

## Securidaca-saponin

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The natural mixture of saponins from *Securidaca longipedunculata* Fresn. [Polygalaceae] can be purified by the formation of a cholesterol complex with subsequent saponin recovery. Crystalline fractions of differing compositions have been obtained from an ethanol-soluble part of the securidaca-saponin. Thin-layer chromatographic analysis of the fractions gives partly overlapping spots. The securidaca-saponin is easily separated into two main fractions by paper chromatography with water-free solvent mixtures containing a substantial fraction of pyridine: one moving fraction consisting of several overlapping and tailing hemolytic substances, and one fraction, also hemolytic, which remains at the starting-point. The hemolytic index of securidaca-saponin is about 100 times larger at pH 5.8 than at pH 8.8. The dose-response curve for hemolysis differs profoundly from that of other saponins by showing a maximum and a minimum, the level of which differs for different samples of human blood.

The presence of saponin in *Securidaca longipedunculata* Fresn. [Polygalaceae], a shrub of tropical Africa, was first reported by Lenz,<sup>1,2</sup> and his results have been confirmed.<sup>3-6</sup> A review on the medicinal use of this plant has been published.<sup>7</sup>

The primary aim of this investigation is the isolation of the various native saponins in quantities which are sufficient for pharmacological research and for further chemical study of the native saponin substances.

Crude securidaca-saponin is easily prepared from a dried water extract of the plant material by dissolving the extract in methanol and precipitating the saponin with ether. The preparation so obtained is a mixture of several saponins, and it contains also substances of other chemical classes.

If a preparation of crude securidaca-saponin is extracted with boiling ethanol, part of it dissolves easily, and part appears as a sticky gum. When the solution is allowed to cool, crystals appear, and several fractions may be obtained which differ in solubility in methanol, and in melting-point. The

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ethanol-insoluble fraction may be dissolved in hot methanol; ethanol is added, and the solution is cooled, whereby an amorphous fraction is obtained. Further fractions may be obtained by partial evaporation of the solution.

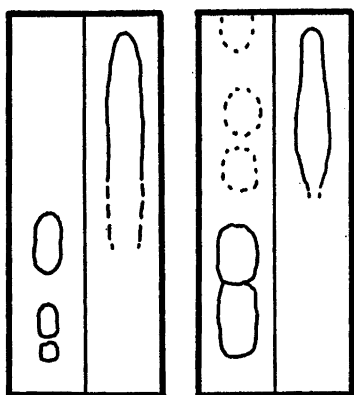
The cholesterol method for purifying saponins is useful for the purification of securidaca-saponin.<sup>8,9</sup> If a preparation of the saponin, dissolved in ethanol, is mixed with an ethanol solution of cholesterol, a precipitate is formed. This precipitate dissolves easily in pyridine, from which the saponin may be precipitated with ether.

Thin-layer chromatography of various fractions of securidaca-saponin on silica gel G chromatoplates does not give separation with most solvents tested, but mixtures containing 20 % butanone, 55–65 % isopropanol or tertiary butanol, and 15–25 % water give spots which are only partly overlapping. The saponin spots on thin-layer chromatograms may be identified with the following technique: the plate is covered with a piece of filter paper, dipped into an isotonic phosphate buffer suspension of washed red blood corpuscles, and after a few minutes the paper is covered with a glass plate. After a few hours the paper is removed and dried. Permanent spots may be seen on the paper. The chromatoplate should be inspected on the under side, through the chromatoplate glass, for tailing spots. In UV light the spots from securidaca-saponin give a weak blue fluorescence. The trimethylsilyl derivative of the securidaca-saponin is too lipophilic for regular thin-layer chromatography on silica gel, as it follows the solvent front closely.

