PHARMACOKINETICS OF GINSENG COMPOUNDS

Shiow-Edith Chen, Ronald J. Sawchuk and E. John Staba
Dept. of Pharmacognosy, College of Pharmacy, University of Minnesota
Minneapolis, U.S.A.

Abstract

Five ginsenosides (A_1, A_2, B_1, B_2, C) and a yellow pigment were isolated from American ginseng stems and leaves. Ginsenoside A_2, B_1, B_2 and C were proven to be identical with Korean ginseng root ginsenoside Rg_1, Rd, Re and Rb_2, respectively.The yellow pigment proved identical with panasenoside isolated from Korean ginseng leaves. Ginsenoside A_1, which was also present in American ginseng roots, was not identical to any of the known root (ginsenoside R_0 — Rg_2) and leaf (ginsenoside F_1—F_3) Korean ginseng saponins.

A gas-liquid chromatographic method was developed to analyze ginsenosides and sapogenins in rabbit plasma and urine samples. Panasenoside and stigmasterol were found to be the best internal standards for ginsenosides and sapogenins, respectively.

Ginsenoside C had a significantly longer half-life, higher plasma protein binding, lower metabolic and renal clearance than ginsenoside A_1, A_2 and B_2. Ginsenosides were not found in rabbit plasma and urine samples after oral administration.

Ginsenoside C had a higher toxicity than ginsenoside A_2 after intraperitoneal administration to mice. Toxicity was not observed after oral administration of the ginsenosides.

Introduction

The pharmacological effects reported for ginseng and its extracts are varied and controversial. It was not until recently that the purified ginsenosides were studied and differences in pharmacological activities noted between the ginseng protopanaxadiol and protopanaxatriol groups. Ginsenoside Rb_1, a protopanaxadiol group ginseng saponin, is reported to be a central nervous system (CNS) sedative and ginsenoside Rg_1, a protopanaxatriol group ginseng saponin, a CNS stimulant (1). The protopanaxadiol saponins will also protect blood from hemolysis, and the protopanaxatriol saponins will be hemolytic (2).Differences among the purified ginsenosides exist with respect to gonadotropism (3), antistress (4), nuclear ribonucleic acid (RNA) synthesis (5), deoxyribonucleic acid (DNA), lipid and protein synthesis (6).

The pharmacokinetics of ginseng saponins and their genins were investigated in this study with the expectation that the data would partially explain the diverse ginseng pharmacological responses known. However, before the pharmacokinetic studies were begun, it was necessary to extract and purify gram quantities of the ginsenosides and develop a biological fluid assay method. Semi-preparative high pressure liquid chromatography (HPLC) was used to obtain

gram quantities of five ginsenosides from the stems and leaves of *Panax quinquefolium* L. (American ginseng). A gas-liquid chromatography (GLC) method was developed to analyze ginsenosides and sapogenins in rabbit plasma and urine samples. With purified ginsenosides and a sensitive assay method available it was possible to determine the pharmacokinetic differences between protopanaxadiol and protopanaxatriol ginsenosides.

Results and Discussion

1. Isolation and identification of ginsenosides and sapogenins

Isolation: A yield of 7.1% (3.23 kg) of crude saponin extract was obtained from dried American ginseng leaves and stems and it is therefore a convenient and economic source of ginseng saponins. Total crude saponin concentrations reported are: dried Korean ginseng roots - 3.4% (7), 7.53% (8), leaves 12% (9), American ginseng roots - 3.13% (10), undried Korean ginseng callus - 0.56% (11) and suspension cells - 0.41% (12), and American ginseng callus - 0.38% (12).

The crude saponin extract contains in large amount ginsenoside A_1, A_2, B_1, B_2, C and a yellow pigment, in small amount ginsenoside Ra, Rb_1 and no ginsenoside R_0 and Rf. From 824.5 g crude saponin extract purified on silica gel columns, semi-preparative HPLC columns, and/or crystallization, a total of 10 g ginsenoside A_1, 9.5 g ginsenoside A_2, 18.3 g ginsenoside B_1, 2.5 g ginsenoside B_2, 6.35 g ginsenoside C and 0.95 g yellow pigment were isolated. Some of the overlapping column fractions obtained were not processed (13). Comparable results for the ginsenosides in American ginseng roots (panaquilins D, E-1 and G-1) and above-ground parts (panquilin (c), (d), C and G-2) were previously reported (10). Ginsenoside Rg_1, Re and Rd have been isolated from Korean ginseng leaves and are present in American ginseng leaves. However, ginsenosides F_1, F_2 and F_3 isolated from Korean ginseng leaves by Yahara *et al.* (9) were not detected in American ginseng stems and leaves.

Panaxadiol (2.5 g) and panaxatriol (1 g) were crystallized from 33.34 g of acid hydrolysate obtained from 67.86 g crude saponin extract. Oleanolic acid, which is present in ginsenoside R_0, was not found in the acid hydrolysate.

Identification: When spotted on TLC plates alongside standard ginsenosides, ginsenoside A_2, B_1, B_2 and C appeared identical to ginsenoside Rg_1, Rd, Re and Rb_2, respectively. However, ginsenoside A_1 did not correspond to any of the standard Korean ginseng root saponins or to ginsenoside F_1, F_2, F_3 from the leaves of Korean ginseng. These identities were further confirmed by elemental analysis, IR spectra, and the mass spectra of acetate derivatives.

Ginsenoside A_1—mp. 197-199°C. Anal. Calcd. for $C_{42}H_{70}O_{13}\cdot 4H_2O$: C, 59.02; H, 9.13. Found: C, 59.21; H, 9.08. IR KBr cm^{-1}: 3400 (broad, OH); 1720 (C = O); and 1620 (C = C). Mass fragments of ginsenoside A_1-acetate, m/e (%): 561(2.5), 465(0.3), 405(1.8), 331(1.1), 273(44.8), 213(19.5), 169(9.0), 153(39.3), 149 (41.0), 143(79.6), 127(21.3), 125(47.2), 109(28.6), 97(27.0), and 43(100). On acid hydrolysis, ginsenoside A_1 showed panaxatriol on TLC.

