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PILOT PRODUCTION OF MEDICINES USING INDIGENOUS RAW MATERIALS

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VIET NAM

Technical Report:
Analysis of and improvement on traditional drugs and laboratory processes for some drugs and intermediates based on plants and their analysis*

Prepared for the Government of Viet Nam
by the United Nations Industrial Development Organization,
acting as executing agency for the United Nations Development Programme

Based on the work of Dr. S.K. Banerjee
Organic Chemist/Analytical Chemist Consultant

Backstopping Officer : R.O.B. Wijesekera
Chemical Industries Branch

United Nations Industrial Development Organization
Vienna

2/76

* This document has not been edited.

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1. INTRODUCTION

The Institute of Materia Medica under the Ministry of Health is engaged in the task aimed at promoting the health of the people of Viet Nam using the vast plant resources of the country. It has done a commendable job towards this objective. However, the bulk of the people of Viet Nam depend on crude traditional drugs for solving their health problems as very few can really afford to use modern medicines. The formulation of the present project is based on the need to evaluate and rationalise this system of medicine in the light of modern knowledge and mordernise production of medicines using the indigenous plant materials. The Institute of Materia Medica is the most suitable national agency to execute this project in view of its background in the field of development of new drugs and technologies for drugs and intermediates based on plants.

2. OBJECTIVES

The objectives of the project are to increase the R & D capabilities of the Institute of Materia Medica, Hanoi. This will enable the Institute not only to develop improved traditional drugs and technologies for drugs and intermediates but also evaluate them.

3. DUTIES ASSIGNED TO THE CONSULTANT (ORGANIC CHEMIST/ANALYTICAL CHEMIST) AS PER JOB DESCRIPTION

The expert will function under the direction of the UNIDO CTA and project co-ordinator and work in collaboration with national counterparts and other international experts so as to accomplish the following:

a Developing laboratory scale processes for the extraction, separation and isolation where deemed necessary of selected medicinal plants.

b Initiating the up-scaling of laboratory processes developed by the Institute of Materia Medica Hanoi with a view to developing industrial scale process protocols.

c Providing training to national counterparts in all relevant aspects concerning organic phytochemical methodology and in particular in

- Methods of steroid chemistry and steroidal transformations
- Methods of instrumental analysis
- Analytical methods of process monitoring and drug standardisation.

The expert will at the end of his first mission write a brief preliminary report on any interim recommendations he wishes to make and at the end of his final mission he will furnish a terminal report. This latter report will further set out his findings and long-term recommendations to government as well as UNIDO for the future activities for the project.

4. WORK REQUIRED TO BE CARRIED OUT BY THE CONSULTANT

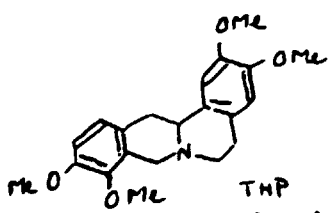
The consultant arrived in Hanoi on the 4th of November, 1987. He duly reported for duties at the Institute of Materia Medica. The first few days were spent by the consultant in apprising himself of the various R & D activities of the Institute through meetings and discussions with its scientists including group leaders, vice-directors and the director. During this period he also had briefing from the CTA in regard to the work he was expected to carry out during the present assignment. The CTA asked the consultant to look into the following problems faced by the Institute in regard to modernising its indigenous production of medicines and work towards their solution.

1. Removal of the colour of the l-tetrahydropalmatine (THP), a tranquillosedative produced in the Institute; facile production of l-THP and its hydrochloride from the *Stephania* species.

2. Facile production of Berberine chloride from Vietnamese plant material Vang Dang (Coscinium fenestratum)
3. Production of tetrahydroberberine (THB), a tranquillosedative from berberine chloride.
4. To streamline the use of Achyranthes bidentata in the Vietnamese system of medicine; preparation of enriched fraction having antiinflammatory and antilipidemic (cholesterol lowering) activities; isolation of the principles active against chronic inflammation.
5. Production of an effective antihepatoxic drug based on Adenosma indianum (Cay Bo Bo) and its evaluation; also use of other species of Adenosma viz. A.bilabiatum and A.capitata for the production of an antihepatoxic drug.
6. Production of Diosgenin
7. Production of 16-DPA
8. Training on the standardisation/analysis of the above products. Discussions & lectures to different research groups on alkaloids, glycosides, coumarins, steroids and essential oils.

5. WORK ACTUALLY CARRIED OUT BY THE CONSULTANT

5.1. Tetrahydropalmatine (THP) and its hydrochloride (THP,HCl):



THP

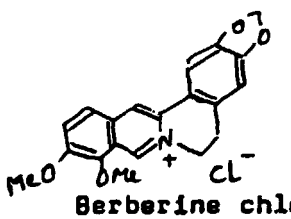
l-Tetrahydropalmatine (THP) is a tranquillosedative occurring in large quantities in the tubers of some Stephania species. The consultant examined some of the samples of THP isolated by the scientists of the Institute of Materia Medica. He was also given a dark-coloured product, called the total bases of Stephania by the scientists. The consultant

told the scientists that the formation of yellow colour in the sample of THP was due to the fact that THP is oxidised by air (atmospheric oxygen) and slowly becomes yellow owing to the formation of palmatine (a yellow alkaloid). The yellow colour in a sample of THP can be easily removed by treating a methanolic solution of the sample with sodium borohydride (The process was demonstrated by the consultant : Vide Annexure A). He also explained the theory of the oxidation of the tetrahydro bases into the quarternary compounds (colour formation is due to extension of conjugation) and how $NaBH_4$ reduces the quarternary compounds into the tetrahydro bases. However, the consultant told the scientists that THP does not become so yellow as is commonly believed provided it is properly stored. In support of his statement he asked the authorities of the Institute to bring the sample of THP isolated by him in India and supplied to the Institute of Materia Medica more than two and a half years ago. The sample had been stored in a brown bottle which was properly capped. The data of the product conformed to the ones given in the Merck Index in regard to its colour which was almost white, m.p. of which was 142-143°, specific rotation $[\alpha]_D^{26}$ which was -289.7° (0.8% in EtOH) and solubility. It also gave a single spot on TLC (Rf 0.69, silica gel G, Et_2O : MeOH (49 : 1). It gave the hydrochloride which melted at 214-217°. On U.V. spectrophotometric analysis it was found to contain 99.03% THP (Vide Annexure B). The processes for the production of THP and its hydrochloride were developed by the consultant and passed on to them (Vide Annexures C and D). The processes were also demonstrated to them. The consultant also told the scientists that the physical constants and other properties of THP and THP, HCl as recorded in the Merck Index should be followed in evaluating the samples of these products. For the quantitative analysis of the samples of THP and THP, HCl U.V. spectrophotometry (vide Annexure B) and HPLC with a UV-detector (at 282 nm) can be used. As THP, HCl is administered as the drug and this being more stable than the base towards aerial oxidation the consultant asked the scientists not to store the base as such for long but instead convert it into hydrochloride within a few days of its isolation. He further advised that both THP and THP, HCl be stored in dark - coloured bottles. As THP is rather a weak base being liberated in the acidic pH the formulations should exclude the exipients which would consume the HCl of THP, HCl thereby liberating the free base. The consultant isolated more than 500 g of THP and converted it into the hydrochloride for use by the industrial pharmacist consultant.