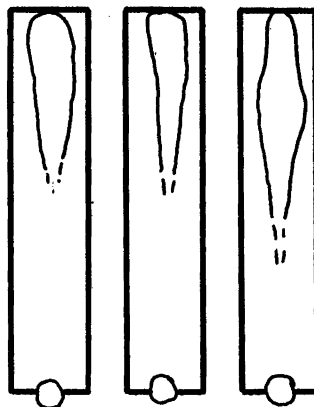
By means of paper chromatography, preparations of securidaca-saponin, including crude saponin preparations and saponin preparations purified with cholesterol, can be separated into one group of substances which have low  $R_F$ -values and which give strong reactions with the common silver reagents but no hemolysis, and one group of overlapping substances which have high  $R_F$ -values and which give only weak reactions with silver reagents but which give strong hemolysis. A suitable solvent mixture for the development is ethyl formate, formic acid, and water 75:10:15. The hemolytic substances can also easily be separated into two groups by means of paper chromatography, using water-free solvent mixtures containing a large proportion of pyridine, *e.g.*, chloroform and pyridine 40:60 (Figs. 1 and 2).

Saponins have been separated by countercurrent technique into an acidic and a neutral fraction,<sup>10</sup> and securidaca-saponin can be similarly separated into two fractions at pH-values above 10 with the solvent system chloroform-ethanol-water (1:2:3), but the fractions appear to overlap, and have not yet been sufficiently characterized.

Preliminary attempts at analytical separation of the components of securidaca-saponin by means of gas chromatography of the trimethylsilyl derivative have indicated that four main components can be distinguished from a preparation purified according to the cholesterol method. Although gas chromatography will be of significant value for the analysis of saponins, one can expect it to be limited to those components which have only few sugar rests. Several saponins with known structure have more than four sugar rests,<sup>11</sup> and trimethylated tetrasaccharides are already so large molecules that their retention time in gas chromatography is of the order of magnitude of an hour.<sup>12</sup> The improvement obtained by substituting dimethylsilylation for



*Fig. 1.* Paper chromatograms (from start to front) developed with 80 % isopropanol and 20 % water (left chromatogram), and with 75 % ethyl formate, 10 % formic acid, and 15 % water (right chromatogram). Spots were visualized with silver nitrate and potassium hydroxide (left side), and with washed red blood corpuscles (right side).



*Fig. 2.* Paper chromatograms (from start to front) showing two fractions of hemolytic substances, developed with 40 % chloroform and 60 % pyridine (left), with 60 % isopropanol and 40 % pyridine (middle), and with 30 % ethyl methyl ketone and 70 % pyridine (right). Spots were visualized with washed red blood corpuscles.

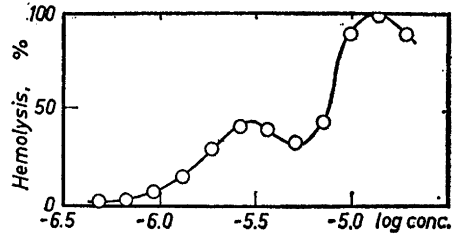
trimethylsilylation can be expected to only enable the gas chromatography of saponins with one additional sugar rest.<sup>13</sup>

The hemolysis of human red blood corpuscles by the natural mixture of substances in a preparation of securidaca-saponin does not follow the usual dose-response relationship. The dose-response curve shows a maximum and a minimum, and the reason for this has not yet been elucidated. The various preparations of securidaca-saponin may contain substances with different hemolytic qualities, and, conjecturally, even substances with the capability of preventing hemolysis.

For an optimum low concentration of saponin the percentage of hemolysis of washed human red blood corpuscles showed a maximum value ranging between 38 and 75 in 16 samples. For a saponin concentration about 1.5 times as large as that for the maximum, there was a minimum, several per cent lower than the maximum. For still more increased concentration of securidaca-saponin, the percentage increases steeply (Fig. 3). Two samples of a total of 18 did not show a maximum and a minimum but a flattened curve. It seems to be particularly noteworthy that the observed variation obtained with blood samples from different patients concerns the percentage of hemolysis, whereas the concentration of the securidaca-saponin preparation for the maximum differs only insignificantly for the various samples.

