

Secundatia peruviana

I. Toxic Substances, and Conditions for their Separation

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Secundatia peruviana [Apocynaceae] contains at least six toxic substances whose partition ratios between isobutanol and water have a range greater than 0.05–6.25. The particularly hydrophilic parts with partition ratios less than 0.05 contain about half of the toxic activity, and can be divided into two main components by a simple manual countercurrent extraction using equal volumes of butanone and buffered water pH 6. The substances constituting the less hydrophilic half of the toxic activity are separated from the most hydrophilic substances, and are fractionated into groups of one or a few main components, by countercurrent extraction between isobutanol and water at the volume ratios 16, 6.8, 2.0, and 0.5. The least hydrophilic toxic fraction studied, with a partition ratio larger than 6.25, is almost insoluble in water.

Thin-layer chromatographic analysis of the toxic substances can be done with silica gel G and a mixture of ethyl acetate, formic acid, and water 10:2:3. This solvent mixture is also useful for paper chromatography, as is a mixture of butanol, acetic acid, and water 4:1:5.

The toxic material is glycosidic. It absorbs UV light with a maximum at about 280 μ . On hydrolysis a red precipitate is formed; the supernatant is not toxic.

Some fractions of the toxic material (LD₅₀ about 0.1 g/kg intravenously in mice) had pharmacological effects resembling those of anticholinesterases.

Secundatia peruviana Poeppig and Endl. is a tropical vine, belonging to the Apocynaceae family; subfamily Echitoideae.¹ Among plants classified in the same subfamily may be mentioned *Nerium oleander* L., *Strophanthus hispidus* DC., and *Strophanthus kombé* Oliv., the glycosides of which are of medicinal value. During the last decade many plants of the family Apocynaceae have been found to contain alkaloids, heart glycosides, and other

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substances of potential medicinal interest.^{2,3} Such substances are somewhat randomly distributed among the various subfamilies and genera of Apocynaceae.

Pharmacological screening of Peruvian plants in this laboratory has indicated that the water-soluble fraction of a crude methanol extract of *Secondatia peruviana* is toxic (LD₅₀ about 0.1 g/kg intravenously in mice) with death occurring within a few seconds or minutes, and with post-mortem movements in many animals. The toxicity of the extract was associated with the presence of glycosides with UV absorption.

The plant material was originally obtained from the Chanchamayo region of Peru and subsequently in large amounts from the Huanuco province of Tingo Maria, Peru.

A METHOD FOR RATIONAL PREPARATIVE SEPARATION

The first goal in this investigation is the isolation of the various toxic substances in such quantities as are sufficient for pharmacological research and for further chemical study. An initial variable solvent solubility test indicated the presence of at least five different substances, but only half of the lethal activity had been precipitated by 0.9 saturation with ammonium sulfate.⁴ Further separation attempts were done with liquid extraction methods.

Theoretical. A countercurrent separation of two substances can be repeated easily and exactly, regardless of the quantity to be separated, and is therefore particularly suitable for many preparative separations. If the partition coefficients of the two substances between the phases in the countercurrent extraction differ so much that the quotient between them is larger than 6, a countercurrent separation carried out manually with such simple means as only five separating funnels can be expected to be so efficient that more than 99 % of each component can be recovered from its appropriate solvent phase, contaminated with less than 1 % of the other component.⁵⁻⁸ If the quotient is much smaller, say less than 1.5, a train of cells is necessary for a reasonably good separation.⁸

Two substances with partition ratios K_1 and K_2 are separated most efficiently⁵ if the volume ratio of the solvent phases equals $1/\sqrt{K_1 K_2}$. Many naturally occurring mixtures, e.g., plant extracts, contain substances whose partition ratios differ over a wide range. It may be advantageous for the determination of the partition ratios of the components, first to divide the extract into fractions with smaller partition ratio ranges. This can be done by simple manual countercurrent extractions at arbitrarily chosen volume ratios,⁹ and these volume ratios correspond to partition ratio border lines between the fractions. The partition ratios of substances can obviously be measured more easily in each of the various fractions than in the whole extract.