On examination of the so called "total bases of Stephania" the consultant found it to be full of starchy, cellulosic and inorganic materials. Not more than 5% of the product was soluble in $CHCl_3$ and ethanol (as seen by repeated boiling with these solvents). The method of isolation of this product was highly crude and cumbersome. Later on the consultant was told that this product was not prepared at the Institute but was supplied by some other agency in Vietnam. The consultant asked the scientists to follow his process for the production of THP and THP, HCl and discontinue the work on processing the so called "total bases" for the manufacture of THP and THP, HCl.

The consultant also advised the scientists to ensure a steady supply of raw materials, Stephania tubers, of good quality containing at least 1% of THP. For this purpose all the nine species of Staphania growing in Vietnam should be evaluated for THP and the areas for the collection of Stephania tubers should be mapped based on the report on the evaluation of the tubers.

5.2. Berberine chloride and the other salts of berberine :



Berberine chloride

Berberine chloride is used in the treatment of non-specific diarrhoea. Because of its toxicity to lower forms of life berberine is used in the treatment of oriental sore. Because of its intense bitter taste it is used in bitter tonic and stomachics. Berberine is also used in eyewashes.

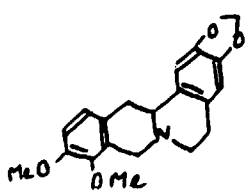
The major source of berberine in Vietnam is Coscinium fenestratum Colebc.(Menispermaceae) (Vang Dang). The powdered plant is used in the treatment of diarrhoea the dose being 8 g of plant material/person/day.

The process being followed in the Institute of Materia Medica for the production of berberine chloride consists in the extraction of the ground roots of C.fenestratum with 0.25% aq. H_2SO_4 for 24 hours at room temperature followed by basification of the aq. acid extract with caustic soda to pH 8.5 - 9 and removal of the precipitate which separates out. The basified solution (after removal of the ppt.) is treated with conc. HCl to pH 1 and the mixture left for 24 hours. The precipitate of berberine chloride which separates out is collected by filtration. This process involving one extraction with aq. H_2SO_4 in the cold does not leach out all the berberine. Secondly, introduction of mineral ions through the addition of NaOH is undesirable. The samples thus obtained did not conform to J.P. specifications on analysis.

An improved process developed by the consultant was passed on to the scientists and demonstrated to them (see Annexure E). The process is economical and facile and excludes one step from the one followed in the Institute, that of addition of NaOH. The sample of berberine chloride prepared following the process of the consultant conformed to the specifications of Japanese Pharmacopoeia - JP. - 10 (Annexure F).

On request from the authorities of the Institute including the Vice-Minister of Health, Prof. Dr. Nguyen Van Dan the consultant developed the processes for the production of berberine acid sulphate (berberine bisulphate) and the neutral berberine sulphate and demonstrated them to the concerned scientists (Annexures G and H).

5.3. Tetrahydroberberine (THB) and its hydrochloride (THB, HCl)



Tetrahydroberberine

Tetrahydroberberine is a tranquillosedative. The naturally occurring form is optically active and the dl - form is conveniently obtainable from berberine by reduction with a number of reducing agents of which $NaBH_4$ seems to be most facile. Being a tetrahydro base like tetrahydropalmatine, THB is oxidised by atmospheric oxygen to berberine (yellow colour). Therefore, like THP and THP,HCl, THB and THB,HCl are to be stored in dark - coloured bottles and THB should preferably be converted into the more stable THB, HCl (the drug which is administered) shortly after its synthesis from berberine chloride.

Processes developed by the consultant for the production of THB and THB,HCl were demonstrated to the concerned scientists (Annexure I and J). The physical constants of these products corresponded well with those recorded in the literature. THB,HCl prepared under the supervision of the consultant also gave a single peak on HPLC analysis using a UV - detector (at 286 nm).

5.4. Achyranthes bidentata :

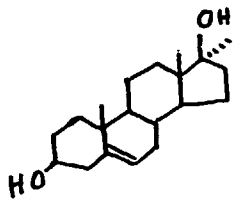
The roots of this plant are used in the treatment of a variety of diseases in the Vietnamese system of medicine. The two most important activities of A. bidentata are its antilipidemic (cholesterol lowering) and antiinflammatory activities. The saponins isolated from the plant have been found to be the saponins ofoleanolic acid. Although the saponin fraction shows no antilipidemic activity but in combination with the sugars of this plant or Piper nigrum (dried berries called pepper) it is very effective as an antiinflammatory agent. Therefore, the consultant concluded that the question was to promote the bioavailability of the saponins by mixing it with the sugars (of the roots of the plant) or better still the active principle of pepper, piperine which is well known for its activity as a promoter of bioavailability. The consultant was requested to give the method of isolation of piperine from Piper nigrum to the scientists. He demonstrated to the scientists the method of isolation of piperine (Annexure K). However, as Piper nigrum is rather expensive in Vietnam he suggested that the cheaper source of piperine, Piper longum be used for the production of piperine. The consultant advised that 90 - 95% alcohol be used for the extraction of the active fractions because

extraction with water or 40% alcohol brings forth a lot of sugars thereby increasing the bulk of the drug formulation. Extraction with 90 - 95% alcohol will extract the saponins and also the desirable amount of sugars thereby yielding a cleaner product. The consultant also suggested that the estimation of the saponin fraction be carried out on the basis of the haemolytic index of the saponins or hydrolysing the saponins with acid to yield oleanolic acid which can be determined by actual isolation. Oleanolic acid was found not to have any antiinflammatory properties as observed by the scientists of the Institute. However, the consultant's experience was otherwise. Some recent preliminary investigation carried out in Regional Research Laboratory, Jammu, India with which the consultant is associated showed that oleanolic acid has marked activity against chronic inflammation. The selection of oleanolic acid for evaluation as an anti-inflammatory agent was based on the discovery of Boswellic acid by C.K. Atal et al. as an effective and safe antiinflammatory agent. This drug called Sallaki, has a very good market in India. The consultant and the CTA asked the scientists of the Institute to produce the right experimental animal models of chronic inflammation for the evaluation of oleanolic acid as an antiinflammatory agent. The CTA is now arranging the procurement of the bacterium required to induce chronic inflammation in animals. The consultant isolated about 6 g of oleanolic acid and handed it over to the scientists for the study and also gave the process (Annexure L) to the scientists following which they isolated some quantity of oleanolic acid.

55. Adenosma indianum and two other spp. of Adenosma :

A.indianum as also two other species of Adenosma viz. A.caeruleum and A.bracteosum enjoy good reputation in Vietnam as a very effective drug for the treatment of liver diseases. The alcoholic extracts of these plants have pronounced choleretic effect although the essential oils have significant choleretic activity. A. indianum being the most abundant species has been extensively studied and is the most commonly used species of Adenosma for the preparation of liver drugs. A.indianum yields saponins

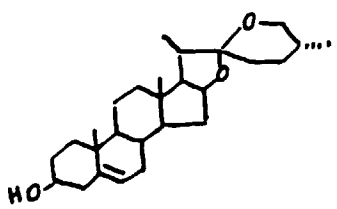
hydrolysis or which gives four sapogenins one of which has been characterised as



by the scientists or the Institute. The consultant asked the scientists to subject this sapogenin to Oppenauer oxidation and find out whether the oxidation product is 17 alfa-methyltestosterone, an anabolic steroid and authentic sample of which, prepared by the consultant, was passed on to the Institute for comparison. This would not only confirm the structure of the sapogenin but may offer a source for 17 alfa - methyltestosterone.

The consultant was requested to suggest methods for the standardisation of these plants. As the potent fractions of A.indianum contains flavonoids with phenolic hydroxyl groups and other phenolics he suggested that the standardisation of the plant be made on the basis of isolating the phenolics as the lead salt (vide Annexure M). He also gave the scientists the methods for the isolation of saponins and phenolics from these three spp. of Adenosma.

