between 18 and 70 years of age were included if they had: serum antibodies to hepatitis C virus (HCV), a positive result for HCV-RNA; serum alanine aminotransferase (ALT) at least 1.5 x the upper limit of normal (ULN) (ULN = 41 IU/l for males and 31 IU/L for females) within the 8 weeks prior to treatment and at the start of treatment; and liver biopsy consistent with mild to moderate fibrosis, or cirrhosis with mild to moderate active hepatitis.

Patients were not eligible for inclusion if they met one or more of the exclusion criteria: other causes of liver disease (for example: chronic hepatitis B, alcohol abuse); decompensated cirrhosis (ascites), hepatocellular carcinoma or hypokalemia.

The study was conducted according to the Declaration of Helsinki and Good Clinical Practice. The protocol was approved by the Medical Ethical Committee of the University Hospital Rotterdam and all patients gave their written informed consent.

Treatment

Patients were treated with intravenous glycyrrhizin (Minophagen Pharmaceutical Company Tokyo, Japan) 6 x 200 mg / week, or 3 x 240 mg / week or 3 x 0 mg /week (placebo) for 4 weeks; the total weekly dosage of glycyrrhizin was 1200 mg, 720 mg or 0 mg, respectively. Some of the patients who received 3 x 240 mg / week or 0 mg glycyrrhizin were treated with 6 x 200 mg glycyrrhizin / week after a wash-out period of at least 6 months. The follow-up after treatment was also 4 weeks. Patients were not allowed to eat liquorice during the study period. Patients were treated on an out-patient basis.

Assessments

Body weight, blood pressure, and plasma concentrations of sodium, potassium, cortisol, DHEA-S (dehydroepiandrosterone sulphate), renin and aldosterone were measured before start of treatment, at the end of treatment and at the end of follow-up. Samples were taken between 08.00 and 10.00 a.m..

Sodium (reference value 135-145 mmol/l) and potassium levels (reference value 3.6-5.1 mmol/l) were determined by routine assays. Cortisol (reference value: 200-800 nmol/l) and DHEA-S concentrations (reference values: males, age 40-49: 2.5-14 µmol/l and females, age 40-49: 0.8-7 µmol/l) were determined in heparin plasma using immulite 1 (Diagnostic Products Corporation, Los Angeles, CA, USA). Renin (reference value: 5-45 µU/ml) was determined in serum by radio immunoassay of generated angiotensin I.[12] Aldosterone (reference value: 139-693 pmol/l) was determined in serum by a commercially available radio immunoassay kit (Coat-A-Count, Diagnostic Products Corporation).

Statistical analysis

Results are expressed as mean ± standard deviation unless stated otherwise. Changes within groups were assessed using the Wilcoxon signed-rank test. Changes between the three dosage groups, taking into account the fact that the group with 6 x week administration consisted partly of patients who also participated in the placebo and 3 x week glycyrrhizin group, were compared with ANOVA.[13] Correlation coefficients given are Spearman's. P-values ≤ 0.05 (two-sided) were considered to be significant, except for within-group comparisons. These were tested at p=0.017 (i.e. 0.05/3) in accordance with Bonferroni's principle.

Results

Table I shows the baseline characteristics of all patients. The three groups were comparable. Each group consisted of 14 or 15 patients; most of the patients were male. The mean age was 47 years (range 25 - 70).

Table II shows the results in detail. The baseline values for the three groups were comparable. Within the placebo group no significant changes were observed. Within the 720 mg per week group, cortisol was significantly lower at the end of follow-up than at baseline (304±153 vs. 407±143 nmol/l). DHEA-S was significantly decreased at the end of treatment and increased till baseline levels after cessation of treatment.

Within the 1200 mg per week group, bodyweight was significantly increased with respect to baseline at the end of treatment as well as at the end of follow-up (82.1±14.3 and 81.7±14.6 vs. 78.6±11.5 kg at baseline). The systolic blood pressure was significantly higher at the end of treatment than at baseline (142±14 vs. 132±15 mmHg). Renin was significantly higher at end of follow-up than at the end of treatment (12.9±10.0 vs. 8.4±11.4 μU/ml). Aldosterone was decreased significantly at the end of treatment and returned to baseline levels at the end of follow-up.

Changes with respect to baseline at the end of treatment were compared between the three groups. Systolic and diastolic blood pressure showed a significant rise with increasing dosage (Figure 3). The decrease in aldosterone and potassium increased with

Table I

Baseline characteristics of the patients per dosage group, expressed as mean ± SD.

group (number of patients)	0 mg (14)	720 mg (15)	1200 mg (15*)
male / female	13 / 1	11 / 4	12 / 3
age (year)	48±7	43±10	51±9
height (cm)	178±8	173±7	175±8
weight (kg)	84±14	78±16	79±11
cirrhosis / noncirrhosis	6 / 8	6 / 9	8 / 7
ALT (upper limit of normal)	3.1±1.5	2.5±1.0	3.7±2.7
systolic blood pressure (mmHg)	131±13	126±12	132±15
diastolic blood pressure(mmHg)	82±9	82±5	79±10

*9 patients received 0 mg or 240 mg glycyrrhizin 3 x week at least 6 months before the 6 x week treatment

Table II

Symptoms of pseudo-aldosteronism after 4 weeks of glycyrrhizin treatment are shown per dosage group, before treatment (BT), at the end of treatment (EOT) and at the end of 4 weeks of follow-up (EOF). Results are expressed as mean ± standard deviation unless stated otherwise. Since there were 3 groups, within group comparisons were tested at p = 0.017 (i.e. 0.05/3) in accordance with Bonferroni's principle.

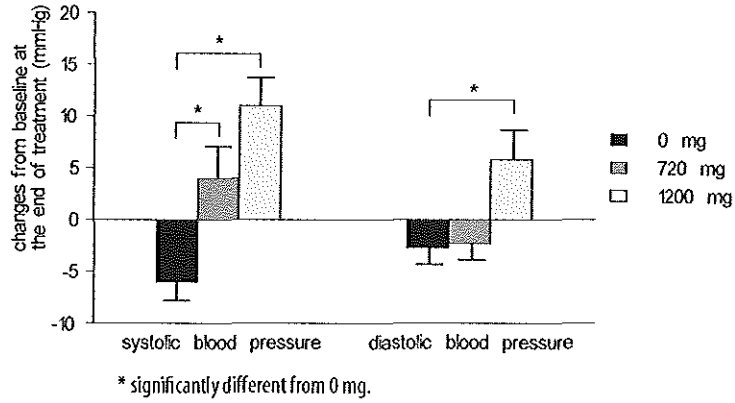
Total weekdose	**0 mg (n=14)**			**720 mg (n=15)**			**1200 mg (n=15)**		
	BT	EOT	EOF	BT	EOT	EOF	BT	EOT	EOF
bodyweight (kg)	84.4±13.6	85.3±14.5	85.0±14.1	78.4±16.0	78.9±15.9	84.4±13.6	78.6±11.5	82.1[b]±14.3	81.7[b]±14.6
systolic blood pressure (mmHg)	131±13	125±13	126±15	126±12	130±14	128±13	132±15	142[b]±14	137±24
diastolic blood pressure (mmHg)	82±9	79±8	80±7	82±5	80±8	79±7	79±10	85±7	81±10
sodium (mmol/l)	140±2	140±1	141±2	141±2	141±2	140±2	141±1	141±2	140±1
potassium (mmol/l)	4.1±0.5	4.1±0.3	4.1±0.2	4.1±0.5	4.0±0.4	4.0±0.4	4.2±0.3	4.0±0.3	4.2±0.2
cortisol (nmol/l)	313±125	307±94	309±123	407±143	313±80	304[a]±153	305±129	326±95	343±118
DHEA-S (µmol/l)	2.1±1.4	1.9±1.1	1.9±1.4	2.8±2.1	2.4[a]±1.9	2.8[c]±2.0	1.9±1.4	1.6±1.2	1.7±1.3
renin (µU/ml)*	15 (6,33)	17 (3,55)	15 (5,29)	13 (6,90)	10 (3,128)	21 (9,120)	11 (3,25)	4 (0.1,41)	8[c] (2.9,32)
aldosterone (pmol/l)*	177 (39,551)	130 (89,485)	139 (47,532)	222 (30,1177)	136 (25,950)	213 (86,1299)	163 (64,701)	97[a] (33,302)	172[c] (61,488)

[a] significantly lower than before treatment;

[b] significantly higher than before treatment;

[c] significantly higher than end of treatment

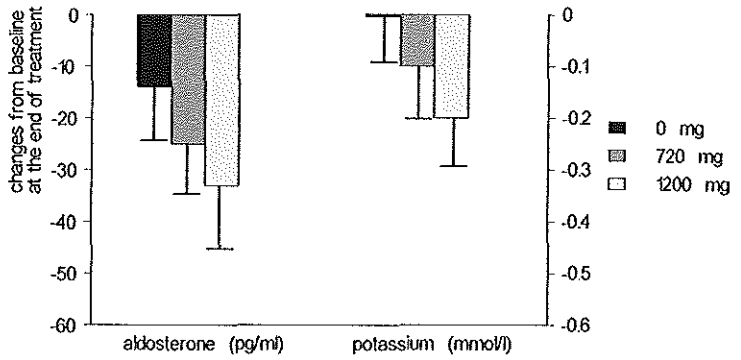
* median (range)



* significantly different from 0 mg.