The influence of pH on the hemolysis of red blood corpuscles differs for various kinds of saponins:<sup>14,15</sup> when the pH value is increased from about 5.6 to 10.4 the hemolytic activity of a saponin may either increase about a hundred times or decrease several hundred times or decrease a few times or show a

Fig. 3. The hemolysis of washed human red blood corpuscles after 3 h in solutions of a saponin preparation from *Securidaca longipedunculata* at various concentrations and at pH 7.4.



weak minimum. The classical saponin standard, Merck's *Saponinum purum albissimum*, belongs to the last-mentioned group, whereas the hemolytic activity of securidaca-saponin decreases more than hundred times when pH is increased from 5.8 to 8.8. (Fig. 4). A time study of the influence of time on hemolysis by securidaca-saponin suggests that results are read after about 3 h, whereas results normally are read after 24 h.<sup>16,17</sup>

The hemolytic index at pH 7.4 for six fractions of securidaca-saponin, using washed red human blood corpuscles pooled from 4 patients ranged between 100 000 and 140 000, considering the lowest concentration which gives complete hemolysis. A sample of Merck's saponin, supposed to have a hemolytic index of 25 000 (by definition) for bovine red blood corpuscles, had a hemolytic index of 95 000 when measured in the same way. However, the hemolytic activity of securidaca-saponin cannot generally be expressed in relation to that of the standard saponin because of the difference in dose-response, and the different influence of pH and time on the hemolysis.

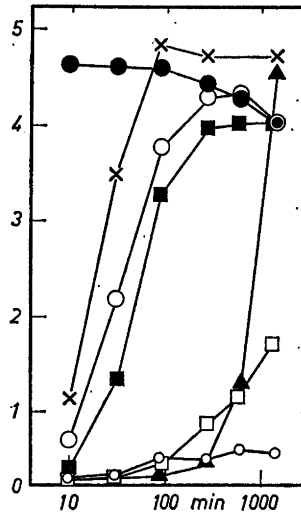


Fig. 4. The influence of time, pH, and concentration on the hemolysis of washed human red blood corpuscles in a solution of a saponin preparation from *Securidaca longipedunculata*. Concentration (g/ml) and pH: ×  $10^{-4}$  and 8.8; ●  $10^{-5}$  and 5.8, ■  $10^{-5}$  and 7.4, ▲  $10^{-6}$  and 8.8; ○  $10^{-6}$  and 5.8, □  $10^{-6}$  and 7.4; ○  $10^{-7}$  and 5.8. Ordinates: colorimeter readings in arbitrary units.

## EXPERIMENTAL

*Crude securidaca-saponin.* About 1.8 g of a dried water extract of the plant material was dissolved in 15 ml of methanol and precipitated by adding a mixture of methanol and ether, followed by ether until the mixture contained about 10 times as much ether as methanol. After a few hours about half of the liquid was removed as a clear supernatant, and it was replaced by ether. A few hours later most of the liquid was removed, and it was replaced by pentane, and the precipitate was then filtered, washed with pentane, and dried in a vacuum desiccator. Yield 1.2 g.

*Fractionation of the crude saponin.* The 1.2 g of crude material was extracted with 40 ml of boiling absolute ethanol. The extract was removed and allowed to cool to room temperature slowly overnight, whereafter 154 mg of crystalline material was filtered off, washed with cold absolute ethanol and dried in a vacuum desiccator. The mother liquor was reduced to about 8 ml, heated so that the precipitate formed during the evaporation dissolved, and left to cool for several hours, whereupon 136 mg of crystalline material was filtered off. The mother liquor was further reduced and left in the cold room for some time, whereupon 109 mg of material were filtered off. The melting points of these preparations were 229, 203, and 199°C, respectively. The material which was not dissolved during the treatment with boiling ethanol was dissolved in boiling methanol, and the solution was left overnight, whereupon 164 mg of apparently amorphous material was filtered off. The mother liquor was partly evaporated in a rotating vacuum evaporator, and ethanol was added in just the amount possible without causing a precipitate to form when the mixture was heated to nearly boiling. The mixture was left to cool down, and 88 mg of amorphous material was filtered off. The mother liquor was further reduced and the mixture was left in the cold room overnight; then 130 mg of precipitate was obtained. The melting points of these preparations were 226, 226, and 210°C, respectively. Prista and Alves found melting points between 140 and 150°C for their various securidaca-saponin preparations which were reprecipitated with ether from an alcoholic solution.<sup>8</sup> The first fraction, both of the material extracted from the crude saponin preparation and of the sticky remainder, was less than 10 % soluble in methanol. A 10 % solution of each of the second fractions was made in warm methanol, and a 10 % solution of the third fraction was prepared at room temperature. These solutions were used for thin-layer chromatography and for paper chromatography, as were also 30 % solutions.