5.6. Diosgenin :

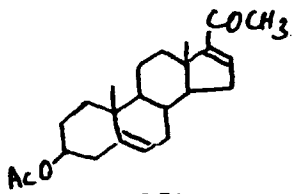


Diosgenin ($C_{27}H_{42}O_3$)
Mol. wt. 414.61

Diosgenin is one or the most important and versatile starting material for the manufacture of steroid drugs including oral contraceptives. The steroid industry of many countries relies almost entirely on diosgenin as the starting material, the most important and only commercially exploitable source being the yams of Dioscorea. A bench scale process for the isolation of diosgenin has been demonstrated to the scientists of the Institute (Annex.N). The process could not be

carried out on larger scale in the Institute for the lack of pilot plant facilities. It is proposed that when the pilot plant becomes operational this process will be demonstrated by the consultant on the pilot plant.

5.7. 16 - Dehydropregnenolone acetate (16-DPA)



16 - DPA

16-DPA is one of the most important intermediates for steroid drugs. A process for the production of this intermediate starting from diosgenin has been demonstrated on the laboratory scale. 16 - DPA thus obtained (Annex.O) conformed to the specifications (Annexure P). It is proposed to carry out pilot production of this intermediate when the plant is commissioned.

5.8. Training of scientists on analysis and standardisation of drugs. Discussions and lectures:

The consultant gave extensive training to scientists of different groups of laboratories including analytical, standardisation, phytochemistry and semisynthetic divisions. The training included analysis of drugs and intermediates based on chemical methods, U.V. spectrophotometry, GLC (gas liquid chromatography) and HPLC (high performance liquid chromatography). He gave a large number of lectures to small groups of scientists on isolation and characterisation of alkaloids, glycosides, steroids, essential oils and coumarins. He took part in about ten meetings in the Institute with scientists some of which were attended by Prof. Nguyen Van Dan, Vice-Minister of Health, the Director, the National Project Co-ordinator, the CTA and the Vice-Directors.

The consultant gave the process for isolation of blood pressure lowering alkaloids of Rauwolfia. He also gave processes for the isolation of ajmalicine (raubasine) and serpentine from Catharanthus roseus and Rauwolfia spp. as also the conversion of serpentine into ajmalicine

(Annexure Q). He also gave the design for a hydrogenator which is very simple and inexpensive and can be used for medium scale production of drugs (Annexure R). He advised the scientists to get it fabricated locally and explained how to operate it including as to how the flask containing the material to be hydrogenated be placed on a horizontal shaker. He also asked them to get the shaker fabricated.

6. CONCLUSIONS AND RECOMMENDATIONS

1. The Institute should strengthen the infracture of the laboratories so as to handle large bench scale operations of processes. For this purpose flasks of different sizes (upto 20 litres), heating mantles (upto 20 L), Soxhlets (upto 10 L), large percolators, water-baths, hot plates, electrical stirrers etc. should be procured.

2. The processes of various drugs and intermediates given by the consultant should be run on large bench and pilot scales when the facilities are created.

3. Procurement of raw materials including plant materials, chemicals and solvents should be streamlined.

4. The work on the production of steroid drugs and intermediates should be encouraged. Necessary fund may please be provided for fabrication and commissioning of plants, raw materials and development of agrotechnologies for the cultivation of Dioscorea spp. (of good quality), the raw material for diosgenin.

7. ACKNOWLEDGEMENTS

The consultant gratefully acknowledges with thanks

1. The valuable guidance received from Dr. C.K. Atal, the Chief Technical Advisor of the Project.

2. The kind co-operations received from Prof. Dr. Nguyen Van Dan, the Hon'ble Vice-Minister of Health and Prof. Nguyen Gia Chan, the Director of the Institute, Mme. Prof. Doan Thi Nhu, the National Project Co-ordinator, Mr. Le Tung Chau and Mr. Truong Canh, the Vice-Directors.

3. The valuable help and co-operations received from scientists including project leaders of which special mention may be made of Mr. Ngo Ngoc Khuyen, Prof. Do Viet Trang, Dr. (Mrs.) Nguyen Thi Nghia, Mrs. Ng.Khoa Dieu Hang, Mr. Nguyen Xuan Cuong, Mrs. Nguyen Hoang Anh, Mrs. Khuong Bang Tuyet, Mrs. Nguyen Thi Thuyet, Mrs. Nguyen Minh Quy, Mr. Pham Kim Man, Mrs. Nguyen Bich Thu, Mrs. Vu Kim Thu, Mrs. Nguyen Kim Bich and Mr. Nguyen Van Thanh.

4. The kind help and co-operation received throughout the execution of work on the project from Mr. Nguyen Tuong Dung, the Project Secretary.

ANNEXURE A

REMOVAL OF YELLOW COLOUR FROM SAMPLES OF TETRAHYDROPALMATINE (THP)

A yellowish sample of THP (50 g) was dissolved in hot methanol (200 ml) and the solution treated with a solution of sodium borohydride (500 mg) in ice-cold water (3 ml) [The amount of $NaBH_4$ to be used would depend on the extent of oxidation the sample had suffered from; the end point can be determined from the colour of the solution - when the yellow colour would not fade further]. The solution was concentrated to about half its volume on a water-bath. On cooling the solution crystals (almost white) of THP separated out which were collected by filtration. The crystals were washed with cold aq. methanol (50%, 10 ml) and then with water (to free it from alkali, tested with pH paper) and finally with cold methanol (5 ml). The mother liquor on concentration gave a further crop of THP (almost white) (which was collected by filtration and washed with aq. MeOH, water and MeOH as above). The total yield was 46 g. The product melted at 142-143° and gave a good optical rotation $[\alpha]_D^{27}$ - 287.9°(c, 0.8% in alcohol).

ANNEXURE B

ASSAY OF A SAMPLE OF TETRAHYDROPALMATINE (THP) OR THP,HCl

Accurately weighed 0.01 g sample of THP (=0.01103 g of THP, HCl) is dissolved in 25 ml of absolute alcohol. From it 0.02 ml of accurately measured solution is transferred to 10 ml volumetric flask and the volume made up to 10 ml with absolute alcohol and D^{282}(absorbance) is measured at 282 nm.

$$\text{Percentage of THP,HCl} = \frac{V_1 \cdot V_2 \cdot D^{282} \cdot 1.103 \cdot K}{V_2 \cdot E^{1\%}_{1cm} \cdot a}$$

where K = Instrument correction factor

V_1 = 25 ml

V_2 = 0.2 ml

V_3 = 10 ml

a = 0.01 g

$$1.103 = \frac{\text{Mol. wt. THP,HCl}}{\text{Mol. wt. of THP}}$$

Example : Assay of a two and a half years' old sample of THP

$$\% \text{ THP, HCl} = \frac{25 \times 10 \times 0.12 \times 1.103 \times 1.03}{0.2 \times 156 \times 0.01103} \quad / K = 1.03 /$$