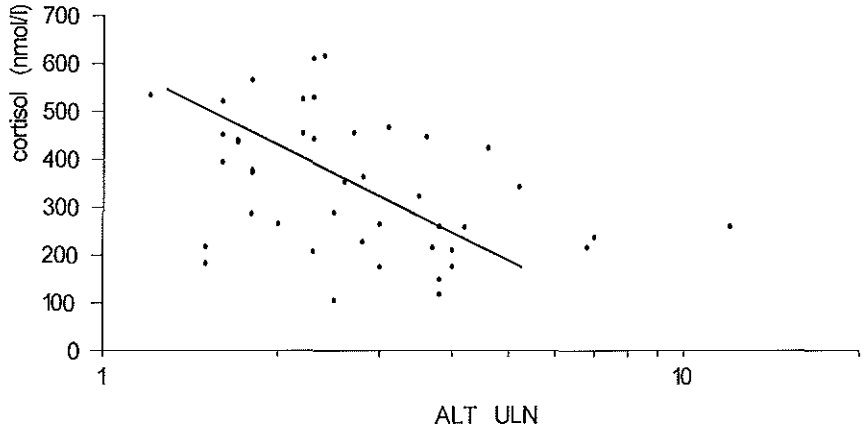
The changes (mean, SEM) from baseline at the end of treatment in systolic and diastolic blood pressure after four weeks therapy with 0 mg, 720 mg or 1200 mg glycyrrhizin weekly.

Figure 3



The changes (mean, SEM) from baseline at the end of treatment in aldosterone (left y-axis) and potassium (right y-axis) after four weeks therapy with 0 mg, 720 mg or 1200 mg glycyrrhizin weekly. The data suggest that with increasing dosage aldosterone and potassium decrease more. Because the variations within the three groups were large, no statistically significant difference was found.

Figure 4



The ALT at baseline (expressed as times the upper limit of normal (ULN); ULN = 41 IU/l for males and 31 IU/l for females) shows a negative correlation with the cortisol level at baseline (rho=-0.5, p=0.002)

Figure 5

increasing dosage although the changes were not significantly different between the three group (Figure 4). The changes in bodyweight, sodium, cortisol, DHEA-S and renin did not differ significantly between the three groups.

Cortisol at baseline was negatively correlated with the ALT at baseline (r=-0.5, p=0.002) (Figure 5). There was no correlation between the cortisol level at baseline and stage of fibrosis. No correlations were observed between ALT at baseline or stage of fibrosis and DHEA-S, aldosterone or renin.

Discussion

Treatment with 1200 mg glycyrrhizin weekly caused a significant increase in systolic blood pressure and bodyweight and a significant decrease in aldosterone; after cessation of therapy systolic blood pressure and aldosterone returned to baseline values. These observations suggest the occurrence of pseudo-aldosteronism caused by glycyrrhizin treatment. The increasing changes with respect to baseline at the end of treatment in aldosterone and potassium levels and systolic and diastolic blood pressure with increasing dosages (Figure 3, 4) confirm the impression of the occurrence of pseudo-aldosteronism. The change in the systolic and diastolic blood pressure was significantly higher in the 1200 mg group compared to the placebo group. It is likely that the large variations in

outcome within the three groups explain why no significant changes in the aldosterone or potassium levels between the three groups were observed.

At the end of follow-up bodyweight within the 1200 mg group was still higher than before treatment. Because all other symptoms of pseudo-aldosteronism returned to baseline values it is unlikely that this increased bodyweight is a remainder of pseudo-aldosteronism.

To prevent the occurrence of pseudo-aldosteronism in the general population, the recommended maximum oral intake is 200 mg glycyrrhizin per day which is 1400 mg per week.[14] On basis of our study results we recommend that patients with chronic hepatitis or compensated cirrhosis should not be treated with a dose exceeding 1200 mg glycyrrhizin weekly; at this dosage some minor symptoms of pseudo-aldosteronism occur and if the dosage is further increased the symptoms of pseudo-aldosteronism are likely to increase.

Patients with cirrhosis and ascites have elevated plasma renin and aldosterone levels.[15] It is likely that this secondary aldosteronism, caused by a hyperkinetic circulation in decompensated cirrhosis with portal hypertension, will be reinforced by treatment with glycyrrhizin. In our study only patients with compensated cirrhosis were included; none of these cirrhotic patients had elevated renin or aldosterone levels before treatment. Patients using thiazide diuretics, which enhance potassium loss, are at risk of developing severe hypokalemia during glycyrrhizin treatment.[16] Hypokalemia, induced by glycyrrhizin, can cause cardiac arrhythmias, cardiac arrest, rhabdomyolysis with clinical presentation of muscle weakness, paraparesis or quadriplegia.[17-21] Therefore, patients with cirrhosis and ascites and patients using thiazide diuretics should be monitored carefully during glycyrrhizin treatment. In Japan hypokalemia and hypertension during glycyrrhizin treatment are successfully treated with the mineralocorticoid receptor antagonist spironolactone.[4]

We observed a negative correlation between ALT levels and cortisol at baseline: the severity of liver disease (ALT) was inversely correlated with cortisol. This finding is in agreement with the observation reported by McDonald *et al.*.[11] They found a negative correlation between the Child-Pugh score (range 5-15) and baseline cortisol levels. Because we included only patients with compensated cirrhosis (with a Child-Pugh score of 5-7) we correlated baseline cortisol levels with ALT and stage of fibrosis at baseline instead of the Child-Pugh score. Ninety percent of the serum cortisol is bound to the liver derived protein cortisol binding globulin (CBG). In patients with liver disease the CGB levels are significantly lower than in healthy persons.[11] Therefore the most likely explanation for the negative correlation between serum cortisol at baseline and severity of

liver disease is a fall in hepatic CBG production. To keep the unbound "active" plasma cortisol level unchanged total cortisol levels should be lower, which is in agreement with our findings that relatively low levels of plasma cortisol were found. On the other hand, in rats with liver cirrhosis the activity of hepatic and renal 11β-hydroxysteroid dehydrogenase is downregulated;[22] this might eventually lead to increased plasma cortisol levels.

The hypothesis that inhibition of the renal conversion of cortisol to cortisone by glycyrrhizin might cause a decrease in cortisol metabolism, which would lead to an inhibition of the hypothalamic-pituitary-adrenocortical feedback mechanism and thereby a decrease in DHEA-S concentrations, was not confirmed in this study; DHEA-S decreased only in the lowest GL dose group not in the highest GL group. This could be explained by the fact that the rise in cortisol in the kidney during GL treatment was sufficient to stimulate the mineralocorticoid receptor, whereas the actual plasma level of cortisol hardly increased during GL treatment so that the decrease in DHEA-S found in the lowest GL group might be considered to be a coincidence.

In conclusion patients with chronic hepatitis or compensated cirrhosis show minor reversible symptoms of pseudo-aldosteronism after treatment with 1200 mg glycyrrhizin weekly for 4 weeks.

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Chapter 4.2

Side effects

Daily intake of 200 g licorice does not reduce testosterone levels in men

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unpublished data

Daily intake of 200 g licorice does not reduce testosterone levels in men

Glycyrrhizin, extracted from the roots of *Glycyrrhiza glabra* is used as flavoring agent and as treatment for chronic hepatitis C.[1] A well known side effect of glycyrrhizin use is pseudo-aldosteronism.[2] Recently Armanini *et al.* reported in a letter in the New England Journal of Medicine that daily oral intake of 500 mg glycyrrhizin significantly reduced serum testosterone levels in seven men.[3] These authors suggested that the activity of 17β-hydroxysteroid dehydrogenase was inhibited. However, levels of androstenedione were not affected; which should be the case if such an enzyme block would be induced.[4] In order to investigate alternative mechanisms behind the decreased testosterone levels we evaluated testosterone, FSH and LH levels in thirty-five male patients with chronic hepatitis C treated with 0 mg glycyrrhizin, three times per week 240 mg glycyrrhizin or 6 times per week 200 mg glycyrrhizin for four weeks. Medication was administered intravenously, follow-up after treatment was also 4 weeks. The effects on body weight, systolic blood pressure, serum potassium, aldosterone and renin in our patients receiving 200 mg glycyrrhizin six times per week were comparable to those in the group receiving 500 mg glycyrrhizin daily described by Armanini *et al.*[3, 5] (data not shown). However, none of our groups showed a significant change of testosterone, FSH or LH serum levels during the study period (Table I). Armanini *et al.* stated that 500 mg of glycyrrhizin daily is eaten by many people.[3] However, the mean daily intake of glycyrrhizin in the United

Table I

Serum testosterone, FSH and LH concentrations in male chronic hepatitis C patients before treatment (BT) with 0 mg, 3 times 240 mg or 6 times 200 mg glycyrrhizin per week, at the end of treatment (EOT) and at the end of follow up (EOF).

Within none of the groups a significant change of testosterone, FSH or LH serum levels during the study period was observed.

Results are expressed as mean ± standard deviation.

Treatment week dose	0 mg (n=12)			720 mg (n=11)			1200 mg (n=12)		
Hormone (ref. value male)	BT	EOT	EOF	BT	EOT	EOF	BT	EOT	EOF
Testosterone									
(nmol/l) (10-30)	29±7	29±6	29±6	26±8	26±9	24±7	28±8	30±8	27±8
FSH (U/l) (2.0-7.0)	7±5	7±5	8±5	7±7	7±7	7±8	8±6	9±6	9±6
LH (U/l) (1.5-8.0)	4±3	6±4	6±4	4±3	4±2	5±3	6±3	6±4	6±4

Kingdom, USA and Belgium is 1, 3 and 5 mg per person, respectively. Depending on the quality of confectionery 1 g of licorice contains approximately 1 mg glycyrrhizin.