Ginsenoside A_2—mp. 178–180°C. Anal. Calcd. for $C_{42}H_{72}O_{14}\cdot 4H_2O$: C, 57.78; H, 9.24. Found: C, 58.20; H, 8.76. IR KBr cm^{-1}: 3400 (broad, OH); and 1620 (C = C). Mass fragments of ginsenoside A_2-acetate, m/e (%): 797(0.3), 770(0.9), 752(11.2), 743(0.6), 525(0.4), 465(10.9), 405(18.3), 404(24.1), 389(8.8), 331(39.2), 295 (9.1), 269(9.4), 202(36.2), 187(27.6), 169(100), 135(53.7), 109(60.3) and 43(98.5). On acid hydrolysis, ginsenoside A_2 yielded panaxatriol.

Ginsenoside B_1—mp. 193-195°C. Anal. Calcd. for $C_{48}H_{82}O_{18}\cdot 5H_2O$: C, 55.58; H, 8.94. Found: C, 55.18; H, 8.35. IR KBr cm^{-1}: 3400 (broad, OH); and 1620 (C = C). Mass fragments of ginsenoside B_1-acetate, m/e (%): 755(1.7), 754 (4.3), 740(0.9), 712(1.1), 619(4.3), 577(0.3), 517(0.4), 467(0.6), 407(32.4), 406(27.5), 391(5.3), 331(82.2), 297(12.1), 271(15.3), 202(21.2), 189 (17.1), 169(100), 135(62.2), 127(7.0), 109(40.0) and 43 (36.2). On acid hydrolysis, ginsenoside B_1 yielded panaxadiol.

Ginsenoside B_2—mp. 195–197°C. Anal.

Calcd. for $C_{48}H_{82}O_{18}\cdot 2H_2O$: C, 58.64; H, 8.82. Found: C, 58.61; H, 8.95. IR KBr cm^{-1}: 3400 (broad, OH); and 1620(C = C). Mass fragments of ginsenoside B_2-acetate, m/e (%): 561(2.4), 525(0.1), 465(4.2), 405(6.9), 404(6.8), 389(2.4), 331(1.9), 295(3.1), 273(45.0), 269(2.7), 213(13.8), 202(8.2), 187(9.1), 169(6.7), 153(21.8), 135(14.3), 122(19.9), 115(23.8), 109(16.7) and 43(100). On acid hydrolysis, ginsenoside B_2 yielded panaxatriol.

Ginsenoside C—mp. 198-200° C. Anal. Calcd. for $C_{53}H_{90}O_{22}\cdot 4H_2O$: C, 55.29; H, 8.58. Found: C, 55.39; H, 8.46. IR KBr cm^{-1}: 3400(broad, OH), 1620(C = C). Mass fragments of ginsenoside C-acetate, m/e (%): 740(0.5), 712(0.6), 619(2.8), 577(0.2), 517(0.2), 467(0.3), 407(18.2), 406(18.8), 391(3.4), 331(54.6), 297(8.3), 271(15.8), 259 (10.3), 202(13.3), 199(7.8), 189(11.9), 169(55.4), 135(24.1), 127(4.8), 109(19.2) and 43(100). On acid hydrolysis, ginsenoside C yielded panaxadiol.

Melting points of the isolated ginsenosides were not always identical to those obtained by Shibata (7,14,15), Kaku (8), Yahara (9) and Kim (10). The melting point of ginsenoside A_2, however, was identical to that of ginsenoside Rg_1 obtained from Pharmaton. Differences in the melting points are believed due to the use of an uncorrected melting point apparatus, different crystalline forms, and/or the number of water molecules present in the ginsenosides.

The broad hydroxyl group band at 3400 cm^{-1} and C = C group at 1620 cm^{-1} in the IR spectra of isolated ginsenosides were also observed by Shibata *et al.* (7,14,15). The carbonyl group in ginsenoside A_1 (1720 cm^{-1}) is believed to originate from the carbohydrate portion as no oleanic acid was found in the acid hydrolysate of crude saponin extract.

Molecular ions were not seen in all ginsenosides, and fragments of higher mass usually showed rather low intensity because of their unstability. The sapogenin portion of ginsenosides were characterized by the fragment ions, m/e 525, 465 and 405 (protopanaxatriol group), and m/e 467 and 407 (protopanaxadiol group) as reported by Komori (16). The sapogenin portion of ginsenosides is further confirmed by the presence of fragments at m/e 404, 389, 295, 269, 202, 187 and 135 for protopanaxatriol group, and fragments at m/e 406, 391, 297, 271, 202, 189 and 135 for protopanaxadiol group. The carbohydrate portion of ginsenosides were characterized by fragments at m/e 331, 169 and 109 (glucose); m/e 619, 577, 517, 331, 169, 127 and 109 (di-glucose); m/e 561, 273, 213, 169 and 109 (rhamnose-glucose); m/e 299 and 199 (arabinose)(9).

Panaxadiol and panaxatriol—On TLC comparison, panaxadiol and panaxatriol had identical Rf values as that of the standards. Their identities were further confirmed by their IR spectra, elemental analysis, and mass spectra of molecular ions at m/e 461 (m+1, panaxadiol), 477 (m+1, panaxatriol), and the fragment ion at m/e 127 (17,18).

Absorption maximum of 545-550 nm was observed when ginsenosides and sapogenins isolated were reacted with vanillin solution and sulfuric acid. The maximum absorbance varied linearly with the amount of ginsenosides (20–100 μg) or sapogenins (10–50 μg) in the reaction mixture and were similar to those obtained by Hiai *et al.*(19) with ginsenosides Rb_1, Rg_1, Rd, panaxadiol and panaxatriol.

Yellow pigment—The yellow pigment isolated was identical to panasenoside isolated from Korean ginseng leaves with respect to absorption maxima in ethanol at 268 nm and 353 nm, elemental analysis, IR spectra, and genins obtained from acid hydrolysis (kaempferol) (20). Mass spectra of its acetate derivative showed major fragments, m/e (%), at 619(1.7), 331(20.1), 286 (19.9), 169(90.4), 127(12.3), 109(36.2) and 43 (100). Fragment ion at m/e 619 confirmed the presence of a disaccharide group (glucose-galactose) in the molecule.