Exploratory countercurrent separation. Exploratory countercurrent separations of the *Secondatia peruviana* extract were carried out at volume ratios in a geometric series with the quotient about 2.5. The weight of the various fractions, and their toxicity in mice were determined. With one exception

Table 1. Exploratory countercurrent fractionation of a *Secondatia peruviana* extract between isobutanol and water.

Fraction No.	Partition coefficient borderlines	Weight mg	LD ₅₀ mg/kg I.V.	Behaviour of mice, killed by a dose close to LD ₅₀
1	pH about 4 0.00—0.05	395	81	Rapid death. Post mortem movements in one out of three.
2	0.05—0.15	148	115	Excitation. Rapid death. No post mortem movements.
3	0.15—0.38	56	93	Excitation. Rapid death. Post mortem movements.
4	0.38—1.00	47	126	Excitation. Rapid death. Post mortem movements.
5	1.00—2.50	38	89	Excitation. Rapid death. Post mortem movements.
6	2.50—6.25	21	150	Tremor, paralysis and convulsion. Post mortem movements.
7	6.25— further fractionated at pH 7.5 —6.25	33	450	Long sedative action before death. No post mortem movements.
8	6.25—	69	*	Tremor and paralysis after 1 min; death (respiratory failure) after 5 min. Post mortem movements. Salivation.

* A solution of 28 mg of the material in a few drops of ethyl alcohol was diluted with 1/2 ml of a 6 % gum arabic solution. The suspension thereby formed showed no signs of instability for 1/2 h; it was then injected intraperitoneally into one mouse.

the LD₅₀ values were rather similar in all fractions. This is one indication among several others, that the extract consists of many toxic substances with a wide range of partition ratios. Different symptoms were noticed at dose levels close to LD₅₀. The results are summarized in Table 1, and some data are represented in Fig. 1.

We can conclude from the number of separatory funnels used, from the number of portions of solvents used in each of the separations, and from the quotient between the volume ratios, that although any of the fractions may contain almost half of the total amount of a substance with a partition ratio just slightly outside the range for which the fraction was taken, the amount of substance whose partition ratio corresponds to the next nearest fraction is less than 11 % of the total amount of that substance.

The main toxic substances in the first two fractions differ from each other, since only fraction 1 (the most hydrophilic), but not fraction 2, causes post mortem movements in mice. However, the toxic substance or substances in fractions 3, 4, and 5 also cause long lasting post-mortem movements. About the same yield in as many as three consecutive fractions over the partition range 0.15—2.50 can hardly be caused by a single substance. The different properties of fraction 8, the least hydrophilic fraction, and of fractions 6, and

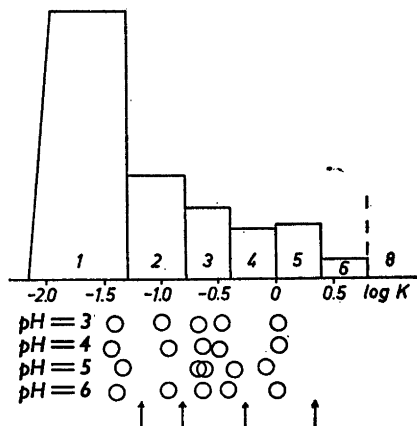


Fig. 1. Countercurrent separation of a *Secodontia peruviana* extract using isobutanol and water at various volume ratios corresponding to partition ratios K , the logarithms of which are indicated in the figure. The areas outlined show the relative amount of toxic material in the various fractions, calculated as the quotient between the weight of the fraction and its LD_{50} value. Rings indicate partition ratios of various UV absorbing substances at various pH values. Arrows indicate suggested new limits for fractions in counter-current separations.

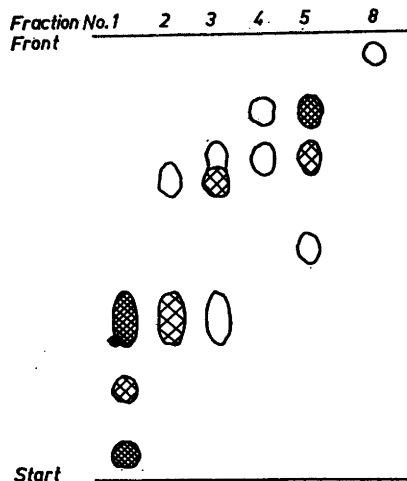


Fig. 2. Thin-layer chromatography of *Secodontia peruviana* extract fractions on silica gel G, with the solvent mixture ethyl acetate, water, and formic acid 10:3:2. Strong colour intensity of the spots is indicated by crosshatching.