We conclude that daily administration of 200 mg glycyrrhizin does not influence testosterone levels. Therefore, the occurrence of reduced serum testosterone levels due to daily intake of 200 g licorice is unlikely.

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Chapter 5.1

Mechanism

The effect of glycyrrhizin and its metabolite glycyrrhetinic acid on proliferation of peripheral blood mononuclear cells

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unpublished data

Summary

Background: ALT decrease during glycyrrhizin treatment for chronic hepatitis C might be caused by an inhibiting effect on the immune mediated damage of hepatocytes. It is unknown whether glycyrrhizin or its metabolite glycyrrhetinic acid is responsible for the ALT decrease.

Aim: To evaluate the effect of glycyrrhizin and glycyrrhetinic acid *in vitro* on the proliferation of peripheral blood mononuclear cells (PBMCs) of a healthy donor and of an untreated patient with chronic hepatitis C.

Methods: PBMCs were incubated with increasing dosages of glycyrrhizin (1 – 1000 µg/ml), glycyrrhetinic acid (1 – 235 µg/ml) or dexamethasone; cells were stimulated with phytohemagglutinin (PHA) and after three days pulsed with tritium thymidine. Proliferation was measured after 16 hours and expressed as counts per minute.

Results: Increasing dosages of dexamethasone inhibited proliferation. Glycyrrhizin did not influence proliferation. Glycyrrhetinic acid turned out to be toxic at higher concentrations (100 µg/ml); it is unlikely that these concentrations can be reached *in vivo*. The responses after incubation of the healthy donor's PBMCs were comparable to those of cells of the untreated patient.

Conclusions: This pilot study did not provide support for a role of glycyrrhizin on PBMCs nor a clue whether glycyrrhizin or glycyrrhetinic acid is the active molecule with regard to ALT decrease.

Introduction

In Japan, glycyrrhizin - extracted from the roots of the plant *Glycyrrhiza glabra* (licorice) - has been used for the treatment of chronic hepatitis for more than 20 years.[1] Glycyrrhizin treatment significantly reduces alanine aminotransferase (ALT) levels in patients with chronic hepatitis C.[2,3] ALT normalization induced by long-term intravenous glycyrrhizin treatment (median 10.1 years) prevented the development of hepatocellular carcinoma in chronic hepatitis C patients in Japan.[4] After intravenous administration glycyrrhizin is metabolized in the human body to glycyrrhetinic acid;[5] after oral administration glycyrrhizin is metabolized to glycyrrhetinic acid in the intestinal lumen before absorption,[6] hardly any glycyrrhizin will be taken up in the body.[7]

It is unknown whether glycyrrhizin, glycyrrhetinic acid or both molecules are responsible for the ALT decrease / ALT normalization in chronic hepatitis C patients. Both glycyrrhizin and its metabolite glycyrrhetinic acid are pharmacologically active substance.[8] If glycyrrhizin is the active substance with regard to inducing ALT decrease intravenous administration is essential, if glycyrrhetinic acid is the active substance oral administration may also be effective.

There is some evidence that glycyrrhizin inhibits immune mediated damage of hepatocytes;[9, 10] glycyrrhizin reportedly induced apoptosis of T-lymphocytes.[11] We evaluated in this *in vitro* study the effect of increasing dosages of glycyrrhizin and glycyrrhetinic acid on proliferation of peripheral blood mononuclear cells of a healthy donor and of an untreated hepatitis C virus (HCV) patient.

Material & methods

Peripheral blood mononuclear cells (PBMCs) from an untreated HCV-patient and from a healthy donor were separated from heparinized blood by Ficoll-Hypaque gradient centrifugation. Cells were seeded into 96-well round bottom microtitre culture plates at 1 x 10^5 cells / well in the presence of phytohemagglutinin (PHA) (1 µg/ml) (Sigma, Zwijndrecht, the Netherlands). Cells were at the same time incubated with medium (negative control), dexamethasone (0.1, 1, 10 or 50 µM, positive control), glycyrrhizin (1, 5, 10, 50, 100, 500, 1000 µg/ml) or glycyrrhetinic acid (1, 5, 10, 50, 100, 235 µg/ml).

Glycyrrhizin (glycyrrhizic acid ammonium salt, Fluka Chemie, Steinheim, Switzerland) was dissolved in phosphate buffer saline (PBS) (Sigma, Zwijndrecht, the Netherlands) by lowering the pH to 4 followed by pH normalization to 7.6; 18β-Glycyrrhetinic acid (Fluka Chemie, Steinheim, Switzerland) was dissolved in dimethyl sulphoxide (DMSO) (Sigma, Zwijndrecht, the Netherlands) and subsequently further diluted with Roswell

Park Memorial Institute culture medium (RPMI) (Gibco BRL, Breda, the Netherlands) with 10% human serum (R10H); dexamethasone (di-Na-phosphate) (Pharmacy AZR, Rotterdam, the Netherlands) was dissolved in R10H.

After three days of incubation, cultures were pulsed with 0.25 μCi ^{3}H-thymidine (Amersham, Breda, the Netherlands) and after 16 h the cells were harvested and counts per minute (cpm) determined using a scintillation counter (Beckman Instruments, Leusden, the Netherlands) Tests were performed in triplicate; each assay was repeated three times.

Results

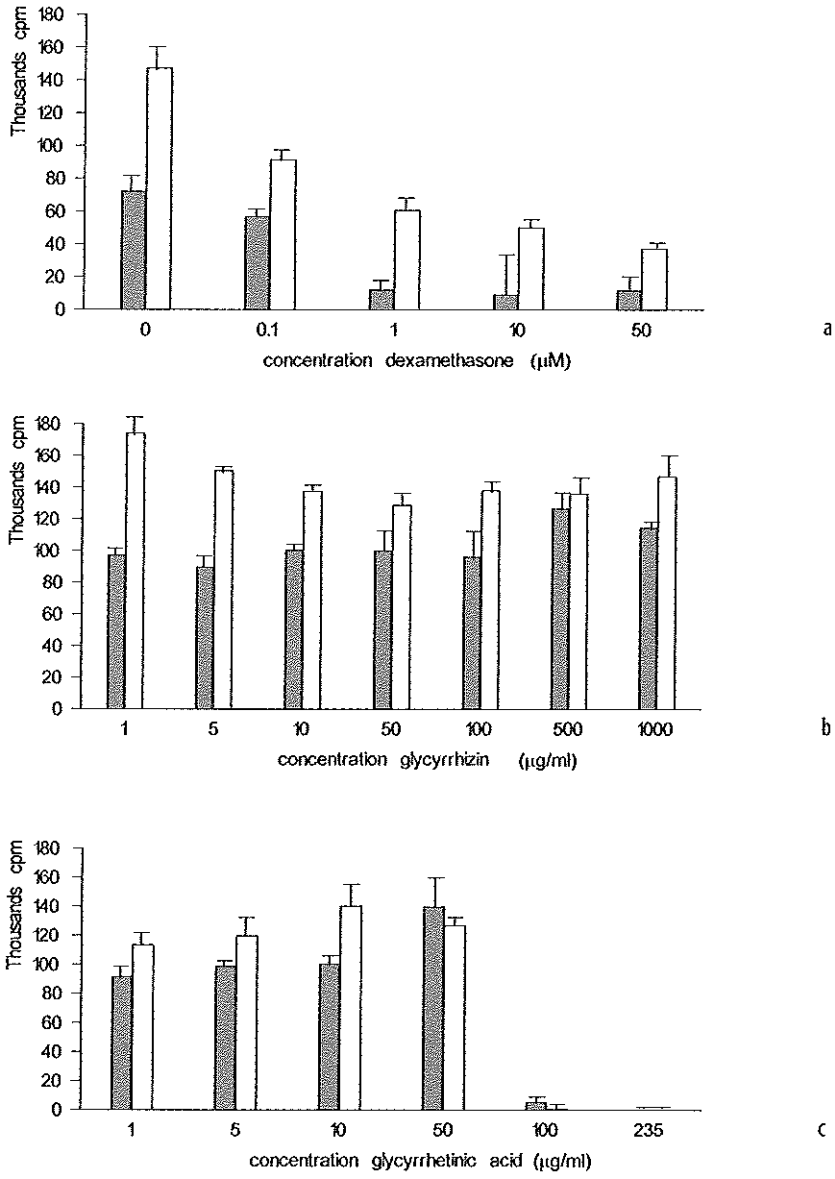
Figure 1 a-c shows the results for the healthy donor and the naive HCV patient. The PBMCs of the untreated HCV patient reached higher levels of ^{3}H-thymidine incorporation after stimulation compared to the healthy donor. The responses after incubation with increasing concentrations dexamethasone, glycyrrhizin and glycyrrhetinic acid of the PBMCs of the healthy donor were comparable to those of the naive HCV-patient. Increasing dosage of dexamethasone inhibited the proliferation of the PBMCs. The addition of increasing dosages of glycyrrhizin had no effect on proliferation. Up to 50 μg/ml the addition of glycyrrhetinic acid did not influence proliferation. At higher concentrations (100 μg/ml and 235 μg/ml) proliferation was almost completely inhibited. Viability tests with trypan blue revealed that at these concentrations the cells were dead.

Discussion

We performed this *in vitro* study to evaluate whether increasing dosages of glycyrrhizin or glycyrrhetinic acid could inhibit the proliferation of PBMCs.