2. Pharmacokinetic studies in the rabbit

Analysis of ginseng saponins and sapogenins in biological fluids: Water-saturated butanol and chloroform satisfactorily extracted and purified ginsenosides and sapogenins from collected plasma and urine samples. However, panasenoside, the internal standard of ginsenosides,

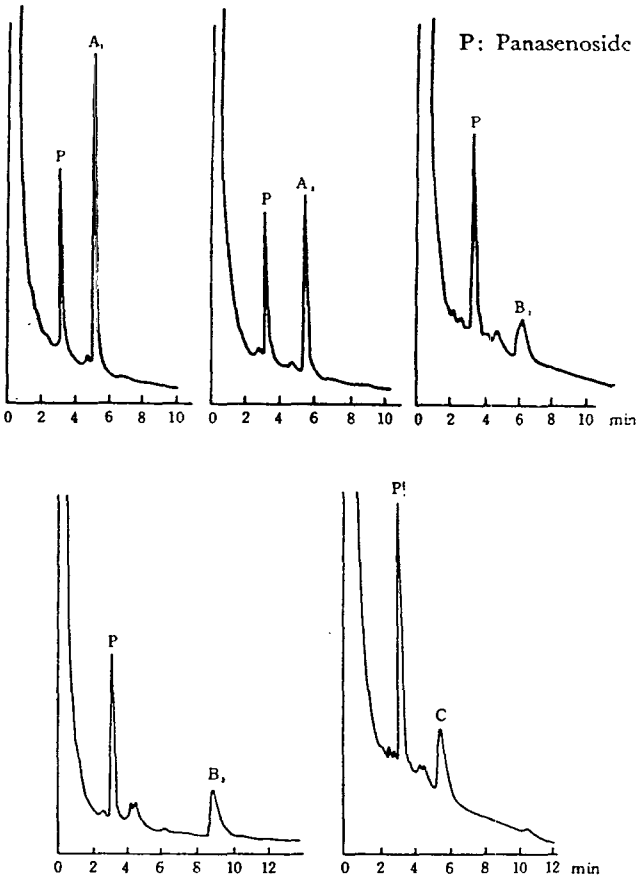

Fig. 1A. Gas-liquid chromatogram of ginsenoside A_1-, A_2-, B_1-, B_2- and C-TMS derivatives in rabbit plasma and urine samples.

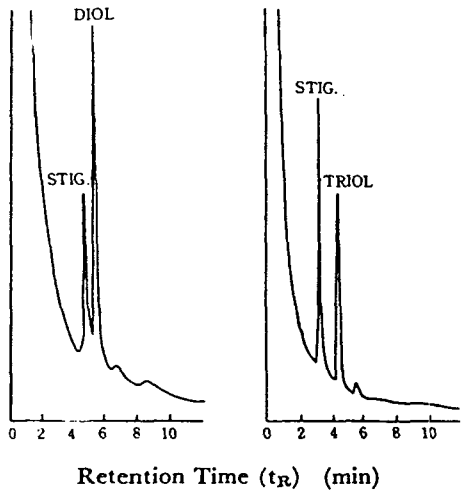

Fig. 1B. Gas-liquid chromatogram of panaxadiol- and panaxatriol- TMS deriv. in rabbit plasma and urine samples.

was not butanol extractable and had to be added to the butanol extract of standards and samples.

The GLC method developed enabled rapid, sensitive estimation of ginsenosides and sapogenins in plasma and urine samples. Linear relationships of peak height ratio to weight ratio were obtained when the amount of ginsenosides assayed was in the range of 20 μg to 300μg (B_2), to 350 μg (A_1), to 400 μg (A_2), to 500 μg (C), and that of sapogenins in the range of 10 μg to 200 μg in 0.1 ml of the silylation mixture(Fig.1). When concentration of ginsenosides and sapogenins exceeded these limits, peak heights were reduced and the retention time increased.

As only 1 μl of the 100 μl reaction mixture was injected into the GLC column, the assay method developed was sensitive to 0.2 μg of ginsenosides and 0.1 μg of sapogenins. Sensitivities of the reported colorimetric method are 10 μg of sapogenins and 20 μg of ginsenosides (19). The qualitative GLC method developed by Bombadelli *et al.*(21) allowed the detection of 2 mg of ginsenosides in 0.5 ml of the silylation mixture (4 μg).

Pharmacokinetic studies: Plasma concentration -time data for the ginsenosides were best fitted with an one-compartment model except in one instance in which the data were best fitted

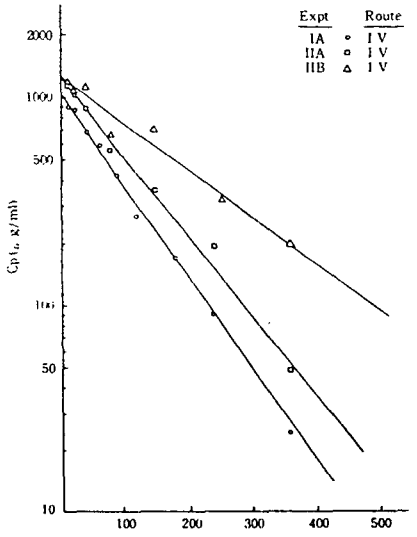

Fig. 2A. Plasma concentration-time profile of semi-puri ginsenoside A_1 (Fr. II).

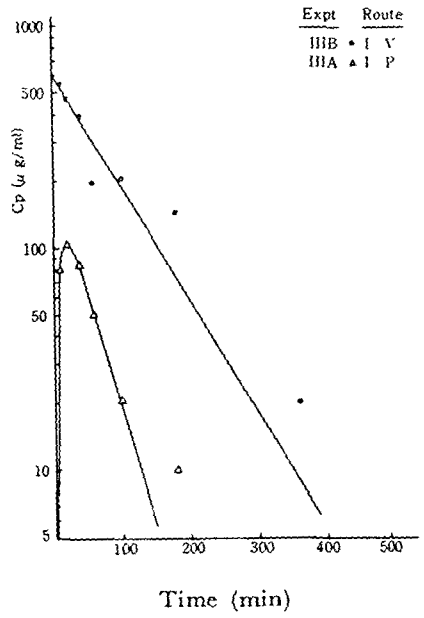

Fig. 2B. Plasma concentration-time profile of ginsenoside A_1.

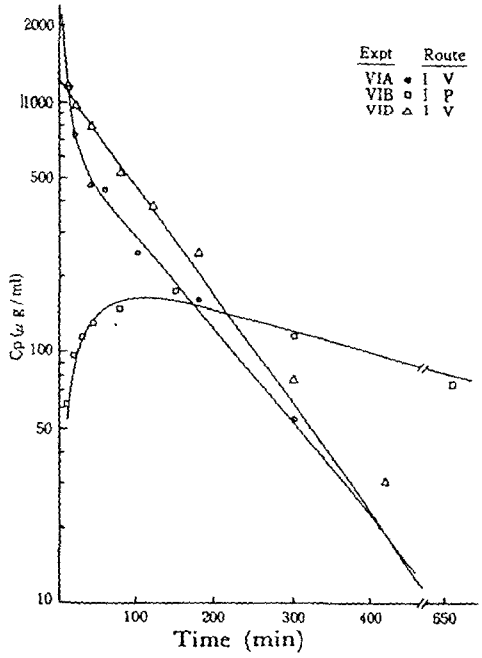

Fig. 2C. Plasma concentration-time profile of semipurified ginsenoside A_2.

with a two-compartment open model (Fig. 2).