7, apparently must be caused by different substances. Consequently, at least six toxic substances are probably present in the *Secodontia peruviana* extract.

Separation conditions. As previously mentioned, the toxicity was associated with the presence of glycosides which absorb UV light. The partition ratios of the main UV absorbing substances were measured in the pH range 3–6 for partition ratios between 0.03 and 1.^{10,11} The results are given in Fig. 1, in which arrows indicate limits for so called “completion of squares” or “diamond” countercurrent separations as calculated from the expression for maximum efficiency mentioned in the theoretical part. These limits correspond to the volume ratios 16, 6.8, 2.0 and 0.5 between isobutanol and water. The pH value of the water phase does not affect the separation within the range 3–6.

About half the toxic activity in the *Secodontia peruviana* extract was caused by substances whose partition ratios between isobutanol and water were less than 0.05. Preliminary experiments indicated the presence of two main components in this part. Because an efficacious countercurrent separation is hardly feasible at a volume ratio considerably larger than 15:1, a few other solvent systems were tested. A mixture of chloroform and ethanol 3:2

has been suggested and extensively used for extracting glucosides from a water solution, half saturated with sodium sulfate,¹² but no noticeable amount of material could be extracted with this mixture. However, butanone and buffered water turned out to be a suitable solvent system. The pH value had a considerable influence upon the value of the partition ratios of the two main UV absorbing components. At pH 6 their partition ratios were 3.4 and 0.31, and subsequently they were separated by a simple manual countercurrent procedure, using equal volumes of butanone and buffered water at pH 6. The separation between these two substances can theoretically be expected to be better than 99 % even if only four separating funnels are used for the procedure.

METHODS FOR ANALYSIS

Thin-layer chromatographic and paper chromatographic methods were worked out for the analytical separation of the constituents of the various fractions. The best overall separation with distinct spots was obtained with silica gel G chromatoplates and, for development, ethyl acetate, formic acid, and water 10:2:3. The same solvent was also useful for paper chromatographic analysis of the middle fractions which gave post mortem movements in mice, as was also the mixture butanol, acetic acid, and water 4:1:5.^{13,14}

PHARMACOLOGY

Suitable doses of preparations of the *Secondatia peruviana* extract produced convulsions and death in mice. Normally, if a mouse does not start to breathe within a minute or less after a convulsion, the damage to the central nervous system is irreversible. In these mice, however, marked post-mortem twitching was seen which went on for at least ten minutes. Since the central nervous system had long since suffered irreparable damage, the twitching must have been peripheral, and the convulsions looked like those produced by anticholinesterases. An almost identical effect was produced by fatal doses of eserine. No other known classes of substance do this.

The action of acetylcholine on the cat blood pressure and heart rate was increased by the extract and, more important, it was prolonged. As far as is known, this can only be achieved with an anticholinesterase.

The action of curare on the cat gastrocnemius was antagonized by the extracts in a way quite similar to the effect of neostigmine. No other class of substance does this.

The action of the extract visible on chick electroencephalograms resembled that of eserine very closely but was of shorter total duration.

EXPERIMENTAL

Exploratory separation. About 0.9 g of water soluble material in a methanol extract of *Secondatia peruviana* was dissolved in 6 ml of water saturated with isobutanol. The pH value of this solution was 3.3. The solution was countercurrent extracted in separatory funnels, using 40 ml portions of isobutanol phase and 6 ml portions of water phase in a so called "completion of square" or "diamond" separation. The top layers were pooled

and reduced in vacuum to a small volume, as were the bottom layers. The countercurrent extraction was repeated with both fractions until the material had been separated into seven fractions with the volume ratios 20:1, 20:3, 13:5, 1:1, 5:2 and 25:4; the inverse value of these ratios are the partition ratios which define the borderlines between the fractions. Five funnels and five portions of each solvent phase were used in these separations. For a second separation at volume ratio 25:4 the pH value was adjusted to 7.5. The fractions were partly evaporated in vacuum, recovered as concentrated water solutions (except the most lipophilic one) and freeze-dried. They were tested for toxicity and specific pharmacologic effects in mice.