Treatment of chronic HCV patients with corticosteroids reduces ALT levels and increase HCV-RNA levels.[12] A reported mechanism of action of glucocorticoids is the induction of apoptosis in human monocytes.[13] Treatment with glycyrrhizin reduces or normalizes ALT without influencing the viral load.[2] Glycyrrhizin is a conjugate of one molecule of glycyrrhetinic acid and two molecules of glucuronic acid; glycyrrhetinic acid has a steroid like structure. It might therefore be possible that glycyrrhizin or glycyrrhetinic acid act in the same way as corticosteroids by inhibiting the immune mediated damage of liver cells.

Dexamethasone was successfully used as a positive control: a dose dependent inhibition of proliferation was observed. Incubation with glycyrrhizin did not influence the proliferation of PBMCs. The plasma levels of glycyrrhizin in HCV patients treated with the maximal recommended therapeutic intravenous dosage (=200 mg glycyrrhizin daily)



PHA induced proliferation expressed as counts per minute (cpm) of peripheral blood mononuclear cells of a healthy donor (gray bars) and an untreated HCV-patient (white bars) after incubation with increasing dosages of dexamethasone (a), glycyrrhizin (b) and glycyrrhetinic acid (c).

Figure 1 a-c

were between 0.5-200 mg/l.[14] Therefore we used in this *in vitro* study concentrations of 1, 5, 10, 50, 100, 500 and 1000 µg/ml glycyrrhizin.

Glycyrrhetinic acid inhibited proliferation almost completely at higher concentrations (≥ 100 µg/ml), this was a cytotoxic effect. Plasma concentrations of glycyrrhetinic acid after intravenous treatment with 120 mg glycyrrhizin were between 0.5-1.7 µg/ml.[15] After oral administration of glycyrrhizin the plasma levels of glycyrrhetinic acid are less than 0.2 µg/ml.[16] Based upon these literature data it is unlikely that after intravenous administration of 200 mg glycyrrhizin plasma levels of more than 100 µg/ml are reached, indicating that the inhibitory effect of glycyrrhetinic acid observed *in vitro* is most likely not the explanation for the ALT lowering effect during glycyrrhizin treatment *in vivo*.

Oh *et al.* reported that glycyrrhizin induced apoptosis in mice splenocytes and thymocytes *in vitro* as well as *in vivo*.[11] We used PBMCs to evaluate the effect of glycyrrhizin and glycyrrhetinic acid on proliferation *in vitro*. Probably PBMCs are not the most appropriate cells to evaluate the effect of glycyrrhizin and glycyrrhetinic acid on the immune system; splenocytes or Kupffercells from the liver might be a more appropriate choice.

Conclusions

This pilot study did not provide support for a role of glycyrrhizin on PBMCs nor a clue whether glycyrrhizin or glycyrrhetinic acid is the active molecule with regard to ALT decrease. Further investigation is warranted to determine whether glycyrrhizin or glycyrrhetinic acid acts as an inhibitor of immune mediated damage in chronic hepatitis C infection and whether glycyrrhizin or glycyrrhetinic acid is the active substance with regard to ALT decrease *in vivo*.

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Chapter 5.2

Mechanism

ALT lowering effect of glycyrrhizin treatment might be caused by an indirect effect on serum cortisol

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unpublished data

Summary

Background: The mechanism by which glycyrrhizin lowers ALT without affecting viral replication has not yet been established. There are many *in vitro* and animal studies which suggest an array of different mechanisms.

Aim: To gain some insight into the mechanism of action of glycyrrhizin in humans treated with glycyrrhizin. We evaluated whether glycyrrhizin might act by its interference with cortisol metabolism, inhibition of soluble vascular adhesion protein-1 (s-VAP-1) formation, increasing antioxidant capacity of plasma or by acting as an immunomodulator.

Methods: Forty-four patients with chronic hepatitis C or compensated C cirrhosis were treated with intravenous glycyrrhizin 6 x 200 mg / week, or 3 x 240 mg / week or 3 x 0 mg / week (placebo) for 4 weeks. Plasma concentrations of ALT, cortisol, DHEA-S (dehydroepiandrosterone sulphate), s-VAP-1, IFN-γ, total antioxidant capacity of the plasma and total number of leukocytes were measured before and at the end of treatment, and 4 weeks after cessation of treatment. Statistical analysis were performed using MANOVA and an analysis of repeated ALT measures as a linear function of the other variables.

Results: A trend towards a negative correlation between ALT and cortisol during the study period was observed (r=-0.32, p=0.06; r=-0.16, p=0.03). We did not find a significant correlation between ALT and the other variables.

Conclusions: ALT reduction induced by glycyrrhizin treatment in patients infected with chronic hepatitis C has been linked to a subtle elevation of serum cortisol. Further investigation of the mechanism of action of glycyrrhizin is warranted before we can state that the effect of glycyrrhizin on serum ALT is caused by its interference in corticosteroid metabolism.

Introduction

Glycyrrhizin, a natural compound extracted from the roots of *Glycyrrhiza glabra*, has been used for more than three decades to treat chronic hepatitis in Japan.[1] Treatment with glycyrrhizin significantly lowers serum alanine aminotransferase (ALT) levels.[2, 3] ALT normalization induced by long-term treatment with glycyrrhizin reportedly reduces progression to hepatocellular carcinoma in patients infected with chronic hepatitis C.[4] We observed a significant decrease in ALT levels during treatment while HCV-RNA levels did not change.[5] The mechanism by which glycyrrhizin lowers ALT without affecting viral replication has not yet been established. There are many *in vitro* and animal studies which suggest an array of different mechanisms.[6] Glycyrrhizin might have a direct protective effect on the hepatocyte. It might stabilize the cell membrane of hepatocytes by preventing penetration by viral particles[7] or by reducing cell membrane permeability which is increased by immuno-activation during viral infection;[8] it might also act in the cytosol as a radical scavenger.[9, 10] Another possibility is that glycyrrhizin inhibits immune-mediated cytotoxicity against hepatocytes by suppressing T-cell-mediated cytotoxicity[11] or by inhibiting NFκB binding activity;[12] NFκB is a transcription factor which seems to play a key role in the regulation of immune cell functions.[13]

The aim of this study was to gain some insight into the mechanism of action of glycyrrhizin *in vivo* in humans. We evaluated four potential mechanisms of action in our patients treated with glycyrrhizin:

1. Exogenously administered corticosteroids significantly reduce ALT levels in patients with chronic viral hepatitis without viral clearance.[14] The glycyrrhizin metabolite glycyrrhetinic acid inhibits the enzymatic (11β-hydroxy-steroid-dehydrogenase) conversion of cortisol to cortisone in the kidney:[15] this may lead to increased cortisol levels with inhibition of the hypothalamic-pituitary-adrenocortical feedback mechanism and thereby a decrease in dehydroepiandrosterone sulphate (DHEA-S) concentration. To evaluate this hypothesis cortisol and DHEA-S levels were measured.
2. Vascular adhesion protein-1 (VAP-1) is a transmembrane molecule, which is expressed among others on endothelial cells within liver sinusoids.[16] s-VAP-1, the soluble form, enhances lymphocyte binding to endothelial cells.[16] Recently, it was shown that s-VAP-1 is most probably identical to an enzyme known as semicarbazide-sensitive amine oxidase (SSAO).[17] s-VAP-1 is a sialylated glycoprotein; the sialic acids are indispensable for the function of s-VAP-1.[18] Glycyrrhizin can inhibit sialylation,[19]

therefore it is possible that glycyrrhizin inhibits the formation of active s-VAP-1. To evaluate this hypothesis s-VAP-1 levels were measured.

3. Hepatitis C related liver damage is often associated with increased iron storage, which elicits free radical-mediated peroxidation.[20] Glycyrrhizin can act as an anti-oxidative agent according to *in vitro* and rat studies.[9, 10, 21] To evaluate this hypothesis the antioxidant capacity of plasma was measured.
4. It is likely that hepatocyte damage during HCV infection is -at least partly- caused by the immune system.[22] Glycyrrhizin can induce apoptosis of T-lymphocytes,[23] which might cause inhibition of immune-mediated damage during HCV-infection. On the other hand it has also been reported that glycyrrhizin induces interferon-γ (IFN-γ) production;[24, 25] if this is true the immune system would be stimulated instead of inhibited by glycyrrhizin.[26] To evaluate the hypothesis that glycyrrhizin might act as an immunomodulator, the total number of leukocytes and the IFN- γ levels (a reflection of the Th_1 cell population) were measured.

We used blood samples of our patients on glycyrrhizin therapy, to evaluate whether there is a correlation between reduced ALT levels on the one hand and changes in cortisol, DHEA-S, s-VAP-1, anti-oxidant capacity of the plasma, total number of leukocytes and IFN-γ levels on the other.

Material & Methods

Patients

Patients between 18 and 70 years with a chronic hepatitis C infection, a positive hepatitis C virus-RNA titre, serum ALT at least 1.5 times the upper limit of normal (ULN), and liver biopsy findings consistent with mild to moderate liver fibrosis or cirrhosis were included. Patients were not eligible for the study if they had other conditions which caused the liver disease, decompensated cirrhosis (Child-Pugh score > 7) or hepatocellular carcinoma.

The study was conducted according to the Declaration of Helsinki and Good Clinical Practice. The Medical Ethical Committee of the University Hospital Rotterdam approved the protocol and all patients gave their written informed consent.