Ginsenoside A_1—Ginsenoside A_1 exhibited a mean elimination half-life of 74.4 min. Previous i.v. administration of the same drug prolonged

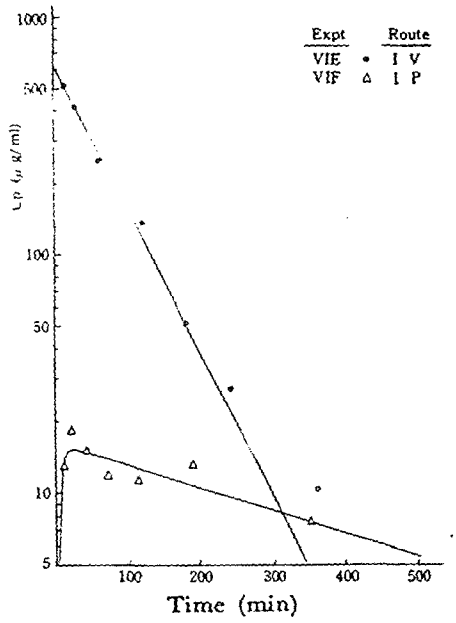

Fig. 2D. Plasma concentration-time profile of ginsenoside B_2.

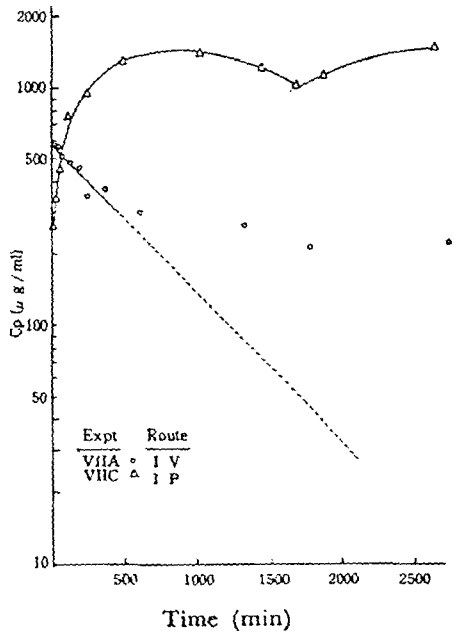

Fig. 2E. Plasma concentration-time profile of ginsenoside C.

its elimination half-life, resulting in a half-life of 136 min. The prolonged elimination suggested possible drug effects on plasma protein binding and hepatic and/or renal function. It is also conceivable that the elimination kinetics of this compound may be described by a two-compartment

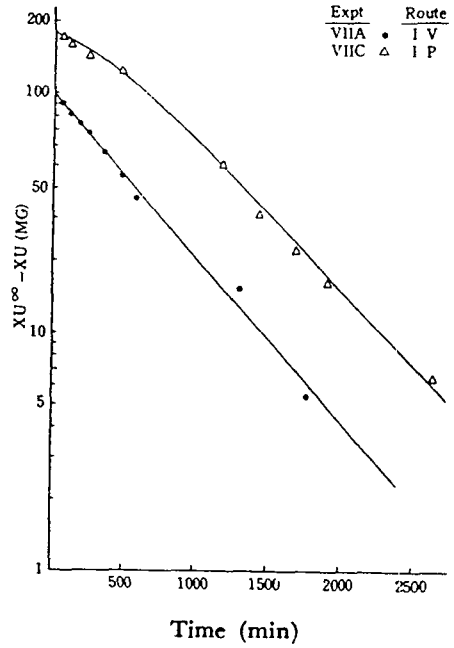

Fig. 2F. Amount remaining to be excreted vs. time plot of ginsenoside C.

model, and that the terminal half-life had not been reached during the sampling period following the first dose. The apparent elimination half-life on a subsequent dose would therefore appear to be somewhat prolonged.

Ginsenoside A_1 demonstrated a shortened mean elimination half-life of 22.7 min as compared to that of ginsenoside A_1 administered as the semi-purified form. Previous drug administration also prolonged elimination of the second dose. Ginsenoside A_1 was rapidly absorbed with a mean absorption half-life of 10.6 min following i.p. administration to the rabbit, and 27.5% of the dose was absorbed into the systemic circulation. It had a mean volume of distribution (Vd) of 141 ml/kg with a range of 96.7 to 171 ml/kg. Previous drug administration did not significantly change its volume of distribution.

Ginsenoside A_2—Semi-purified ginsenoside A_2 had a β-phase elimination half-life of 82.6 min. A shorter elimination half-life was obtained following administration of the second dose with less drug effect after oral dosing than after i.v. dosing. Absorption of ginsenoside A_2 after i.p. administration was much slower than that of ginsenoside A_1 (absorption half-life 363 min), and 80.3% of the i.p. dose was absorbed into the systemic circulation. Ginsenoside A_2 had a Vd of 134 ml/kg. However, when the pharmacokinetic behavior of ginsenoside A_2 was fitted with a two-compartment open model, it was found to have a central compartment volume (Vc) of 70.0 ml/kg, and that during the post-distributive phase (Vd_β) a value of 202 ml/kg, and that at steady state (Vd_{ss}) a value of 173 ml/kg.

Ginsenoside B_2—Ginsenoside B_2 had a shortened elimination half-life of 49.8 min as compared to that of semi-purified ginsenosides A_1 and A_2. Absorption of ginsenoside B_2 after i.p. dose (absorption half-life 324 min) was slightly faster than that of semi-purified ginsenoside A_2. About 35% of the i.p. dose was absorbed into the systemic circulation. Ginsenoside B_2 had a Vd of 246 ml/kg.

Ginsenoside C—Pharmacokinetic parameters of ginsenoside C were estimated from the early plasma samples after i.v. administration and from urinary excretion data after i.v. and i.p. doses, as the presence of a suspected metabolite in the plasma interfered with its assay. Ginsenoisde C showed a significantly longer mean elimination half-life of 445 min as compared to that of ginsenoside A_1, A_2 and B_2. Elimination half-life of second dose ginsenoside C (412 min) was slightly shorter than that of the first dose. It had an absorption half-life of 318 min similar to that of ginsenoside B_2, and 86.6% of the dose was absorbed into the systemic circulation after i.p. administration. The volume of distribution of ginsenoside C was smaller than ginsenosides A_1, A_2 and B_2 (114.9 ml/kg).