/ 0.01 g of THP $\equiv$ 0.01103 g of THP, HCl /

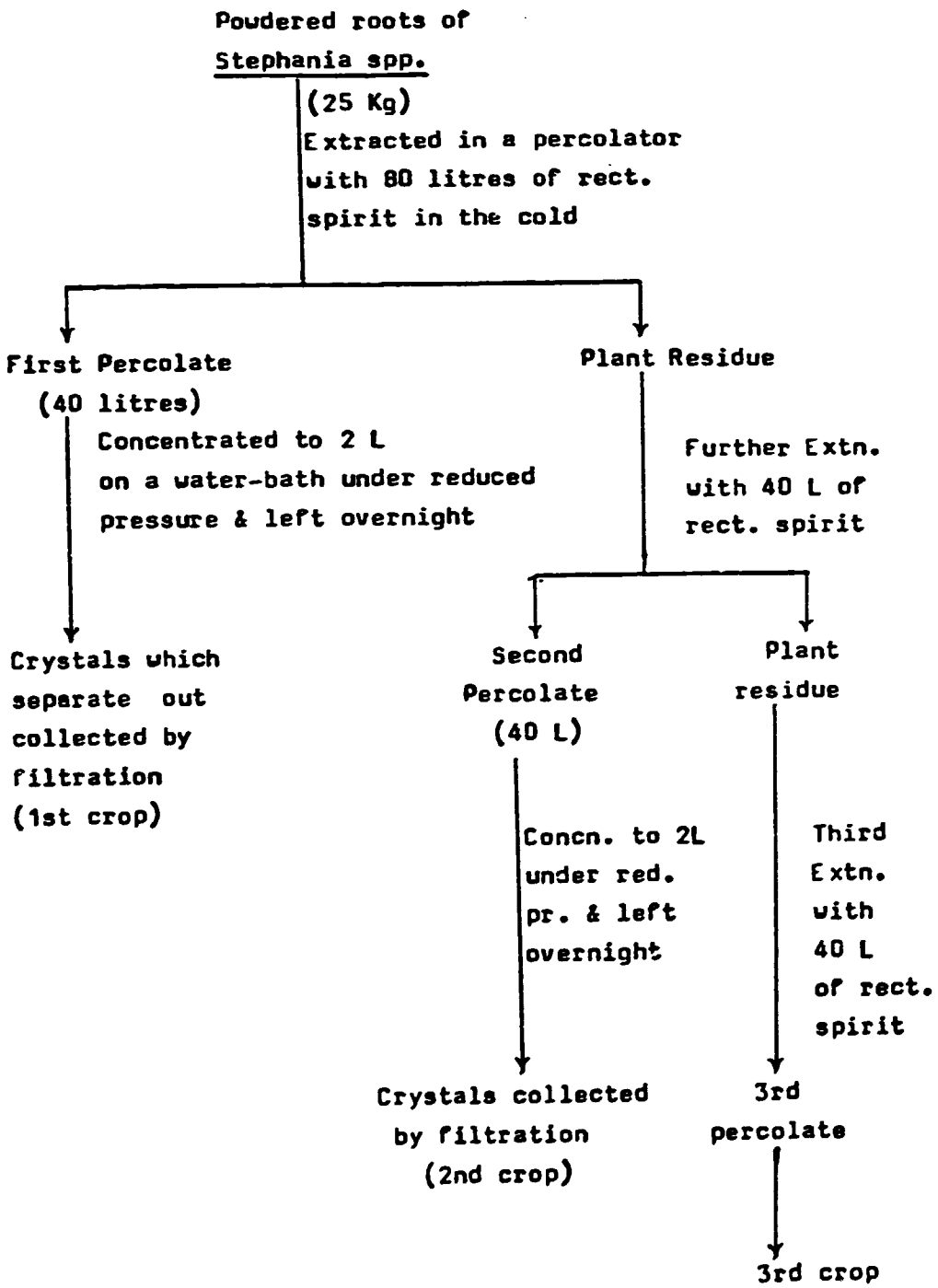
For the analysis of a sample of THP, HCl base be liberated from an accurately weighed sample of THP, HCl by treating its chloroform soln. with NH_3 and the residue obtained on evaporation of the solvent be dissolved in 25 ml of absolute alcohol and proceeded as above.

HPLC can also be used to assay a sample of THP or THP,HCl using MeOH as the mobile phase and UV detector at 282 nm. For this purpose a calibration graph (plotting peak height against concentration) may be drawn using different concentrations of solutions of standard products. Then comparison of heights of peaks of samples with those in calibration graph would indicate the percentage of THP or THP,HCl in the samples.

ANNEXURE C

ISOLATION OF TETRAHYDROPALMATINE (T.H.P.)

$[C_{21}H_{25}NO_4]$ Molecular weight 355.42



In all, six extractions are carried out each time charging the plant residue with 40 L of rectified spirit. The first three percolates are concentrated on a water-bath under reduced pressure (water-pump), each time to 2 L. The separated crystals are collected by filtration and the crystals washed with alcohol. The mother liquors from the first three crops (6 L) on concentration give a further crop of crystals.

All these crops of crystals are mixed and dissolved in chloroform. The chloroform solution is washed in a separating funnel with 2% aq. NaOH soln. (2x 250 ml) and then with water (2 x 300 ml) and dried (Na_2SO_4). The washed chloroform solution is passed through 30-40 g of celite to remove suspended impurities. The residue obtained on removal of chloroform on crystallisation from methanolgives THP. Themother liquor on concn. gives a further crop.

The 4th, 5th and 6th percolates are concentrated separately on a mantle to 10 L each and then on a water-bath to 2 L each under reduced pressure (water pump). These concentrates on keeping may not deposit any crystals. The concentrates are combined and the alcohol removed completely under vacuum. The residue is extracted with 2% aq. HCl till the extract give tests for alkaloids. The aqueous extract is washed with ether twice and then extracted with chloroform in which THP hydrochloride is soluble. The chloroform extract is then washed twice with 2% aq. NaOH solution which not only liberates the free base but also removes the phenolic bases. The chloroform layer is then washed with water and dried (Na_2SO_4). The residue obtained on removal of the solvent is then crystallised from methanol when a further crop of THP is obtained.

The treatment given to the 4th, 5th and 6th percolates can be applied in general for the isolation of THP from the roots of Stephania spp. If the THP content of the tubers is high (around 1% or more) then THP separates out on concentration of the first three percolates.

Roots —(Rect. spirit, Cold)→ Extract —(Removal of solvent under reduced pressure)→ Dry extract —(2% aq. HCl)→ Acid aqueous ext.

Acid aqueous ext. —(Washed with ether and then extracted with chloroform)→ Chloroform extract (containing THP,HCl)

Chloroform extract —(Washed with 2% aq. NaOH, dried and distilled)→ Residue

Chloroform extract —(Crystallisation from MeOH)→ THP

The above method is based on the principle that THP, HCl is soluble in chloroform.

The consultant has worked on Stephania spp. of India for the isolation of THP and the process is based on that. The amount of Stephania tubers processed under the supervision of the consultant at the Institute of Materia Medica was much less than what is described in this process owing to the infrastructural deficiencies of the Institute. However, the consultant gave this large bench scale process so that the Institute can make necessary arrangements for handling operation at this scale.

Melting Point of l - THP (cryst. from methanol) = 147°;
$[\alpha]_D^{20}$ - 291° (c = 0.8 in 95% alcohol).

ANNEXURE D

PREPARATION OF TETRAHYDROPALMATINE HYDROCHLORIDE (THP,HCl)

Tetrahydropalmatine (120 g) was dissolved by refluxing it in methanol (300 ml) and to the hot solution concentrated hydrochloric acid (37 ml) was added, shaking the flask during the addition. On cooling the flask in the fridge for 2 hours white crystals of tetrahydropalmatine hydrochloride separated out. These were collected by filtration and the crystals washed with cold methanol (30 ml) and dried (yield 99 g, m.p. 214 - 217^{0}). The mother liquor on concentration to 100 ml gave a further of THP,HCl (22 g). THP, HCl thus obtained on HPLC analysis gave a single peak (UV detector at 282 nm, mobile phase MeOH).