Treatment

Glycyrrhizin was given as Stronger Neo-Minophagen® C (SNMC®, Minophagen Pharmaceutical Co., Ltd., Tokyo, Japan). This preparation is a clear solution for intravenous administration, consisting of 2 mg glycyrrhizin, 1 mg cysteine and 20 mg glycine per

ml of physiological saline. Patients received placebo or 240 mg glycyrrhizin 3 times/week or 200 mg glycyrrhizin 6 times/week for 4 weeks; the total weekly dosage was therefore 0, 720 or 1200 mg glycyrrhizin, respectively. Follow-up after treatment lasted also 4 weeks.

Assays

Samples were taken before treatment (BT), at the end of treatment (EOT) and at the end of follow-up (EOF).

ALT was determined by standard assay (reference values: male 41 IU/L, female 31 IU/L, upper limit of normal).

For cortisol and DHEA-S assessments heparin plasma samples were taken between 8 - 10 a.m.. Cortisol (reference value: 200-800 nmol/l) and DHEA-S (reference values male, age 40-49: 2.5-14 µmol/l and females, age 40-49: 0.8-7 µmol/l) were assayed by means of Immulite 1 (Diagnostic Products Corporation, Los Angeles, CA, USA). Interassay coefficients of variation were below 8.5 and 9.1%, respectively.

SSAO (s-VAP-1) was assessed in serum by a functional assay in which benzaldehyde, generated from the substrate benzylamine, was quantitated by high performance liquid chromatography with fluorimetric detection after derivatization with dimedone.[27] The coefficient of variation was <7%.

The total antioxidant capacity of deproteinated blood was determined with a spectrophotometric assay that was based on the ability of antioxidants to neutralize the free radical of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate)(ABTS). First plasma was deproteinized by adding an equal volume of 10% trichloroacetic acid. After centrifugation, an aliquot of the supernatant was added to a solution of ABTS radicals. The reduction of absorption at 734 nm, which reflects ABTS radical consumption, was determined. The reduction was related to that of a series of trolox calibration solutions. The antioxidant capacity of the deproteinated plasma was expressed as the concentration of trolox solution that has similar antioxidant capacity as the deproteinated plasma sample: TEAC (trolox equivalent antioxidant capacity). The coefficient of variation was 5%.[28]

Total number of leukocytes was determined by routine tests (reference value 4.0 - 10.0 x 10^9/l).

Levels of IFN-γ were determined by ELISA (CLB, Amsterdam, the Netherlands); the detection limit was 1 pg/ml.

Statistical analysis

Univariate analyses were performed using Stata 5.0 software (Stata Corporation, College

Station, Texas, USA); multivariate analysis was performed with SAS release 6.12 (SAS Institute Inc., Cary, North Carolina, USA).

Univariate analysis using Mann-Whitney's test was performed to evaluate comparisons between the three treatment groups; the Wilcoxon test was used to evaluate significant differences within groups.

The correlation between the change in ALT and the change in other variables (cortisol, DHEA-S, SSAO, TEAC, leukocytes and IFN-γ) was evaluated by multivariate analysis. Only 5 of 44 patients were complete responders (=reached normal ALT levels) at the end of treatment; if we were to compare responders (n=5) with nonresponders (n=39) information about partial ALT response would be lost. Therefore we chose to evaluate the correlation/dependency between ALT and the other variables by:

1. A multiple analysis of variance (MANOVA) taking into account the repeated measures design of the study. As a result, the correlation matrix for responses (and especially the correlation between ALT and the other responses) was adjusted for dose and time.
2. An analysis of the repeated ALT measures at the end of treatment and at the end of follow-up as a linear function of the other measurements adjusted for dose, time and baseline levels of all responses.

Results

The baseline characteristics of the patients are shown in Table I. Each treatment group consisted of 14 - 15 patients; the median age was 47 years (range: 25-70). Most patients (85%) had not responded to interferon or interferon / ribavirin combination therapy.

Table II shows the median (0.05, 0.95 percentile) values for ALT, SSAO, TEAC, total number of leukocytes and IFN-γ before treatment, at the end of treatment and at the end of follow-up per dosage group. Cortisol and DHEA-S values are shown in chapter 4.1 (Table II, page 61).

Univariate analysis revealed that groups were comparable at baseline except for IFN-γ which was significantly lower in the 1200 mg group compared with the two other groups (p=0.03). This statistically significant difference is due to the use of another batch for IFN-γ determination of the 1200 mg group; clinically the IFN-γ values did not differ significantly.

ALT levels at the end of active treatment were significantly lower than before treatment (p=0.002); at the end of follow-up the ALT levels had returned to baseline. At the end of treatment 5 patients (2 of the 720 mg group and 3 of the 1200 mg group) had normal ALT values; 10 patients had ALT levels above normal but less than 1.5 times ULN (2 of the placebo, 4 of the 720 mg and 4 of the 1200 mg group); in another 3 cases the mean

Table I

Baseline characteristics of all 44 patients

placebo / 720 mg / 1200 mg	14 / 15 / 15
male / female	36 / 8
age (year) #	47 (34,66)
cirrhosis / noncirrhosis	20 / 24
previous interferon yes / no	38 / 6
ALT (upper limit of normal)#	2.5 (1.5,6.7)
HCV-RNA 10^6 gen.eq.#*	10.7 (1.2,51.4)
genotype-1 / non-1	22 / 22

median (0.05,0.95 percentile). *genome equivalent

percentage decrease in ALT at the end of treatment was more than 50% while ALT was more than 1.5 times ULN. Within the 720 mg group DHEA-S was significantly decreased at the end of treatment and increased to baseline levels after cessation of treatment. None of the other variables changed significantly during the study period.

For multivariate analysis all variables except s-VAP-1 were transformed by means of a natural logarithm to achieve a better approximation of the normal distribution. We found a trend toward a negative correlation between ALT and cortisol. Analysis according to the first method (MANOVA) resulted in r=-0.32, p=0.06. Analysis according to the second method resulted in r=-0.16, p=0.03. Multivariate analysis of the fixed effects (second method) showed a borderline significant positive correlation (r=0.6, p=0.05) between ALT and TEAC. We did not observe a significant correlation between ALT and DHEA-S, vascular adhesion protein (s-VAP-1), total number of white blood cells (leukocytes), or IFN-γ. Table III shows the results of the multivariate analysis.

Discussion

Glycyrrhizin treatment significantly reduced ALT levels in patients with chronic hepatitis C. However, the average cortisol levels remained unchanged during the study period; only after multivariate analysis did a trend toward a negative correlation between ALT and cortisol during glycyrrhizin treatment become clear. This trend might support the hypothesis that the mechanism of action of glycyrrhizin is indirect by increasing endogenous cortisol levels. Glycyrrhetinic acid, a metabolite of glycyrrhizin, inhibits the enzymatic (11β-hydroxy-

steroid-dehydrogenase) conversion of cortisol to cortisone;[15] this may lead to increased cortisol levels. These increased cortisol levels might be responsible for ALT decrease.

Table III

The results per parameter (multivariate analysis) in relation with ALT (ln).

We observed a trend to a negative correlation between ALT and cortisol and a borderline positive significant correlation between ALT and TEAC.

	General Linear Models MANOVA		Solution for fixed effects (MIXED)		
	Correlation coefficient	p-value	Estimate of x effect	standard error	p-value
Cortisol (ln)	-0.32	0.06	-0.16	0.07	0.03
DHEA-S (ln)	0.21	0.24	0.06	0.09	0.5
s-VAP-1	0.14	0.4	0.0002	0.0002	0.4
TEAC (ln)	-0.02	0.9	0.6	0.3	0.05
Leukocytes (ln)	0.08	0.7	0.08	0.1	0.6
IFN-γ (ln)	-0.05	0.8	0.08	0.1	0.5

(ln) = natural logarithm

At baseline we observed a negative correlation between ALT levels and cortisol: the severity of liver disease (ALT) was inversely correlated with cortisol (chapter 4.1, Figure 5). Multivariate analysis revealed that during glycyrrhizin treatment this negative correlation remained. Therefore another explanation for the trend toward a negative correlation between ALT and cortisol might be that an improvement in liver function might lead to higher levels of cortisol binding globulin (CBG) and cortisol. In patients with liver disease the CGB levels are significantly lower than in healthy individuals,[29] while cortisol levels are also lower although not significantly.[30] Hepatocytes contain the enzyme 11β-hydroxysteroid-dehydrogenase type 1 which catalyzes the conversion of cortisone to cortisol; this enzyme is downregulated in cirrhosis.[31] Improvement in liver function might therefore lead to higher CBG and cortisol levels. If this is the case the trend to a negative correlation between ALT and cortisol would be explained by two phenomena, independent of each other but both related to improvement in liver function.

As reported in chapter 4.1, a clinically significant increase in plasma cortisol levels would lead to inhibition of the hypothalamic-pituitary-adrenocortical feedback mechanism and thereby a decrease in DHEA-S levels. If this had occurred we should have observed a negative correlation between ALT and cortisol and a positive correlation between ALT and

Table II

The results before treatment (BT), at the end of treatment (EOT) and at the end of follow-up (EOF) per dosage group. Cortisol and DHEA-S values are shown in chapter 4.1 (Table II, page 61). Results are expressed as median (0.05, 0.95 percentile). (univariate analysis).