Urinary excretion of unchanged ginsenosides: Fractions of ginsenoside A_1 excreted unchanged in the urine ranged from 38.7% to 70.1% with a mean value of 54.5%. A smaller fraction of ginsenoside B_2 was excreted unchanged in the urine (17.4%) as compared to that of ginsenoside A_1, A_2(60.7%) and C(41.2%).

When the urinary excretion rate ($\Delta Xu/\Delta t$) was plotted against plasma concentration at midpoint of collection interval (Cptmid), ginsenosides A_1, A_2 and B_2 showed lower renal clearances

$\left(RC = \frac{\Delta Xu/\Delta t}{Cptmid}\right)$ at higher ginsenoside plasma concentration. This suggested the possible presence of a lag time between the administration of the drug and appearance of unchanged drug in the urine, and/or saturable transport in the urinary excretion of the compound. In contrast, ginsenoside C showed a higher renal clearance at higher ginsenoside C plasma concentrations.

The average renal clearances of ginsenosides in the rabbits were determined by the relationship $RC = \frac{X_U^\infty}{AUC}$ (X_u^∞: total amount excreted unchanged in the urine, AUC: Area under the plasma concentration-time curve). Significantly lower renal clearance and total body clearance were observed with ginsenoside C (RC 0.0714, TBC 0.1733 ml/min/kg) as compared to that of ginsenoside A_1 (RC 0.95, TBC 1.89 ml/min/kg), A_2 (RC 0.85, TBC 1.40 ml/min/kg), and B_2 (RC 0.67, TBC 3.75 ml/min/kg). The low total body clearance of ginsenoside C resulted in its significantly longer elimination half-life.

Oral administration: No ginsenoside was found in plasma or urine samles after oral administration. Analysis of fecal samples of ginsenoside A_1 and A_2 was also negative. Deconjugation of the urine and fecal samples with β-glucuronidase resulted in an unidentified GLC peak in extracts of urine samples.

Possible reasons suggested for the absence of ginsenosides in the plasma and urine samples upon oral administration are : a) poor absorption from the gastrointestinal (G-I) tract; b) binding of ginsenosides to components within the G-I tract; c) metabolism by gut microorganisms before absorption; and d) use of a poor animal model. Absence of ginsenosides A_1 and A_2 in the fecal samples suggested that they were absorbed, bound, or metabolized in the G-I tract. Furthermore, ginsenoside A_3 was found to be stable in rabbit gastric juice for at least 24 hrs. The 48 hrs sample showed the presence of a more non-polar metabolite. Partial conversion of ginsenosides is therefore believed to occur in the G-I tract or immediately upon absorption.

Plasma protein binding: Significant differences in plasma protein binding were observed between the protopanaxadiol group of ginsenosides (B_1 and C >99%) and the protopanaxatriol group of ginsenosides (A_1 72.12%, A_2 31.84%, and B_2 77.19%). The significantly longer elimination half-life observed with ginsenoside C is probably due to its higher plasma protein binding. In addition, high plasma protein binding resulted in a decreased volume of distribution, retarded drug metabolism, and decreased the rate of excretion of drug through the glomerulus for ginsenoside C. The differences in plasma protein binding between ginsenosides A_1, A_2, B_2 and ginsenoside B_1, C were further confirmed with human plasma (A_1 30.23%, A_2 33.24%, B_2 45.54%, and B_1 and C > 99%). Pharmacokinetics of ginsenoside B_1 is probably similar to that of ginsenoside C because of its high plasma protein binding.

Differences in pharmacokinetic behavior of ginsenosides observed are suggested to be primarily structure related. A similar pharmacokinetic-structure relationship has also been observed for the two digitalis glycosides, digitoxin and digoxin. Presence of an additional hydroxyl group in the genin portion of digoxin is responsible for its pharmacokinetic behavior being significantly different from that of digitoxin (22,23), such as half-life (digitoxin 5–7 days; digoxin 1.4 days), volume of distribution (digitoxin 40 liters; digoxin 500-liters), fraction excreted unchanged into the urine (digitoxin 32%; digoxin 60%), and fraction protein bound (digitoxin 95%; digoxin 15%). Differences in the pharmacological responses observed between protopanaxadiol and protopanaxatriol group ginsenosides, such as CNS depressive and stimulating effects, and both causing and preventing hemolytic effects, may be related to their significantly different pharmacokinetic behavior.

Panaxadiol and panaxatriol genins: The pharmacokinetics of panaxadiol and panaxatriol could not be studied because of their toxicity. The volume of distribution was believed to be greater than 1.7 liter/kg, based on the 10μg/

ml assay sensitivity and 50 mg dose of sapogenin administered. Their large volume of distribution suggested the possibility of extensive protein binding.

Toxicity: Ginsenoside C showed greater toxicity than ginsenoside A_2 when they were administered to mice. Deaths were observed with ip dose of 9 mg or 18 mg ginsenoside C, and with 18 mg of ginsenoside A_2. The higher toxicity of ginsenoside C may be related to its lower renal and metabolic clearances.

Death rate was insignificantly greater than that of the controls after oral administration of ginsenosides A_2 and C. Similar results were reported with semi-purified and purified ginsenosides to mice (1) and ginseng preparations to humans (24). Metabolism of ginsenosides during absorption is therefore suggested to occur following oral administration. Absence of ginsenosides in the plasma and urine samples after oral administration may also be explained by this conversion process.

Meterials and Methods

1. Isolation and identification of American ginseng compounds

Plant material: Four-year old American ginseng (*Panax quinquefolium* L.) leaves and stems were collected in September at the Fromm Brothers Ginseng Farm, Hamburg, Wisconsin, U.S.A.

Large scale isolation of ginsenosides: Batches of coarsely ground, dried American ginseng stems and leaves (45.5 kg) were extracted with chloroform in a Lloyd extractor for 24 hrs. The residue was air-dried and extracted again with methanol for 24 hrs. (Methanol Extract I).