ANNEXURE E

PROCESS FOR THE PREPARATION OF BERBERINE CHLORIDE ($C_{20}H_{18}NO_4.Cl,2H_2O$) FROM THE ROOTS OF COSCINIUM FENESTRATUM (VANG DANG)

The roots (100 kg) are finely powdered (8 mesh) and soaked completely with dilute sulphuric acid (0.4%, 500 lit) in a wooden Vat of 1000 lit. capacity. The drug is allowed to macerate for 1 hour at a temp. between 70-80°C. The liquor (approx.300 lit. first wash) is drained completely and the drug is recharged with hot dilute sulphuric acid (0.3%, 500 lit.) and kept for a further one hour at 70-80° as above. The cond wash is collected and combined with the first wash. The extr ed drug is again charged with dilute sulphuric acid (0.20%, 500 lit.) and heated as above for 1 hr at 70-80°.

The third wash (500 lit.) of the vat is collected and combined with the first and second washes. The combined washes (1300 lit.) are treated with concentrated hydrochloric acid (10 L) in the settler tank and the mixture allowed to settle. The supernatant solution is siphoned off or drained out through the taps fitted to the tank at the right heights and the suspension is filtered in a centrifuge to yield crude berberine chloride.

Purification of Berberine chloride :

1 kg of the crude berberine chloride is dissolved in distilled water (20 lit.) by boiling in a stainless steel steam jacketed vessel. Activated carbon (30 g) is added and the solution boiled for 15 minutes. It is then filtered hot through a bed of paper pulp in a hot filtration funnel and about 10 ml of conc. hydrochloric acid is added to the filtrate. Bright yellow precipitate of berberine chloride appears on cooling. It is filtered, dried at room temperature and weighed (yield - 900 g).

N.B. The drug after the third extraction can be leached again with 500 L of 0.4% sulphuric acid at 70-80°C. This solution can be used to extract berberine from a fresh lot of roots.

The percentage of aqueous sulphuric acid used above refers to V/V. The process given here is based on the consultant's experience, because this process developed by him has been released to Industry in India which is producing berberine chloride in large quantities a considerable amount of which is being exported, mainly to Japan. However, the consultant could not carry out an operation of this size as such facilities do not exist in the Institute of Materia Medica. He could give demonstration of this process only on one kilogram batch that too with a lot of improvisation.

The berberine chloride thus obtained conformed to the I.P. - 10 specifications. On HPLC analysis it gave a single peak only (UV detector, 421 nm, MeOH).

The consultant advised the Institute to set up an extraction unit of this size and the details of the procedure were explained to them. Small scale extraction of berberine chloride cannot be economically viable as berberine chloride is not an expensive drug.

ANNEXURE F

BERBERINE CHLORIDE
BERBERINE HYDROCHLORIDE

$C_{20}H_{18}ClNO_4 \cdot xH_2O$

Berberine Chloride, calculated on the anhydrous basis, contains not less than 97.0% and not more than 102.0% of berberine chloride ($C_{20}H_{18}ClNO_4$:371.82).

Description : Berberine Chloride occurs as yellow crystals or crystalline powder. It is odorless or has a faint, characteristic odor. It has a very bitter taste.

It is freely soluble in hot water, soluble in hot ethanol, sparingly soluble in methanol, slightly soluble in ethanol, and very slightly soluble in water.

Identification : (1) Dissolve 0.01 g of Berberine Chloride in 20 ml of water by warming, and after cooling add 1 ml of potassium iodide TS: a yellow precipitate is produced.

(2) Dissolve 0.1 g of Berberine Chloride in 20 ml of water by warming, add 0.5 ml of nitric acid, cool, and filter after allowing to stand for 10 minutes. To 3 ml of the filtrate add 1 ml of silver nitrate TS, and collect the produced precipitate: while the precipitate does not dissolve in dilute nitric acid, it dissolves in an excess amount of ammonia TS.

(3) Dilute the sample solution obtained in the Assay with twice its volume of water, and measure the absorption spectrum of the solution : it exhibits absorption maxima at the wavelength between 226 nm and 228 nm, between 262 nm and 264 nm, between 343 nm and 345 nm, and between 419 nm and 424 nm.

(4) Dissolve 0.4 g of Berberine Chloride in 6 ml of methanol by warming, filter if necessary, add this solution to a solution of 0.2 g

of sulfamine previously dissolved in 2 ml of acetone by warming, and concentrate the mixture by warming in a water bath to about 4 ml : an orange to red-brown precipitate is produced. Collect this precipitate, wash with two 5-ml portions of a mixture of methanol and acetone (3 : 1), and dry the precipitate on a porous sheet : the precipitate melts between 216° and 223° (decomposition).

Purity : (1) Clarity of solution : Dissolve 0.10 g of Berberine Chloride in 50 ml of water by warming on a water bath : the solution is clear.

(2) Acid Shake thoroughly 0.10 g of Berberine Chloride with 30 ml of water, and filter. To the filtrate add 2 drops of phenolphthalein TS and 0.10 ml of 0.1 N sodium hydroxide : the yellow color changes to an orange to red color.

(3) Sulfate Shake 1.0 g of Berberine Chloride with 48 ml of water and 2 ml of dilute hydrochloric acid for 1 minute, and filter, Discared the first 5 ml of the filtrate, take the next 25 ml of the filtrate, add water to make 50 ml, and perform the test using this solution as the test solution. Prepare the control solution with 0.50 ml of 0.01 N sulfuric acid, 1 ml of dilute hydrochloric acid, 5 to 10 drops of bromophenol blue TS and water to make 50 ml (not more than 0.048%).

(4) Heavy metals Proceed with 1.0 g of Berberine Chloride according to method 2, and perform the test. Prepare the control solution with 3.0 ml of Standard Lead Solution (not more than 30 ppm).

Water : Not more than 16.5% (0.3 g).

Residue on ignition : Not more than 0.10% (1 g).

Assay : Weigh accurately about 0.2 g of Berberine Chloride, and dissolve in about 200 ml of water by warming. Cool, and add water to make exactly 1000 ml. Measure exactly 10 ml of the solution, dilute with water to make exactly 100 ml, and use this solution as the sample solution. Weigh accurately about 0.2 g of potassium bichromate (standard reagent), previously dried at 110° for 4 hours, dissolve in water, then add 10 ml of 1 N sulfuric acid and water to make exactly 100 ml, and use this

solution as the standard solution. Read the absorbances of the sample solution and the standard solution, At and As, at 421 nm.

$$\text{Amount (mg) of berberine chloride } (C_{20}H_{18}ClNO_4) = \text{amount (mg) of potassium bichromate (standard reagent)} \times \frac{At}{As} \times \frac{1}{1.006}$$

Storage : Preserve in light-resistant, tight containers.

ANNEXURE G

PREPARATION OF BERBERINE ACID SULPHATE (BERBERINE BISULPHATE, $C_{20}H_{18}NO_4.HSO_4$)

Berberine chloride (100 g) was dissolved in methanol by refluxing and to the hot solution was added with stirring a mixture of conc. H_2SO_4 (19 ml) and methanol (190 ml) (The mixture must be prepared by adding the sulphuric acid slowly to methanol cooled in ice). The mixture was allowed to cool when berberine bisulphate was obtained as yellow needles. This was collected by filtration and washed with a little ice cold water (5 ml) and a little acetone for quick drying. The yield was 98 g. The properties conformed to those described in literature.