*Purification with cholesterol.* A warm solution of 42 mg of a crystallized but still fairly crude securidaca-saponin preparation in 2 ml of ethanol was precipitated with a hot concentrated ethanol solution of 40 mg of cholesterol. The mixture was allowed to cool, and 33 mg of precipitate was recovered; melting range 218–224°C. The preparation was dissolved in 0.2 ml of dry pyridine and the saponin precipitated with 2 ml of ether. After a few hours most of the supernatant solution was removed as a clear liquid and was replaced with pentane. The mixture was filtered, yielding 20 mg of purified amorphous saponin. This material was chromatographed on a silica gel G chromatoplate with 20 % butanone, 65 % isopropyl alcohol, and 15 % water and was then found to be a mixture of saponins, with overlapping spots. Contrary to the results with all other preparations, no material with higher  $hR_F$  value than that of the hemolytic substances could be detected with the aid of iodine vapor, and with UV light. The purified securidaca-saponin was also chromatographed on filter paper. With a solvent consisting of 80 % isopropyl alcohol and 20 % water, and with a solvent consisting of 75 % ethyl formate, 10 % formic acid, and 15 % water it was found to contain material which strongly reduced the regular silver reagents and which had lower  $R_F$  value than the hemolytic substances (Fig. 1). With water-free solvent mixtures containing pyridine, e.g., 40 % chloroform, and 60 % pyridine, or 60 % isopropyl alcohol and 40 % pyridine, or 30 % ethyl methyl ketone and 70 % pyridine, some of the saponin remained at the starting-point, and some moved, however with considerable tailing (Fig. 2).

*Thin-layer chromatographic analysis.* In order to move spots of securidaca-saponin on a silica gel G chromatoplate, the solvent mixture must have a dielectric constant larger than 20 and preferably about 30, except when the mixture contains a substantial proportion of pyridine.<sup>18</sup> The following mixtures were found useful for separating the saponins (as overlapping spots) from other substances: (a) 15 % acetic acid, 60 % butanol, and 25 % water; (b) 40 % chloroform, 40 % methanol, and 20 % ammonia (17 %); (c) 50 % butanone, 20 % tertiary butanol, and 30 % water; (d) 20 % butanone, 60 %

tertiary butanol, and 20 % water; and (e) 20 % butanone, 65 % isopropyl alcohol, and 15 % water. For the last-mentioned solvent mixture, the  $hR_F$  value for all of the saponin preparations tested was between 15 and 50. A two-dimensional thin-layer chromatogram developed in both directions with the last-mentioned solvent showed an elliptic spot oriented along a diagonal, indicating the presence of overlapping spots rather than tailing. Spots were best visualized with iodine vapor, and saponin spots were identified in the following way. The chromatoplate was covered with a filter paper soaked in a buffered suspension of washed red human blood corpuscles (for description see the section on hemolysis). After a few minutes the paper was covered with a glass plate, and after a few hours white spots appeared on the paper. Chlorosulfonic acid reagent and antimony pentachloride reagent were also found useful for the visualization of spots. The trimethylsilyl derivative of securidaca-saponin was prepared according to the method of Sweeley *et al.*<sup>19</sup> and, after evaporation of the reagents in a rotating vacuum evaporator, dissolved in dry tetrahydrofuran. Spots on silica gel G chromatoplates, developed with chloroform, or with a mixture of 95 % pentane and 5 % methanol, followed the solvent front closely. For identification of the saponin derivative the chromatoplates were sprayed with a freshly prepared mixture of conc. HCl and methanol 1:2, heated to about 60°C for a few minutes and kept in a vacuum desiccator over NaOH pellets overnight, whereupon a filter paper, soaked in a suspension of red blood corpuscles, was applied. White spots appearing after a few hours indicated the presence of a saponin.