Methanol Extract I was mixed with silica gel to remove sugars and other impurities. The methanol filtrate was combined with methanol silica gel washings (2x) and concentrated (Methanol Extract II). Water (200 ml) was added to Methanol Extract II (100 ml) and extracted with chloroform (300 ml) to remove chlorophylls. The aqueous layer was diluted with water (300 ml) and extracted with water-saturated butanol (350 ml). The butanol layers were combined and concentrated (Crude Saponin Extract, 825.4 g dry weight). The Crude Saponin Extract was applied to silica gel columns and purified as previously reported (13).

Hydrolysis of crude saponin extract: Crude Saponin Extract (67.86g) was hydrolyzed with a mixture (1 liter) of methanol-30% hydrochloric acid (4:1) in a steam bath for 5 hrs. Methanol was removed from the hydrolysate by evaporation in vacuum. Ethanol (200 ml) was added to the residue and evaporated. The residue was then diluted with 200 ml water and extracted with chloroform 3x (400 ml, 200 ml, 200 ml). The chloroform layers were combined, concentrated, washed with 100 ml of water, and evaporated to form a crude hydrolysate.

The crude hydrolysate obtained (33.34 g) were applied to silica gel columns (5 cm × 83 cm). The columns were eluted with benzene-acetone (3:1) at a flow rate of 16 ml/min. Fractions (50 ml) were collected and assayed by TLC with the eluting solvent system as mobile phase and ceric sulfate as the spray reagent. Fractions showing presence of panaxadiol and panaxatriol were combined and concentrated.

Panaxadiol was crystallized twice as colorless needles in ethylacetate from fraction 33–40. Panaxatriol was crystallized from benzene as colorless needles from fraction 45–64.

Identification of ginseng saponins and sapogenin:

a. Thin-layer chromatography (TLC)

Ginsenosides A_1, A_2, B_1, B_2, C and the yellow pigment isolated were spotted on silica gel G plate (250 μ, pre-coated) along with the standard ginsenosides R_0-Rg_2 and F_1-F_3. Plates were developed with chloroform-methanol-water (65:35:10, lower phase) or n-butanol-acetic acid-water (4:1:5, upper layer) and visualized with ceric sulfate spray reagent (3% ceric sulfate in 3% sulfuric acid). Purity of ginsenosides isolated were examined by two-dimensional TLC utilizing the two solvent systems.

Panaxadiol and panaxatriol were spotted on TLC plates along with standard panaxadiol and panaxatriol. The plate was developed with benzene-acetone (3:1) and visualized as described

above.

b. Analytical

Melting points, infra-red spectrometry, and mass spectrometry were determined as described previously (13).

c. Ultra-violet and visible absorption

The absorption spectra of yellow pigment in ethanol was determined from 200 nm to 380 nm with a Cary 14 Instrument (Cary Instrument, Monrovia, Ca.).

All ginsenosides and sapogenins isolated were reacted with 8% vanillin solution (0.25 ml) and 72% sulfuric acid (2.5 ml) in a 60° C water bath for 10 min. The absorption spectra of each sample was determined from 500–600 nm and relationship of absorption at absorption maximum vs. amount of each saponin and sapogenin were also determined.

d. Acid hydrolysis of ginsenosides and yellow pigment

Ten mg of purified ginsenosides were hydrolyzed in methanol-hydrochloric acid (4:1) for 5 hrs at 65° C. The methanol phase was evaporated. Two ml of ethanol were added to the residue and evaporated in vacuum. The residue was diluted with water (2 ml) and extracted with chloroform three times (15 ml). The chloroform layers were combined, concentrated, and assayed by TLC developed with benzene-acetone (3:1) and visualized as above.

Ten mg of the yellow pigment were hydrolyzed with 5% sulfuric acid (7.2 ml) in an 120° C oil bath for 2 hrs. When the acid hydrolysate was cooled a yellow precipitate appeared. The precipitate was filtered, recrystallized from methanol and analyzed by TLC along with standard kaempferol in the following solvent systems: ethylacetate-ethyl methyl ketone-formic acid-water (5:3:1:1), chloroform-methanol-water (65:35:10, lower phase), and n-butanol-acetic acid-water (4:1:5,upper phase). Kaempferol was detected by either 5% sodium carbonate or a ceric sulfate spray reagent.

2. Pharmacokinetic studies of ginseng saponins and sapogenins in the rabbit

Animals: New Zealand White male rabbits weighing approximately 3 kg were purchased from the Oak Crest Rabbitry, Edina, Mn. They were maintained on Purina rabbit chow and tap water for at least 2 wks before the experiments. The animals were fasted but permitted free access to water for 48 hrs before an oral administration, overnight before an intraperitoneal (ip) administration, and not at all before an intravenous (iv) administration.

Ginsenoside administration: After the hair on the ear was removed with a commercial depillatory the marginal ear vein was cannulated with a Bard I-Cath i.v. placement unit (a 12" radiopaque inside needle catheter fitted with a 19 gauge needle, C. R. Bard, Inc., Murray Hill, NJ) for i.v. administration as well as blood collection. Clotting of blood in the catheter was prevented by infusing 0.3 ml of heparin solution (100 units/ml) after each blood collection.

The i.v. dose was usually given over a 5–10 min period. The i.p. administration was given with a 21 gauge, 1 to 1.5" needle inserted into the peritoneal cavity. Ginsenosides were administered orally in gelatin capsules (size No. 2). The capsules were administered by placing them at the back of the oral cavity with the help of a capsule administration device, followed with 30 ml of water administered by syringe. The rabbit was kept in a restraining cage throughout the experiment.

Blood collection: Blood was collected through the catheter before ginsenoside administration for preparing the standard curve. Blood samples (1.5 ml/sample) were drawn with 3 ml disposable syringes at appropriate intervals after ginsenoside administration. Each blood sample was transferred into a heparinized Vacutainer tube and centrifuged for 15 mins. The plasma layer was carefully removed and stored in the freezer until analyzed.