ANNEXURE H

PREPARATION OF BERBERINE SULPHATE $(C_{20}H_{18}NO_4)_2SO_4 \cdot 3\ H_2O$

Berberine bisulphate (25 g) was taken in a beaker and distilled water (400 ml) was added to it. The beaker was heated to boiling on water-bath (when about 80% of berberine bisulphate had dissolved) and to this was added liquor ammonia carefully with stirring till the pH of the solution became neutral (tested with pH paper) when a clear yellow solution was obtained. The beaker was left in the fridge for 3 hours when shining yellow crystals of (neutral) berberine sulphate were obtained. This was collected by filtration and the crystals washed with a little ice cold water (about 5 ml) and finally with acetone for quick drying. The filtrate on concentration to 50 ml and cooling in the fridge gave a further crop of berberine sulphate (total yield 26 g). The properties of berberine sulphate thus obtained corresponded with those recorded in literature.

ANNEXURE I

PREPARATION OF TETRAHYDROBERBERINE

Materials required : Berberine chloride = 100 g
$NaBH_4$ = 13 g
MeOH = 1000 ml

Berberine chloride (100 g) and aq. methanol (1000 ml, 90%) were taken in a flask fitted with a 2-necked adaptor, one neck being attached to a reflux condenser. The flask was heated on a water-bath and when all the berberine chloride had dissolved the flask was removed from the water-bath and sodium borohydride (8g) was slowly and carefully added through the other neck while shaking the flask during the addition. After this a solution of sodium borohydride (5 g) dissolved in ice-cold water (20 ml) was slowly added to the mixture and the flask placed back on the water-bath. The solution was refluxed for 15 minutes. The crystals which separated out on cooling the flask were collected by filtration and the crystals washed first with cold aq. MeOH (50%, 20 ml) and then with cold water till the washings were free from alkali (tested with pH paper). The mother liquor on concentration gave a further crop (The total yield of THB was found to be 97% of the theoretical). m.p. 171 - 172° UV : 284 nm (E 28,300).

ANNEXURE J

PREPARATION OF TETRAHYDROBERBERINE HYDROCHLORIDE (THB,HCl)

A mixture of tetrahydroberberine (110 g) and methanol (1100 ml) was refluxed for 20 minutes and to the hot solution conc. hydrochloric acid (37 ml) was added and the mixture refluxed for a further 5 minutes. On cooling white crystals of tetrahydroberberine hydrochloride (96 g) separated out. These were collected by filtration and the crystals washed with ice cold methanol (15 ml). On HPLC analysis (UV detector at 286 nm, mobile phase MeOH) it gave a single peak. The properties of this product corresponded with those recorded in literature. The mother liquor on concentration gave a further crop (17 g) of THB, HCl.

ANNEXURE K

ISOLATION OF PIPERINE FROM *PIPER NIGRUM*

Dried and powdered berries of *Piper nigrum* (100 g) were extracted in a Soxhlet extractor for 12 hours. The extract was concentrated to 20 ml and cooled when crystals of crude piperine separated out. These were collected by filtration. Piperine thus obtained on crystallisation from alcohol gave crystals m.p. 127 - 128^{o} (2.1 g). However, the crystals had a very pale yellow colour which was removed by dissolving the crystals in benzene (12 ml) and passing the solution through a column of neutral alumina (10g) and eluting the column with benzene. Removal of benzene gave a white solid residue which on crystallisation from alcohol afforded piperine (1.9 g) as prisms, m.p. 129 - 130^{o}.

ANNEXURE L

ISOLATION OF OLEANOLIC ACID FROM THE ROOTS OF ACHYRANTHES BIDENTATA

The roots (250 g) of Achyranthes bidentata were crushed and extracted successively with petroleum ether and rectified spirit. The alcoholic extract was concentrated to 250 ml and to this was added conc. hydrochloric acid (40 ml) and the mixture refluxed for 2 hours. Water (200 ml) was added to this and the alcohol removed completely by distillation on a water-bath. The mixture was cooled and the dark coloured crude sapogenin was collected by filtration, washed free from acid and dried. The crude sapogenin thus obtained was extracted in a thimble extractor with acetone. The acetone extract on concentration gave crude oleanolic acid (pale brown). It was dissolved in alcohol by refluxing and the solution treated with a little charcoal and filtered hot. The filtrate on concentration gave oleanolic acid as fine white needles (3.7 g), m.p. 303 - 307°.

ANNEXURE M

ESTIMATION OF PHENOLICS IN *ADENOSMA SPP.*

The dried and powdered plant material (20 g) was successively extracted with benzene and methanol in a Soxhlet extractor for 6 hours. The methanolic extract was concentrated to 10 ml and to this, water (10 ml) and aqueous lead acetate solution were added when a greenish precipitate was obtained. This was collected on a sintered glass funnel previously weighed. The precipitate was washed with distilled water and dried (at 50°in an oven and finally in a vacuum desiccator). The weight of the precipitate was found out from the difference in weights (weight of sintered funnel with the ppt. minus weight of the sintered funnel). Let it be A. The ppt. was carefully transferred into a beaker using distilled water (15 ml) as the carrier. To this, methanol (15 ml) and aq. sulphuric acid (10%, 2 ml) were added (tested for complete precipitation of $PbSO_4$) and mixture triturated on a water-bath for 30 minutes. The precipitate of $PbSO_4$ thus obtained (the phenolics went into aq. MeOH) was collected on a sintered glass funnel and thoroughly washed with distilled water, dried and its weight determined. Let it be B.

$$\text{The amount of lead in B} = \frac{207B}{303}$$

The amount of phenolics

$$x = A - \frac{207\,B}{303}$$

$$\% \text{ phenolics} = \frac{100\,x}{20}$$

An analysis of a sample *Adenosma* gave the percentage of phenolics as 0.7%.

ANNEXURE N

PREPARATION OF DIOSGENIN FROM *DIOSCOREA* TUBERS

Disintegration : Air-dried tubers of *Dioscorea* (500 g) are soaked in water for 48 hours. The tubers are taken out and passed onto a disintegrator/crusher to crush the tubers to a mesh size of 5 - 10.

Hydrolysis : The crushed tuber is then taken in a flask. To this is added 3 litres of 2 N hydrochloric acid. The mass is kept at boiling temperature for 6 hours maintaining the volume of the mass constant by adding water. The hydrolysed tubers are collected by filtration. The hydrolysed tuber is washed with water, sodium carbonate solution and water to free it from acid.

Drying : The acid free hydrolysed tuber as obtained above is dried in the sun (or over steam during the rainy season).

Extraction : The above dried material (about 200 g) is packed in a Soxhlet extractor and extracted with solvent oil b.p. 60-80°, 1000 ml. For complete extraction of diosgenin about 36 percolations are needed. The extract is concentrated to about 100 ml and centrifugedto yield flakes of diosgenin. Yield = 13 g, m.p. 193 - 97°.

The diosgenin obtained as above was purified by crystallisation from toluene - acetone mixture (5 : 1, 70 ml) when pure diosgenin, m.p. 201 - 4° (yield = 11.5 g) was obtained. This gave a single spot on TLC (GLC indicated more than 97% purity). This diosgenin is suitable for using as a starting material for the production of steroid hormones and intermediates. However, diosgenin sample containing pennogenin (as seen by the more polar spot on TLC) should be purified by crystallisation from acetone-methanol mixture.

ANNEXURE O

METHOD FOR THE PRODUCTION OF 16 - DPA

Acetolysis : Diosgenin (100 g) (m.p. 202-204°) is refluxed with acetic anhydride (250 ml) and pyridine (0.5 ml) for one hour using a calcium chloride guard tube. To the reaction mixture is added a mixture of pyridine hydrochloride and acetic anhydride (100 ml) (Note I) and the mixture is again refluxed for 4½ hours under anhydrous conditions. The complete conversion of mono-acetate to pseudo diosgenin diacetate is again checked by TLC using the solvent system C_6H_6-EtOAc (9:1) and spray reagent (ceric ammonium sulphate).