Total weekdose	0 mg (n=14)			720 mg (n=15)			1200 mg (n=15)		
	BT	EOT	EOF	BT	EOT	EOF	BT	EOT	EOF
ALT (ULN)	3.0 (1.2, 6.8)	3.0 (1.4, 5.6)	2.8 (1.5, 5.5)	2.3 (1.6, 5.2)	1.6* (0.9, 3.2)	2.0 (0.9, 4.6)	3.0 (1.6, 12.5)	1.6*(0.7, 5.2)	2.7(1.3, 11.8)
SSAO (sVAP-1, mU/L)	567 (285, 913)	548 (302, 791)	530 (355, 890)	461 (97, 1226)	365 (123, 818)	422(102, 1027)	730 (382, 971)	710(300, 1193)	670 (358, 982
TEAC (µmol/mL)	674 (626, 754)	706 (594, 782)	672 (550, 740)	683 (597, 794)	679 (551, 793)	662 (577, 774)	670 (506, 776)	682 (648, 778)	694 (559, 759)
Leukocytes x 10^9/L	5.6 (2.8, 11.5)	5.5 (2.3, 8.0)	5.2 (2.1, 9.3)	5.6 (3.0, 9.2)	5.4 (2.5, 15.3)	5.4 (2.5, 10.5)	5.1 (2.7, 9.4)	5.4 (2.6, 9.1)	5.5 (2.4, 8.3)
IFN-γ (pg/mL)	2.3 (1.5, 11.6)	2.5 (1.6, 11.3)	3.5 (1.6, 10.5)	2.0 (1.5, 5.5)	2.3 (1.5, 5.5)	2.1 (1.6, 15.9)	1.3# (0.7, 3.0)	1.5 (0.7, 3.8)	1.3 (0.7, 3.6)

* significantly lower than before treatment

\# significantly lower than baseline levels of the other two groups (p=0.03)

DHEA-S. Because we did not observe a positive correlation between ALT and DHEA-S, we might conclude that cortisol levels did not increase significantly.

In this study we did not find evidence to support the hypotheses that a decrease in ALT is correlated with a change in s-VAP-1, total number of leukocytes or IFN-γ. We therefore conclude that glycyrrhizin most likely does not influence s-VAP-1 levels, total number of leukocytes or IFN-γ levels.

If glycyrrhizin would increase the total antioxidant capacity of plasma, a negative correlation between ALT and TEAC should be found. Instead, multivariate analysis of the fixed effects of ALT and TEAC resulted in a borderline significant positive correlation ($r=0.6$, $p=0.05$). Therefore it is not likely that glycyrrhizin contributes significantly to the overall antioxidant capacity of plasma.

Most studies describing a possible mechanism of action of glycyrrhizin focussed on one hypothesis and were conducted in animals or *in vitro*.[6] This study is unique since we used human blood samples of HCV patients before, during and after glycyrrhizin treatment. Instead of focussing at one hypothesis, we evaluated four possible hypotheses concerning four different mechanisms of action.

In conclusion the ALT reduction induced by glycyrrhizin treatment in patients infected with chronic hepatitis C has been linked to a subtle elevation of serum cortisol. This might support the hypothesis that ALT reduction is related to inhibition of the conversion of cortisol to cortisone by glycyrrhizin's metabolite glycyrrhetinic acid. Further investigation of the mechanism of action of glycyrrhizin is warranted before we can state that the effect of glycyrrhizin on serum ALT is caused by its interference in corticosteroid metabolism.

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Discussion

Discussion

We started the studies described in this thesis in 1996. At that time the standard treatment for chronic hepatitis C was interferon monotherapy; a minority of patients (20%) responded with viral clearance and ALT normalization.[1] So for the patients who did not respond different treatment strategies were needed. Although the primary aim of antiviral therapy is viral clearance, several long term studies on chronic hepatitis B and C revealed that persistent ALT normalization (biochemical remission) was a key prognostic factor for reduction of complications, regardless of the presence of viral markers in the serum.[2-4] Therefore, if viral clearance could not be achieved, the induction of biochemical remission (ALT-normalization) without viral clearance was considered to be a goal of treatment.[5]

There are several monotherapies, which aim at the induction of biochemical remission without viral clearance in patients infected with chronic HCV, for example ursodeoxycholic acid (UDCA) or ribavirin in the western world. In Asia, Japan, glycyrrhizin has been used as a treatment for chronic hepatitis since 1958.[6]

All three therapies significantly reduce ALT levels during treatment, after cessation of therapy ALT levels increase again. During oral UDCA therapy 14% percent of the patients reached normal ALT levels, side effects hardly occurred, and until now histological improvement after UDCA for chronic HCV has not been reported.[7-10] During oral ribavirin treatment 29-41 percent of the patients reached normal ALT levels, reversible hemolytic anemia occurred in almost all ribavirin treated patients and histological liver improvement (decreased hepatic inflammation and necrosis) was found after treatment of at least 24 weeks.[11-14] During intravenous glycyrrhizin treatment 36% of the patients reached normal ALT levels, side effects were minimal, and long-term treatment (median 10 years) with glycyrrhizin significantly reduced the progression of chronic hepatitis C to hepatocellular carcinoma.[3]

Therefore, the efficacy with regard to ALT normalization of glycyrrhizin and ribavirin appears superior to UDCA. The occurrence of side effects seems most obvious during ribavirin treatment. The oral way of administration of UDCA and ribavirin is more convenient than the intravenous way of administration of glycyrrhizin. However, glycyrrhizin is the only treatment of which it has been reported that long-term monotherapy induces reduction of progression of the liver disease.[3]

For more than 20 years glycyrrhizin was widely used as a treatment for chronic hepatitis in Japan and the East Asiatic region but not in Europe.[15] In 1980 Wildhirt started to use

glycyrrhizin for the treatment of chronic hepatitis B, C and compensated posthepatic cirrhosis in Germany. After 12 years of experience Wildhirt reported uncontrolled prospective studies which showed clinical and biochemical improvement. He was very positive about the treatment with glycyrrhizin and considered glycyrrhizin therapy superior to interferon, although the data shown in his article did not support this conclusion.[15] Eisenburg reported in 1992 also a positive effect of glycyrrhizin treatment in German chronic hepatitis B patients.[16] A search of the Medline database from 1966 till 1999 (keywords glycyrrhizin(e), glycyrrhizic acid) did not reveal more data about the use of glycyrrhizin for chronic hepatitis in Europe. Why are there no continuations of these positive studies by Wildhirt and Eisenburg described in the international literature? In contacts with physicians from Germany they referred to the frequent occurrence of side effects (hypokalemia, rise of blood pressure, retention of body fluid) especially in patients with cirrhosis. However, we could not find published evidence about the occurrence of side effects during glycyrrhizin therapy for patients with chronic hepatitis in Germany. In chapter 4.1 of this thesis the occurrence of side effects in our patients, with chronic hepatitis C or compensated cirrhosis, treated with glycyrrhizin is described. Only minor reversible side effects occurred in patients receiving 200 mg glycyrrhizin 6 times per week, no reduction of treatment was necessary. So our experience with glycyrrhizin treatment is not in agreement with the German experience. As discussed in chapter 4.1, patients with decompensated cirrhosis are more prone to develop severe side effects during glycyrrhizin treatment. Therefore it might be possible that in Germany patients with decompensated cirrhosis who were treated with glycyrrhizin, developed severe side effects; this might be an explanation for the different findings.

Glycyrrhizin is administered as Stronger Neo-Minophagen C (SNMC®), a clear solution for intravenous use which contains 2 mg glycyrrhizin, 1 mg cysteine and 20 mg glycine per mL physiological saline.[3, 17-21]

In Japan, glycyrrhizin is usually administered daily.[3, 17-20] The treatment schedule is not standardized. The dosage varies between 40 ml[17, 18] and 100 ml.[3, 19, 20] After an initial daily treatment of 8 weeks the dosage[19] is often reduced to 20-40 ml daily or the administration frequency is diminished to 2-7 times per week.[3] Long-term treatment appears essential in order to reduce the incidence of hepatocellular carcinoma.[3]

Daily intravenous administration of medicines is very unusual for the outpatient clinic in the Western world. Therefore we started with a 4 weeks placebo controlled feasibility study in which glycyrrhizin was administered three times per week. We found that 56

out of 57 patients completed the treatment and that outpatient intravenous therapy was more easily accepted than anticipated. This study (chapter 2.1) also revealed that active treatment was significantly more effective with regard to the mean percentage ALT decrease than placebo. We found that 10% (4/41) of the patients reached normal ALT levels at the end of treatment. This is in agreement with the finding of Tsubota *et al.*,[21] they found that after three times per week treatment 17.7% (11/62) of the patients reached normal ALT levels. Therefore we conclude that three times per week glycyrrhizin treatment is as effective with regard to ALT normalization in our European patients compared with the Japanese patients.

Arase *et al.*,[3] however, reported that 36% of their patients (30/84) reached ALT normalization with daily treatment. Our second study revealed that it is also feasible to treat European outpatients with intravenous treatment six times per week. Twenty percent (3/15) reached normal ALT values at the end of treatment, which is comparable to the results of Arase *et al.*.

In conclusion, administration of six times per week is more effective than three times per week. This seems logical if the pharmacokinetic parameters of glycyrrhizin are considered. With a half-life of approximately 8 hours, (chapter 3) six times per week treatment was associated with detectable levels of glycyrrhizin at all times, while glycyrrhizin disappeared from the blood with three times per week administration.