*Paper chromatography.* The following mixtures were found useful for separation of saponins from other substances: (a) 75 % ethyl formate, 10 % formic acid, and 15 % water; (b) 80 % isopropyl alcohol and 20 % water; (c) 60 % butanol, 20 % ethanol, and 20 % water. The following mixtures were useful for separating the hemolytic substances into two fractions, one of which remained at the starting-point: (a) 70 % isopropyl alcohol and 30 % pyridine; (b) 40 % chloroform and 60 % pyridine; (c) 30 % ethyl methyl ketone and 70 % pyridine. The paper was either dipped in an acetone solution of silver nitrate and subsequently hung up and sprayed with a solution of KOH in 100 % ethanol,<sup>20</sup> or placed on a glass plate and sprayed with a suspension of washed human red blood corpuscles,<sup>21</sup> and then covered by another glass plate resting on two glass plates placed besides the paper on the bottom glass plate. After a few hours hemoglobin in the areas of hemolysis had moved sufficiently to make these areas discernible.

*Partition coefficients.* For eventual countercurrent separation of the components, partition coefficients were determined for the solvent system chloroform-ethanol-water (phosphate buffer) 1:2:3 at various pH values, using the method of sequential extractions,<sup>22,23</sup> and determining the hemolytic index of the various extracts. At all pH values between 5.8 and 10.2 the partition coefficient of one component was about 2; for another component the partition coefficient decreased with increased pH value from 1 at pH 8.6 to 0.15 at pH 10.2.

*Hemolysis.* A suspension of washed red blood corpuscles from surplus patient blood was used for the determination of hemolytic indices. After the blood corpuscles had sedimented overnight, their volume (usually about 2 ml) was marked off on a clean test tube, and a mark was also drawn indicating twice the volume. The supernatant medium was removed, the blood corpuscles mixed with 25 ml of saline, and the suspension was filtered through cotton and centrifuged. The supernatant was removed, and the blood corpuscles were again suspended in 25 ml of saline and centrifuged. The supernatant was removed, the sediment was poured into the previously mentioned clean test-tube, sufficient saline being added to fill it up to the second mark. Two ml of this suspension were diluted to 100 ml with isotonic phosphate buffer pH 7.4 for the assay of hemolytic activity; equal volumes of this suspension and a saline solution of a saponin preparation were mixed, and after 3 h of hemolysis, the mixture was centrifuged and the light absorption of the supernatant was measured in a colorimeter. If a mixture contained slightly less saponin than necessary for complete hemolysis, the readings increased for about 4 h, but if the mixture contained slightly more saponin than necessary for complete hemolysis, the readings reached a maximum and started to decline after about 2 h. Not only the influence of time but also the influence of pH was measured, using isotonic phosphate buffers or carbonate buffers of various pH. The hemolytic index for one securidaca-saponin preparation was about 2 000 000 at pH 5.8; about 150 000 at pH 7.4; and about 20 000 at pH 8.8. For the assay of hemolytic indices serial dilutions of saponin preparations were used. 4 ml of isotonic saline was poured into each of 12 test tubes. 10 ml of

a solution of a preparation in saline was pipetted into the first tube and the contents mixed; from this mixture 10 ml were transferred to the second tube and so on. A typical example of the results obtained in such experiments with securidaca-saponin is shown in Fig. 3. The reason for the appearance of the maximum and minimum have not yet been elucidated, nor is it known which qualities of the blood samples cause the different hemolysis values at the optimum securidaca-saponin concentration. In similar experiments with Merck's pure white saponin the normal dose-response curve was obtained. For identification of saponin spots in thin-layer chromatography a filter paper was soaked in a tenfold dilution of the washed red blood corpuscles with phosphate buffer pH 7.4 or 5.9 and placed on top of the chromatoplate. For identification of saponin spots in paper chromatography the paper was sprayed with a fourfold dilution of the washed red blood corpuscles with equal volumes of isotonic saline and phosphate buffer pH 5.9.

*Acknowledgements.* The author wishes to express his sincere thanks to Professor Dermot B. Taylor for the privilege of joining his research group and for supplying a dried water extract of *Securidaca longipedunculata*. This work was supported by U. S. Army Contract No. DA-18-108-CML-6613.

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Received March 28, 1967.