Urine collection: The bladder of the rabbit was cannulated for urine collection. The tip of a size 10 Bardex Foley catheter, obtained from C.R. Bard, Inc., Murray Hill, N.J. was lubricated and inserted into the penis of a relaxed rabbit. When the tip of the catheter had been threaded through the urethra into the bladder, 3 ml water was injected into the placement ballon to maintain

Table 1. Summary of ginseng pharmacokinetic studies

Ginsenoside (Sapogenin)	Expt No.	Rabbit No.	Rabbit Wt. (kg)	Route Admin.	Dose (mg)
Semi-purified ginsenoside A_1	IA	I	3.16	iv	500
	IB	I	3.43	oral	500
	IIA	II	2.85	iv	500
	IIB	II	2.85	iv	500
Ginsenoside A_1	IIIA	III	3.95	ip	500
	IIIB	III	3.95	iv	400
	IV	IV	2.77	oral	404
	VA	V	3.25	iv	250
	VB	V	3.25	ip	500
Semi-purified ginsenoside A_2	VIA	VI	3.19	iv	500
	VIB	VI	3.19	ip	500
	VIC	VI	3.02	oral	500
	VID	VI	3.02	iv	500
Ginsenoside B_2	VIE	VI	3.27	iv	500
	VIF	VI	3.27	ip	500
Ginsenoside C	VIIA	VII	3.79	iv	250
	VIIB	VII	3.79	ip	500
	VIIC	VII	3.51	ip	500
	VIII	VIII	3.68	oral	500
Panaxadiol	IX	IX	3.23	iv	50
Panaxatriol	X	X	3.01	iv	50

the catheter in position throughout the experiment. A urine collection was completed after flushing the rabbit bladder with 37° C normal saline (30 ml/flushing) at 5, 10, and 15 min before the scheduled urine collection time. The total volume obtained during each collection interval was measured and an aliquot was frozen until analysis.

Analysis of ginsenosides and sapogenins in biological fluids: The method for preparing ginsenoside and genin standard curves, and the analysis of these compounds in plasma and urine, are as previously described (27).

Pharmacokinetic studies: A summary of studies performed on rabbits is shown in Table 1. Ginsenosides were given in 5 ml 10% ethanol solution or propylene glycol-ethanol-0.9% NaCl (4:1:20) solution for i.v. and i.p. administrations, and in gelatin capsules for oral administration. Panaxadiol and panaxatriol were given i.p. 1.6 ml absolute ethanol intravenously. Blood and urine samples were collected at appropriate times to allow the determination of desired pharmacokinetic parameters.

Ginsenoside A_1—Both pure and semi-purified ginsenoside A_1 were used. The semi-purified ginsenoside A_1 consisits of ginsenoside A_1 and trace amounts of ginsenoside A_2, and was used in the preliminary toxicity and pharmacokinetic studies. Experiment IIB, IIIB, VB were performed 24 hrs, 29 hrs and 48 hrs after Experiment IIA, IIIA and VA, respectively. Experiment IB was performed 33 days after Experiment IA.

Ginsenoside A_2—Semi-purified ginsenoside A_2, consisting of ginsenoside A_2 and a small amount of ginsenoside A_1 (approximate ratio, 8:2) was used. Experiment VIA-D were performed on the same rabbit, with Experiments VIB and VID 24 hrs after VIA and VIC, respectively, and VIC 37 days after VIA.

Ginsenoside B_2—Pure ginsenoside B_2 was used. Experiment VIE and VIF were performed on the same rabbit used to study ginsenoside A_2, with VIE 25 days after VID, and VIF 48 hrs after VIE.

Ginsenoside C—Only 250 mg rather than the usual dose of 500 mg was given i.v. to the rabbit because of the toxicity of ginsenoside C. Experiment VIIB was performed 48 hrs after VIIA, and VIIC 19 days after VIIB.

Panaxadiol and panaxatriol—Much higher toxicity of both sapogenins was observed when given i.v. and therefore only 50 mg was administered. Extensive hemolysis was observed in the plasma samples. The rabbits survived for at least 10 hrs but no more than 20 hrs.

Data analysis: A digital computer program for nonlinear regression analysis (KINA) was used to estimate the basic pharmacokinetic parameters of each ginsenoside and sapogenin. KINA is an interactive program adapted from University of Minnesota, Division of Health Computer Sciences (DHCS) library program, SUMEXP. Plasma concentration and urinary excretion data were fitted to a single exponential or biexponential term using equal weighing of data (25, 26).

Stability of ginseonside A_1 in rabbit gastric juice: Ginsenoside A_1 (50 mg) was incubated in freshly obtained rabbit gastric juice (5 ml) at 37°C. Samples of the incubation mixture were removed at appropriate times and analyzed by TLC developed in chloroform-methanol-water (65:35:10, lower phase) solvent system.

Fecal sample analysis: Fecal samples collected after oral administration of semi-purified ginsenosides A_1 and A_2 were homogenized with water in a Waring blender for 2.5 min, respectively. They were centrifuged and the supernatant was removed and analyzed as that for plasma and urine samples.

Deconjugation of fecal and urine samples by β-glucuronidase: Fecal and urine samples after oral administration of ginsenoside A_1 and A_2 were deconjugated with β-glucuronidase (type H-1, partially purified powder from *Helix pomatia*, β-glucuronidase activity: 400,000 units/g at pH 5.0 at 37°C; sulfatase activity: 1 g will hydrolyze approximately 40,000 μmoles p-nitrocatechol sulfate per hr at pH 5.0 at 37° C, Sigma Chemical Co., St. Louis, Mo). To 1 ml of urine samples or feces supernatant, 1 ml of 0.2 M sodium acetate buffer (pH 5.0) and 0.1 ml of enzyme solution (10,000 units) were added. The mixture was incubated at 30° C for 15 hrs, extracted with water-saturated butanol (3 ml, 2x) and analyzed as before by GLC and TLC.

Plasma protein binding of ginsenosides: Ten and 20 μl of ginsenosides methanol solution (50 μg/μl) were well-mixed with 1.5 ml of freshly prepared rabbit plasma in a sonicator. Twenty μl of the same ginsenoside solutions were also well-mixed with 1.5 ml of freshly prepared human plasma to perform the protein binding studies. An aliquot (0.3 ml) of each sample was saved for total drug content determination. The remaining portion was filtered with an ultrafiltration device obtained from Millipore Co., Bedford, Mass., consisting of 3 ml microsample cell and Pellicon molecular filter (PSED 01310). Flow rate was facilitated with a nitrogen gas pressure of 15 psi. Approximately 0.4 ml of the filtrate was collected over 60 min, and 0.3 ml of it was saved for free drug determination. The free and total drug concentration of each ginsenoside sample were assayed as above by GLC.

Toxicity of ginsenosides A_2 and C: Male Swiss Webster White mice weighing approximately 30 g were obtained from Bio-Lab Co., St. Paul, Mn. They were fed on Purina Laboratory chow and tap water. Groups of 10 were fasted but permitted free access to water for 24 hrs before the experiments. Three dosages (4.5, 9.0 and 18 mg) of ginsenosides A_2 or C dissolved in 0.5 ml of 5% ethanol were administered either i.p. or orally (by incubation) to the mice. Animals were fasted for 24 hrs and feeding was resumed after the first day of administration. Deaths in each group were noted at 24 hrs and over 10 days.