The aim of glycyrrhizin treatment was to induce biochemical remission (ALT normalization) in order to reduce the progression of the liver disease. Only 20% of our actively treated patients reached normal ALT levels during glycyrrhizin therapy (chapter 2.2). If ALT normalization is necessary to reduce progression of the disease, glycyrrhizin therapy might be successful in only 20% of the treated patients. Several studies report now that ALT levels need not to be normal to reduce the disease progression; significantly lower ALT levels were also associated with a lower incidence of hepatocellular carcinoma.[22, 23] Glycyrrhizin might be a successful treatment for more than 20% of the treated patients, since all treated patients showed a significant decrease of ALT (chapter 2.2).

In our first clinical study in Rotterdam (chapter 2.1) glycyrrhizin was administered via an intravenous (i.v.) cannula in a peripheral vein during 15-20 minutes. In the additional study (chapter 2.2) glycyrrhizin was given via a butterfly needle in 5 minutes, in accordance with the methodology used in Japan. After completion of glycyrrhizin administration the way of venular access (cannula or butterfly needle) was removed. A

literature survey revealed that intravenous access by a cannula remained three times longer functional than a butterfly needle (49.5 versus 15.4 hours);[24] extravasation of fluid occurred in 1 out of 71 cases (1.4%) with a cannula compared with 18 out 71 cases (25.5%) with a needle;[25] the bacterial adherence to butterfly needles was less compared with a cannula:[26] a cannula caused significantly more phlebitis (18.8% versus 8.8% with a needle) and butterfly needles were significantly more associated with infiltration (40.1% versus 17.9% with a cannula).[27] In conclusion, a cannula remains longer functional and is associated with less extravasation and infiltration, whereas a butterfly needle is associated with less bacterial adherence and phlebitis. The venular access during glycyrrhizin therapy should be functional for maximal 20 minutes so the occurrence of occlusion or phlebitis in such a short time will be minimal. We observed in our patients no significant differences between administration via a butterfly needle or via a cannula with regard to extravasation of medication and hematoma at the injection site (chapter 2.2). Therefore based upon the literature and our experience we cannot recommend one way of administration above the other.

The mechanism by which glycyrrhizin induces ALT decrease without viral clearance is not known. It is possible that glycyrrhizin or one of its metabolites acts directly protective on the hepatocyte, it might also be possible that glycyrrhizin interferes with the immune system which causes the damage to the hepatocyte (chapter 5.2).

It is even unclear whether glycyrrhizin or its metabolite glycyrrhetinic acid is the active substance with regard to ALT decrease (chapter 5.1).

The elimination half-life of glycyrrhizin is approximately 8 hours (chapter 3); the half-life of glycyrrhetinic acid is 12-39 hours.[28] Based upon the half-life of glycyrrhizin daily administration is warranted to obtain detectable levels in the plasma; for glycyrrhetinic acid administration every other day would be sufficient. Our observation that six times per week administration was significantly more effective than three times per week, makes it likely that glycyrrhizin itself is the active molecule with regard to ALT decrease. If glycyrrhetinic acid would be responsible for the ALT decrease, three times per week administration would suffice.

At this moment the standard treatment for chronic hepatitis C has become combination therapy with interferon / ribavirin.[29] Forty-fifty percent of the patients become sustained responder (=virus is not detectable and ALT is normal six months after cessation of therapy). So 50% still does not respond with viral clearance.

Glycyrrhizin treatment should be further explored for patients who did not respond with viral clearance to standard treatment or patients who are not eligible for or refuse treatment with interferon / ribavirin.

Short-term treatment with glycyrrhizin appeared feasible and effective with regard to inducing ALT decrease. A long-term prospective study with glycyrrhizin in European patients should be conducted to evaluate the feasibility and the effect on liver histology. The treatment schedule should be optimized.

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Summary

Samenvatting

Summary

Chronic hepatitis C infection can be associated with progressive liver disease that may evolve insidiously to cirrhosis and carries the risk of hepatocellular carcinoma. For patients who do not respond with viral clearance to standard therapy (interferon or interferon / ribavirin combination therapy), other treatment options should be sought. Glycyrrhizin, extracted from the roots of *Glycyrrhiza glabra*, has been used in Japan as a treatment for chronic hepatitis for more than 30 years. It induces an alanine aminotransferase (ALT) decrease / normalization and thereby reportedly reduces the progression of chronic hepatitis C to hepatocellular carcinoma. The few reported clinical and pharmacokinetic data about the use of glycyrrhizin as a treatment for chronic hepatitis come from Japan. Because of its sweet taste glycyrrhizin is also used as a food additive in for example licorice. Pseudo-aldosteronism with hypokalemia and hypertension is a well described side effect of an excess intake of licorice, caused by glycyrrhizin's metabolite glycyrrhetinic acid. The mechanism by which glycyrrhizin induces ALT decrease without viral clearance is unknown (chapter 1).

Glycyrrhizin given as treatment for chronic hepatitis, should be administered 2 till 7 times per week intravenously. In Western Europe it is very unusual to treat out-clinic patients with frequent intravenous medication. The studies presented in this thesis describe our experience with short-term (4 weeks) glycyrrhizin treatment in European patients.

We started with a double-blind, randomized, placebo-controlled trial in which medication was given 3 times per week for 4 weeks (chapter 2.1). Fifty-four patients were treated with 0 mg (placebo), 80 mg, 160 mg or 240 mg glycyrrhizin. The mean percentage ALT decrease at the end of active treatment was significantly higher compared with placebo, while there was no significant difference between the three active groups (6%, 23%, 26%, and 29% for 0 mg (placebo), 80 mg, 160 mg or 240 mg glycyrrhizin, respectively). It turned out to be feasible to treat our out-clinic patients with 3 times per week intravenous medication. In order to increase the efficacy, we conducted an additional open trial in which the efficacy and feasibility of 6 times per week i.v. medication administration was evaluated for 4 weeks.

Chapter 2.2 presents the combined results of these two consecutive trials. Sixty-nine out of seventy eligible patients completed treatment according to protocol (compliance

99%). The percentage of patients with ALT normalization at the end of treatment doubled after 6 times per week treatment compared with 3 times per week (20% (3/15) versus 10% (4/41)); due to the relatively small numbers of patients studied, no statistically significant difference was found. The mean percentage ALT decrease at the end of active treatment was significantly more compared with placebo, while six times per week was significantly better than three times per week (6%, 26% and 47% for placebo, three times per week and six times per week treatment, respectively). After cessation of therapy ALT returned to baseline levels within two weeks. Hepatitis C Virus Ribonucleic Acid (HCV-RNA) levels did not significantly change during the study period. There were no clinically significant differences between placebo, 3 times or six times per week treatment with regard to the occurrence of adverse events.

Twenty-four hour pharmacokinetic measurements were performed on the first day of treatment and on or around fourteen days after start of treatment in 35 at random selected patients (chapter 3). The half-life of glycyrrhizin was approximately 8 hours, so with six times per week treatment glycyrrhizin was detectable in the plasma at all moments, in contrast to three times per week administration. Glycyrrhizin plasma levels declined according a three-compartment model with a total clearance of 7.6 mL/h/kg. The first phase of the three-compartment model can be explained predominantly by the distribution of glycyrrhizin; glycyrrhizin was mainly confined to the vascular compartment. The second phase can be explained by metabolism of glycyrrhizin in the liver. The third phase can be explained by an enterohepatic cycle of glycyrrhizin, which would extend the elimination phase. We did not observe a correlation between severity of liver disease and pharmacokinetic parameters.

We evaluated the occurrence of pseudo-aldosteronism in patients who received 0 mg, 720 mg or 1200 mg glycyrrhizin per week (chapter 4.1). Only within the group receiving 1200 mg glycyrrhizin per week minor reversible symptoms of pseudoaldosteronism (significant decrease of aldosterone and significant increase of systolic blood pressure and body weight) occurred.

Although a letter in the New England Journal of Medicine, October 1999, reported that daily intake of 500 mg glycyrrhizin significantly reduced testosterone levels in men, we could not confirm this in our male patients. We concluded that our maximum daily dose (200 mg glycyrrhizin) does not influence testosterone levels (chapter 4.2).

Glycyrrhizin as well as its metabolite glycyrrhetinic acid are pharmacological active substances. It is unknown which of these molecules is responsible for ALT decrease. In a pilot *in vitro* study with peripheral blood mononuclear cells (PBMCs) of an untreated patient with chronic hepatitis C, we evaluated the effect of incubation with increasing dosages of glycyrrhizin and glycyrrhetinic acid on stimulation of these cells. This study did not provide support for an effect of glycyrrhizin on PBMCs, nor whether glycyrrhizin or glycyrrhetinic acid is the active molecule with regard to ALT decrease (chapter 5.1).

In order to reveal the mechanism by which glycyrrhizin induces ALT decrease without viral clearance, we evaluated the relation of several parameters with the course of ALT during glycyrrhizin treatment and follow-up after treatment *in vivo*. We found a just significant negative correlation between changes in ALT and cortisol. Further investigation of the mechanism is warranted before we can confirm that the effect of glycyrrhizin on serum ALT is caused by its interference in corticosteroid metabolism (chapter 5.2).

In conclusion, it is feasible to treat European patients with 3-6 times per week intravenous glycyrrhizin for 4 weeks. Treatment induces a significant ALT decrease. Six times per week is more effective than three times per week treatment. HCV-RNA levels do not change significantly and side effects are minimal and reversible. To evaluate the benefit of treatment on liver histology a prospective long-term treatment study with glycyrrhizin should be performed.