Oxidation : The above product is transferred into one litre beaker and water (60 ml) is slowly added with stirring. It is allowed to cool and when the reaction subsides the beaker is cooled and the temperature is brought down to 10°. The mixture is stirred with a stirrer and chromium trioxide (30 g) dissolved in water (17 ml) and glacial acetic acid (140 ml) is added dropwise during one hour with stirring. During the addition temperature is maintained between 14-19°. The mixture is stirred vigorously after the addition for a further 2 hours at 14-20°. In no case the temperature of reaction mixture is allowed to rise above 20°. Sodium bisulphite (18 g) dissolved in minimum volume of water and 3 ml of alcohol are added. The mixture is stirred for a further 20 minutes. It is then transferred into a one litre flask and refluxed for 1½ hours. The top of the condenser is attached to a rubber tube for the exit of SO_2 gas. The mixture is cooled to room temperature and poured into water (4 L) taken in a bucket slowly with stirring. The yellowish white granular product which separates out on standing overnight is filtered, washed throughly with water and dried at room temperature. The crude 16-DPA is crystallised from methanol to yield 16-DPA (41 g) (m.p. 171 - 173°). The mother liquor is concentrated on a water-bath to remove the solvents completely and then crystallized from methanol to get a second crop (5 g). The mother liquor from the second crop on chromatography on alumina (20 g) gave another 4 g of 16-DPA the elution of the column having been carried out with n - hexane.

Note 1 :

Preparation of the pyridine hydrochloride catalyst for 100 g diosgenin : Conc. hydrochloric acid (12 ml) was taken in a 100 ml R.B. flask fitted with a baby condenser and to this was added pyridine (12 ml) slowly through top of the condenser. After this the condenser was removed and the flask was placed on a water-bath (dipping the flask inside the boiling water) and the liquids (pyridine, HCl, H_2O) in the flask were removed under reduced pressure using a water-pump. The final traces of liquids were removed using a vacuum pump. The solid pyridine hydrochloride thus obtained was completely transferred into the reaction flask using 100 ml of acetic anhydride (for acetolysis mentioned above).

ANNEXURE P

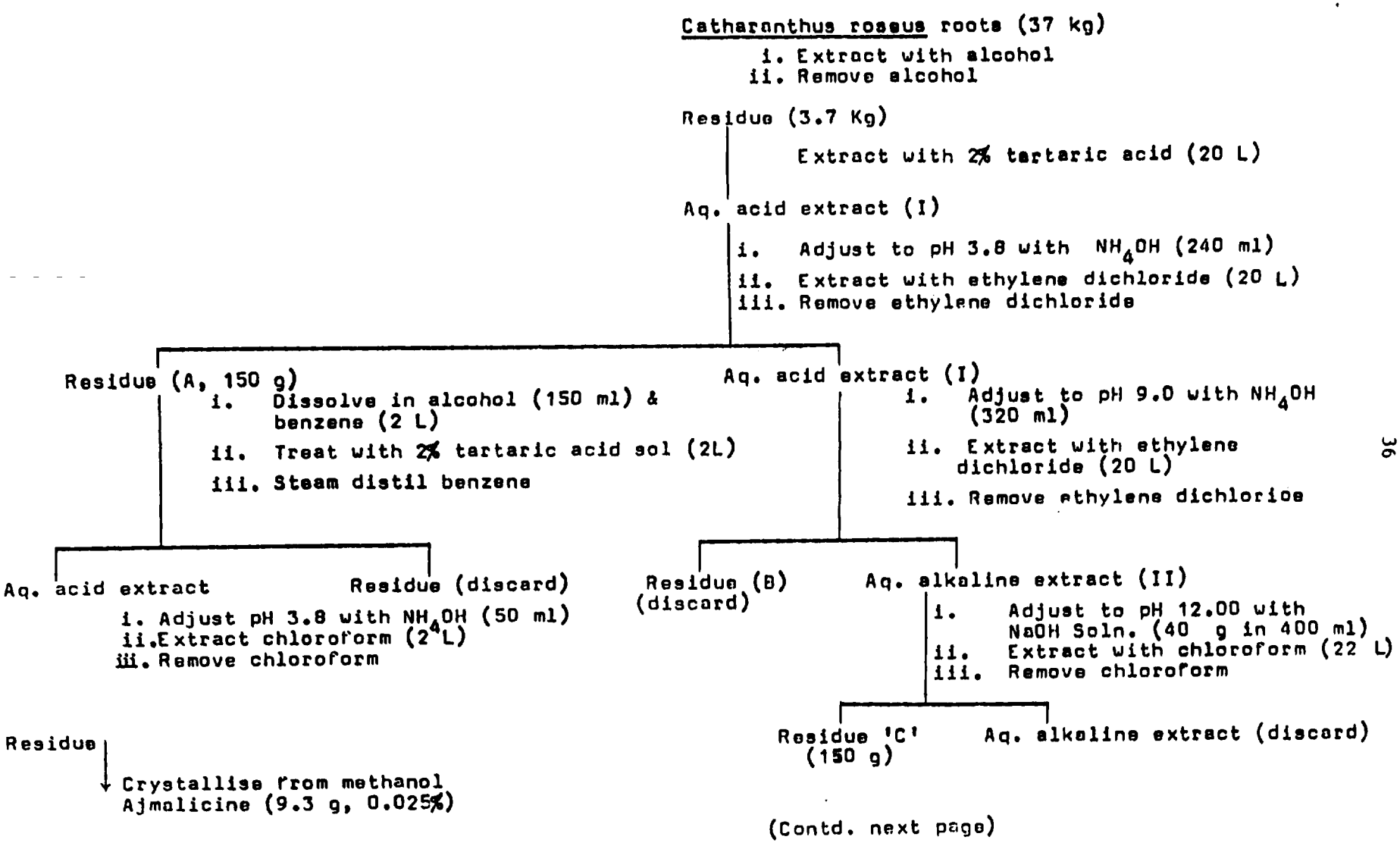
16-DPA

Specifications :

Description	:	Fine white crystalline powder odourless
25% solution in Methylene Chloride	:	Almost clear
Loss on drying in vacuum at 100°C for 3 hrs.	:	Not more than 0.5%
$E^{1\%}$ in Ethanol at 238 mu 1 cm (max)	:	257 - 263
Specific rotation at 20°C of 1% in Chloroform	:	- 39.5° to - 41.5°
Melting Point	:	171° - 178°
I.R. (1% solution in CS_2)	:	Resembles reference standard
Thin Layer Chromatography (T.L.C.)	:	No non-polar apot (non-polar with respect to major spot) should be present. Total impurities should be not more than 2%. Should compare with reference standard.