Determination of partition ratios. The method of sequential extraction was used,^{10,11} and because other experiments had indicated that the toxicity is caused by glucosides absorbing UV light, the partition coefficients of the main UV absorbing substances were measured. Before the sequential extraction was carried out, and in order to increase the accuracy, the fractions were usually divided into two subfractions by means of a countercurrent separation on a microscale, using two or three glass-stoppered, 3 ml test tubes with pointed end, and pipette droppers.

Partition ratios between butanone and water. Because a saturated solution of butanone in water absorbs UV light strongly, the butanone concentration in the sequential extracts had to be reduced to an insignificant level. Hence, the sequential water phase extracts, 5 ml portions, were extracted with 10 ml of diethyl ether, and 2 ml of the remaining water phase were again extracted with 10 ml of diethyl ether. The UV absorption was measured against a blank treated in the same way.

UV absorption. The light absorption of the most hydrophilic toxic fraction of the *Secundatia peruviana* extract was measured in the range 225–580 μ . The results are, of course, preliminary because no pure substance was available at that time. There was only one maximum value, $E_{1\text{ cm}}^{\%} = 14$ at about 280 μ , and only one minimum value, $E_{1\text{ cm}}^{\%} = 6.4$ at about 260 μ . $E_{1\text{ cm}}^{\%}$ was about 100 at 225 μ , 1 at 315 μ , and 0.1 at 520 μ .

Example of countercurrent separation of toxic substances under optimum conditions. 53 mg of the most hydrophilic preparation from a fraction with isobutanol and water was countercurrent extracted at pH 6 in five separatory funnels with 11 portions, 5 ml each, of butanone phase and water phase. The six butanone portions first recovered contained 30 % of the material, and the six water portions first recovered contained 64 % of the material, while 6 % of the material was found in the remaining portions.

Paper chromatography. Paper chromatographic analyses were run using the test tube technique and the small museum jar technique.^{15,16} For the most hydrophilic fraction, solvent mixtures containing butanone, an alcohol, and water gave spots without fixation and tailing, e.g., butanone, butanol, acetic acid, and water 4:2:1:3; butanone, isobutanol, and water 2:1:2; and butanone, butanol, and water 3:1:2. A mixture of tertiary butanol, concentrated ammonia, and water 3:1:1 was also useful. For the middle fractions, the following solvent systems were useful: butanol, acetic acid, and water 4:1:5; and ethyl acetate, formic acid, and water 10:2:3.^{13,14} Spots were visualized by their capability to absorb UV light, and by their power to reduce silver hydroxide.¹⁷ Aluminium chloride reagent did not give a typical reaction for flavon glucosides.¹⁸ Moreover, trichloroacetic acid and chloramine reagent did not give a typical reaction for heart glycosides.^{19,20} Bromocresol green produced a colour on spots, indicating the presence of several acidic substances.

Thin-layer chromatography. Hänsel and Hörhammer's solvent (ethyl acetate, formic acid, and water 10:2:3), previously used only for paper chromatography,¹⁴ gave better separation than any other solvent tested (Fig. 2). However, some of these spots probably contain more than one component, because the relative strength of the reaction of the various spots was different with a silver nitrate and ammonia reagent, with the chloramine and trichloroacetic acid reagent, and with the aluminium chloride reagent.

Determination of LD_{50} . For the determination of the toxicity of various preparations, suitable doses were injected into the tail vein of mice with a weight about 20 g. The doses were chosen on a logarithmic scale with mantissa differences of 0.125. The experiments were done according to the "up-and-down" method. Six values were used for the calculation, the first of which was the last dose without changed response (survival or death) in the beginning of the determination.²¹⁻²³

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