Acknowledgements

The authors thank the Fromm Brothers Ginseng Farm for their generous financial support and providing ginseng plant materials. We also thank Dr. S. Shibata, Meiji College of Pharmacy, and Dr. J. Shoji, Showa University, Tokyo, Japan, for authentic samples of ginsenosides R_0-Rg_2; Dr. K. H. Rueckert, Pharmaton Co., Lugano, Switzerland, for ginsenoside Rg_1; and Dr. O. Tanaka, Hiroshima University, Hiroshima, Japan, for ginsenoside F_1, F_2 and F_3. Technical assistance

of Mr. Edwin Chang and Ms. Linda Cartier in this study is also appreciated.

References

1. Takagi, K.: Pharmacological studies on ginseng. Proc. International Ginseng Sympo., Seoul, Korea, p. 119(1974).
2. Namba, T., Yoshizaki, M., Tomimori, T., Kobashi, K. and Hase, J.: Hemolytic and its protective activity of ginseng saponins. Chem. Pharm. Bull., **21(2)**, 459(1973).
3. Brekhman, I. I., Dardymov, I. V. and Dobryakov, Y. I.: On the pharmacology of individual *Panax ginseng* glycosides. Farm. i. Toksikol., **29(2)**, 167(1966).
4. Brekhman, I. I. and Dardymov, I. V.: Pharmacological investigation of glycosides from ginseng and eleutherococcus. Lloydia, **32(1)**: 46(1969).
5. Iijima, M., Higashi, T., Sanada, S. and Shoji, J.: Effect of ginseng saponins on nuclear ribonucleic acid (RNA) metabolism. I. RNA synthesis in rats treated with ginsenosides. Chem. Pharm. Bull., **24(10)**, 2400(1976).
6. Yamamoto, M., Kumagai, A. and Yamamura, Y.: Effects of active principles in *Panax ginseng* on DNA, RNA, protein and lipid synthesis in bone marrow and testicles of rats. Proc. International Ginseng Sympo., Seoul, Korea, p. 129(1974).
7. Sanada, S., Kondo, N., Shoji, J., Tanaka, O. and Shibata, S.: Studies on the saponins of ginseng. I. Structures of ginsenoside R_0, Rb_1, Rb_2, Rc and Rd. Chem. Pharm. Bull., **22(2)**, 421(1974).
8. Kaku, T., Miyata, T., Uruno, T., Sako, I. and Kinoshita, A.: Chemicopharmacological studies on saponins of *Panax ginseng* C. A. Meyer. Arzneim Forsch., **25(3)**, 343(1975).
9. Yahara, S., Tanaka, O. and Komori, T.: Saponins of the leaves of *Panax ginseng* C. A. Meyer. Chem. Pharm. Bull., **24(9)**, 2204(1976).
10. Kim, J. Y. and Staba, E. J.: Plant biochemistry of ginseng saponins. Proc. International Ginseng Sympo., Seoul, Korea, p. 77(1974).
11. Furuya, T., Kojima, H., Syono, K. and Ishii, T.: Isolation of panaxatriol from *Panax ginseng* callus. Chem. Pharm. Bull., **18(11)**, 2371(1970).
12. Jhang, J. J., Staba, E. J. and Kim, J. Y.: American and Korean ginseng tissue cultures: growth, chemical analysis and plantlet production. In Vitro, **9(4)**, 253(1974).
13. Chen, S. E. and Staba, E. J.: American Ginseng. I. Large scale isolation of ginsenosides from leaves and stems. Lloydia, **41(4)**, 361(1978).
14. Sanada, S., Kondo, N., Shoji, J., Tanaka, O. and Shibata, S.: Studies on the saponins of ginseng. II. Structures of ginsenoside Re, Rf and Rg_2. Chem. Pharm. Bull., **22(10)**, 2407(1974).
15. Nagai, M., Tanaka, O. and Shibata, S.: Chemical studies on the oriental plants drugs. XXIV. Structure of ginsenoside Rg_1, a neutral saponin ginseng roots. Tetrahedron, **27(5)**, 881(1971).
16. Komori, T., Tanaka, O. and Nagai, Y.: Studies of the saponins from the ginseng root extract: mass spectra of ginsenoside Rg_1-decaacetate and related compounds. Org. Mass. Spect., **9**, 944(1974).
17. Shibata, S., Fujita, M., Itekava, H., Tanaka, O. and Ishii, T.: The structure of panaxadiol, a sapogenin of ginseng. Tetrahedron Lett., **10**, 419(1962).
18. Shibata, S., Tanaka, O., Soma, K., Iida, Y., Ando, T. and Nakamura, H.: Studies on saponins and sapogenins of ginseng, the structure of panaxatriol. Tetrahedron Lett., **3**, 207(1965).
19. Hiai, S., Oura, H., Odaka, Y., and Nakajima, T.: A colorimetric estimation of ginseng saponins. Planta Medica, **28**, 363(1975).
20. Komatsu, M., Tomimori T. and Makiguchi, Y.: Studies on the constituents of the herb of *Panax ginseng* C. A. Meyer. II. On the flavonoid constituents. Yakugaku Zasshi, **89(1)**, 122(1969).
21. Bombardelli, E., Bonati, A., Gabetta, B., Martimelli, E. M. and Mustich, G.: Gas-liquid chromatographic and mass spectrometric investigation on saponins in *Panax ginseng* extracts. Fitoterapia, **57(3)**, 99(1976).
22. Okita, G. T.: Distribution, disposition and excretion of digitalis glycosides. *Digitalis*, Fish, C. and B. Surawicz (Ed), Gruze and Stratton, New York, p. 13(1969).
23. Doherty, J. E.: Digitalis glycosides, pharmacokinetics and their clinical implications. Ann. of Internal Medicine, **79(2)**, 229(1973).
24. Stengel, F. and Listabarth, H.: Clinical trials of a new geriatric preparation. Arztliche Praxis, **20(4)**, 130(1968).
25. Hartley, H. O.: The modified Gauss-Newton method for the fitting of nonlinear regression function by least squares. Technometrics, **3**, 269(1961).
26. Hartley, H. O. and Booker, A.: Non-linear least squares estimation. Ann. Math. Stat., **36**, 638 (1965).
27. Chen, S.E. and Staba, E. J.: American Ginseng. II. Analysis in biological fluids. J. Pharm. Sci. (submitted for publication)(1978).