Samenvatting

Chronische hepatitis C infectie is een progressieve leverziekte die langzaam maar zeker kan leiden tot lever cirrose en die een verhoogd risico op hepatocellulair carcinoom met zich mee brengt. Voor patiënten die niet reageren met klaring van het virus op standaard behandeling (interferon of interferon / ribavirine), is het noodzakelijk dat er andere behandelingsmogelijkheden worden gezocht. Glycyrrhizine, afkomstig uit de wortels van de plant *Glycyrrhiza glabra*, wordt al meer dan 30 jaar gebruikt als behandeling voor chronische hepatitis in Japan. Glycyrrhizine induceert een alanine aminotransferase (ALT) daling of normalisatie; het is beschreven dat glycyrrhizine behandeling de progressie van een chronische hepatitis C infectie naar hepatocellulair carcinoom remt. Er is een aantal artikelen over het gebruik van glycyrrhizine als behandeling voor chronische hepatitis C waarin ook de farmacokinetiek beschreven wordt. Deze artikelen komen vrijwel allemaal uit Japan.

Omdat glycyrrhizine een zoete smaak heeft, wordt het ook gebruikt als smaakversterker in voedingsmiddelen, zoals bijvoorbeeld in drop. Pseudo-aldosteronisme met hypokaliëmie en hypertensie is een goed gedocumenteerde bijwerking van een overmaat aan drop. De manier waarop glycyrrhizine een ALT daling induceert zonder klaring van het virus in niet bekend (hoofdstuk 1).

Glycyrrhizine dient 2 tot 7 keer per week intraveneus te worden toegediend als behandeling voor chronische hepatitis. In West Europa is het erg ongebruikelijk om poliklinische patiënten zo frequent te behandelen met intraveneuze medicatie. De onderzoeken in dit proefschrift beschrijven onze ervaring met kortdurende (4 weken) glycyrrhizine behandeling in Europese patiënten.

Wij zijn begonnen met een dubbel-blind, gerandomiseerd, placebo-gecontroleerd onderzoek waarbij de medicatie drie maal per week gedurende 4 weken werd gegeven (hoofdstuk 2.1). Vierenvijftig patiënten werden behandeld met 0 mg (placebo), 80 mg, 160 mg of 240 mg glycyrrhizine. Het gemiddelde percentage ALT daling aan het eind van de behandeling in de groep behandeld met actieve stof was significant hoger dan in de placebogroep; er was geen significant verschil tussen de drie glycyrrhizine groepen (6%, 23%, 26% en 29% voor achtereenvolgens 0 mg (placebo), 80 mg, 160 mg of 240 mg). Het bleek haalbaar te zijn om poliklinische patiënten te behandelen met drie maal per week een intraveneuze toediening. Om de effectiviteit van glycyrrhizine te verhogen, hebben we een

aanvullend onderzoek uitgevoerd waarin de effectiviteit en de haalbaarheid van 6 maal per week intraveneuze medicatie toediening voor de periode van 4 weken werd geëvalueerd.

Hoofdstuk 2.2 beschrijft de gecombineerde resultaten van de twee opeenvolgende onderzoeken. Negenenzestig van de 70 geschikte patiënten doorliepen het onderzoek zoals het in het protocol was voorgeschreven (therapie trouw 99% van de patiënten). Het percentage patiënten met ALT normalisatie aan het eind van de zes maal per week behandeling verdubbelde in vergelijking met de drie maal per week behandeling (20% (3/15) versus 10% (4/41)); vanwege het relatief kleine aantal patiënten werd geen statistisch significant verschil gevonden. Het gemiddelde percentage ALT daling aan het eind van behandeling was significant groter in de met glycyrrhizine behandelde groep dan in de placebo groep; zes keer per week was significant beter dan drie keer per week (6%, 26% en 47% voor achtereenvolgens placebo, drie maal en zes maal per week glycyrrhizine behandeling). Na het stoppen van de glycyrrhizine toediening, stegen de ALT waarden binnen veertien dagen tot het uitgangsniveau. Hepatitis C Virus Ribonucleic Acid (HCV-RNA) waarden veranderden niet significant gedurende de onderzoeksperiode. Er waren geen klinisch significante verschillen in het optreden van nevenverschijnselen tussen placebo, drie maal en zes maal per week glycyrrhizine toediening.

Voor het bepalen van 24 uur farmacokinetische parameters van glycyrrhizine werden, op de eerste dag van behandeling en op of rondom de veertiende dag na het starten van de therapie, bloedmonsters afgenomen van 35 at random geselecteerde patiënten (hoofdstuk 3). De halfwaarde tijd van glycyrrhizine was ongeveer 8 uur. Tijdens de zes maal per week glycyrrhizine behandeling was op elk tijdstip glycyrrhizine te meten in het plasma, dit was niet het geval bij de drie maal per week glycyrrhizine behandeling. Glycyrrhizine plasma waarden daalden volgens een 3-compartimentsmodel met een totale klaring van 7,6 ml/uur/kg. De eerste fase van het 3-compartimentsmodel kan worden verklaard door de verdeling van glycyrrhizine; glycyrrhizine bevond zich grotendeels in het vasculaire compartiment. De tweede fase kan worden verklaard door de omzetting van glycyrrhizine in de lever. De derde fase kan verklaard worden door de enterohepatische kringloop van glycyrrhizine, dit proces vertraagt de eliminatie fase. We hebben geen correlatie gevonden tussen de ernst van de leverziekte en de farmacokinetische parameters.

We hebben het optreden van pseudo-aldosteronisme geëvalueerd in patiënten die waren behandeld met 0 mg, 720 mg of 1200 mg glycyrrhizine per week (hoofdstuk 4.1).

Slechts binnen de groep die 1200 mg glycyrrhizine per week heeft gehad, traden minimale reversibele verschijnselen van pseudo-aldosteronisme op (significante daling van aldosteron, and significante stijging van de systolische bloedruk en het gewicht).

Hoewel een bericht in de New England Journal of Medicine, oktober 1999, vermeldde dat dagelijkse inname van 500 mg glycyrrhizine de testosteron spiegels van mannen significant reduceerde, vonden wij dit verschijnsel niet in onze mannelijke patiënten. Wij concludeerden dat onze maximale dagelijkse dosis (200 mg glycyrrhizine) geen invloed heeft op de testosteron spiegels (hoofdstuk 4.2).

Glycyrrhizine en het metaboliet glycyrrhetine zuur zijn beide farmacologisch actieve moleculen. Het is onbekend welke van deze twee moleculen verantwoordelijk is voor de ALT daling. In een oriënterend *in vitro* onderzoek met perifeer bloed mononucleaire cellen (PBMC's) van een onbehandelde patiënt met chronische hepatitis C en een controle persoon, hebben we het effect van incubatie met oplopende dosering glycyrrhizine en glycyrrhetine zuur op stimulatie van deze cellen geëvalueerd. Wij vonden geen effect van glycyrrhizine of glycyrrhetine zuur op de PBMC's; er werd dus in dit model geen indicatie gevonden of glycyrrhizine dan wel glycyrrhetine zuur het actieve stofje is met betrekking tot de ALT daling (hoofdstuk 5.1).

Om het mechanisme van de door glycyrrhizine geïnduceerde ALT daling zonder klaring van het virus te achterhalen, hebben we de relatie tussen het verloop van ALT en enkele andere parameters geëvalueerd gedurende de behandeling en de follow-up na behandeling *in vivo*. We vonden dat er een negatieve correlatie tussen veranderingen in de spiegels van ALT en cortisol aanwezig was. Verder onderzoek naar het achterliggende mechanisme is nodig voordat we kunnen bevestigen dat het effect van glycyrrhizine op ALT is veroorzaakt door glycyrrhizine's beïnvloeding van het cortisol metabolisme (hoofdstuk 5.2)

Concluderend blijkt het haalbaar te zijn poliklinische patiënten in Europa te behandelen met 3-6 maal per week intraveneus glycyrrhizine. Behandeling induceert een significante ALT daling. Zes keer per week behandeling is effectiever dan drie maal per week. HCV-RNA waarden veranderden niet significant en bijwerkingen waren minimaal en reversibel. Een langdurig onderzoek met glycyrrhizine dient te worden uitgevoerd om het effect van de behandeling op histologisch niveau in de lever vast te kunnen stellen.

Dankwoord

Dankwoord

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Curriculum Vitae

De auteur van dit proefschrift werd op 17 april 1971 geboren te Tricht (gemeente Buurmalsen). Zij volgde het V.W.O. aan het Koningin Wilhelmina College te Culemborg. In 1989 werd begonnen met de studie Geneeskunde aan de Erasmus Universiteit Rotterdam. Van september 1993 tot september 1994 was zij werkzaam als student-onderzoeker op het Laboratorium van Experimentele Chirurgie, Erasmus Universiteit, Rotterdam, onder leiding van Dr. R.L. Marquet en Dr. R.W.F. de Bruin. Op 20 januari 1994 werd het doctoraal geneeskunde met lof afgelegd en op 19 juli 1996 werd het arts-examen eveneens met lof behaald. Van augustus 1996 tot januari 2000 was zij werkzaam op de afdeling Maag-, Darm-, en Leverziekten van het Academisch Ziekenhuis Rotterdam (Dijkzigt). Tijdens deze periode werd onder begeleiding van Prof.dr. S.W. Schalm onderzoek verricht naar glycyrrhizine behandeling bij patiënten besmet met chronische hepatitis C, hetgeen de basis vormde voor dit proefschrift. Sinds januari 2000 is zij werkzaam als research arts bij Good Clinical Practice te Rotterdam. Zij is getrouwd met Jan W. Paul. Samen hebben zij een zoon, Jasper.