ANNEXURE Q

FLOW SHEET SHOWING EXTRACTION OF AJMALICINE AND CONVERSION OF SERPENTINE TO AJMALICINE



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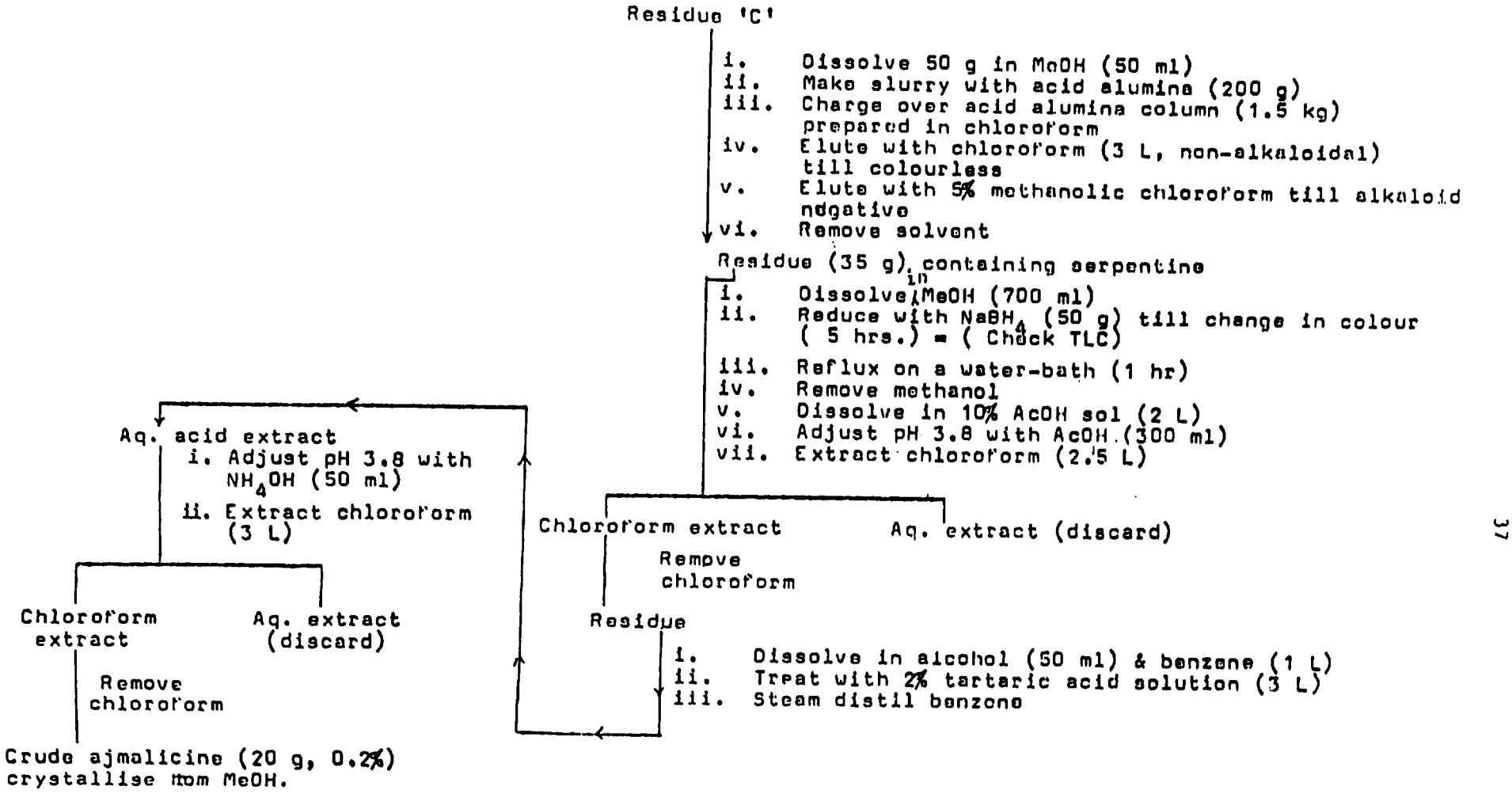
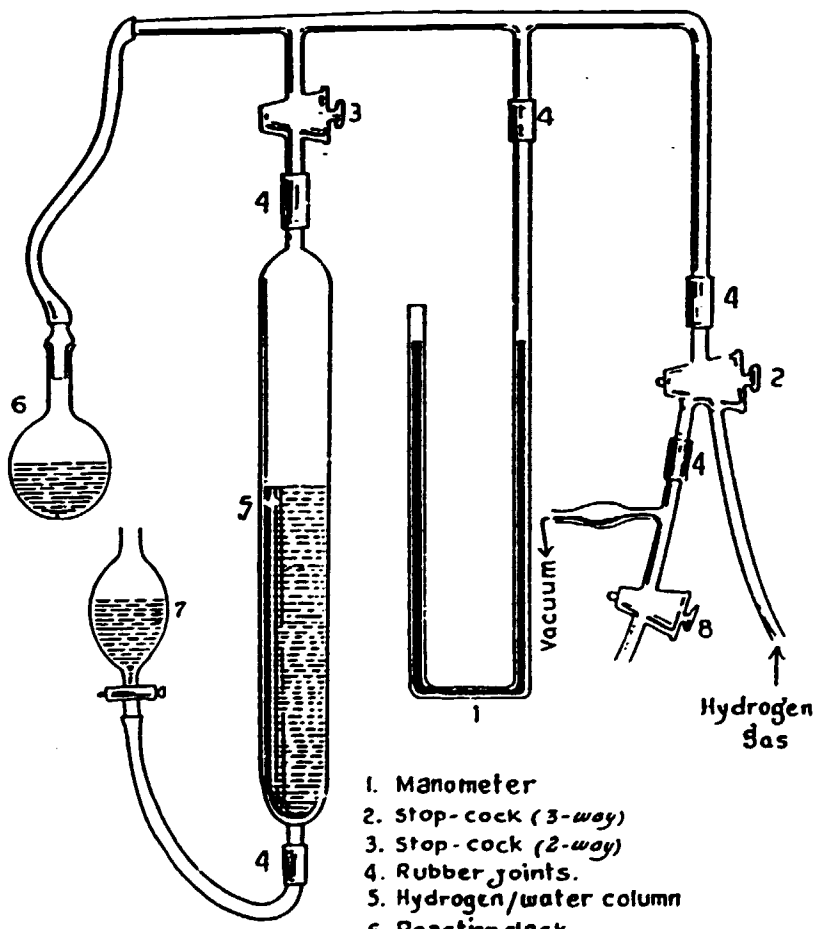

Residue 'C'
i. Dissolve 50 g in MeOH (50 ml)
ii. Make slurry with acid alumina (200 g)
iii. Charge over acid alumina column (1.5 kg) prepared in chloroform
iv. Elute with chloroform (3 L, non-alkaloidal) till colourless
v. Elute with 5% methanolic chloroform till alkaloid negative
vi. Remove solvent
Residue (35 g) containing serpentine in
i. Dissolve in MeOH (700 ml)
ii. Reduce with NaBH4 (50 g) till change in colour (5 hrs.) = (Check TLC)
iii. Reflux on a water-bath (1 hr)
iv. Remove methanol
v. Dissolve in 10% AcOH sol (2 L)
vi. Adjust pH 3.8 with AcOH (300 ml)
vii. Extract chloroform (2.5 L)
Chloroform extract
Aq. extract (discard)
Remove chloroform
Residue
i. Dissolve in alcohol (50 ml) & benzene (1 L)
ii. Treat with 2% tartaric acid solution (3 L)
iii. Steam distil benzene
Aq. acid extract
i. Adjust pH 3.8 with NH4OH (50 ml)
ii. Extract chloroform (3 L)
Chloroform extract
Aq. extract (discard)
Remove chloroform
Crude ajmalicine (20 g, 0.2%) crystallise from MeOH.

FIGURE R

Hydrogenation Apparatus



1. Manometer
2. Stop-cock *(3-way)*
3. Stop-cock *(2-way)*
4. Rubber joints.
5. Hydrogen/water column
6. Reaction flask
7. Separating funnel
8. Stop cock