Review Article

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STANDARDIZATION OF PLANT-BASED MEDICINE

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ABSTRACT: In Pakistan Ministry of Health and the health policy maker are still doubtful in making policy for plant-based medicine. Though WHO recommendations are very clear but there are few points left in standardization of herbal drugs. This brief review article is written with the object to provide base line to ministry for adaptation of procedure and methods.

KEY WORDS: Herbal medicine, Standardization methOds, Microbial tests, Gross Behavioural Changes, Pharmacology.

INTRODUCTION

Nowadays the global interest in herbal remedy is the standardization of it, though medicinal plants are the oldest known source of health care products. This happens due to the availability of thousands of herbal remedies in the market. Some of them are on with synthetic organic compounds or with the name of natural product remedy.' To ensure the purity of the drug material its standardization is necessary.

The standardization of plant-based medicine has two folds apart from compound herbal medicine i.e. plant-based medicine in crude form (powder, extract; liquid, solid and semi-solid), it may be classified as alkaloid, tannin, flavonoid, fixed oil, steroid, volatile oil, carbohydrate, saponin, resin, glycoside, hormone, phenol & phenol-glycoside, vitamin, coumarin, cynogenic glycoside, protein, amino acid containing drugs, and in pure natural chemical compound form such as Ephedrine, Ascorbic acid, Atropine, Colchicine, Morphine, Codeine etc. The crude form herbal medicine requires different types of standardization methods than pure forms. For practical purpose both types are described below.

Standardization Methods of Crude form of PiantBased Medicine

According to WHO recommendations a plant drug must be standardized on the following criteria:

1. Scientific name
2. Family
3. English name
4. Other names
5. Macroscopic (Organoleptic method)
6. Microscopic
7. Reaction of Chemicals
8. Fluorescence method
9. Physical tests (identity, purity, physico-chemical constants, assay)
10. Chemical tests
11. Chromatography
12. Chemical constituents
13. Assay or Standardization by Analytical methods 14. Biological methods
15. Pharmacological methods
16. Toxicological methods

Practical Example of Crude form Plant-Based Medicine: Turmeric is one of the suitable example for the explanation of standardization plant-based medicine, because currently it is used in the cure of different ailments such as arthritis, ulcer, bronchial asthma, conjunctivitis, inflammation, septic etc. and lot of research work has already been carried out on it (Handa *et ai.,* 1998).

1. Scientific *name:Curcuma long a* Linn. (Syn. *Curcuma domestica* Valeton)
2. Family name: Zingiberaceae
3. English name: Turmeric
4. Other name: Haldi, Halada, Curcuma
5. Macroscopic (Organoleptic)
   Observations *Part:* Rhizome
   Shape: Ovate, oblong, round cylindrical or elongate, sometime conical. length variable from 3-8 em, diameter 2-3cm. branches sessile and lateral 7 -11 em in length and 1-1.5 cm in diameter, having nodes. internodes and root scars.
   Coiour: Yellowish to yellowish brown (externally). orange yellow to yellow.
   Odollr: Aromatic
   Taste: Aromatic and bitter
   Fractllre:Short. broken surface is blackish yellow in colour

Surface: Rough and uneven

6. Microscopic (drug in powder form) Observations: For microscopic ex amination powder drug slide can be prepared either in chloral hydrate or glycerin, or in water.
   Epidermis: It consists of single layer thick walled cubical or slightly elongated cells of different sizes, which also bear scattered simple multicellular, uniserriate trichomes.
   Cortex: It starts below epidermis and consists of 6-8 layers thin walled, rounded parenchymatous cells, which contains gelatinised starch grains with yellow colour pigments.
   Vascular Bundles: They are scattered and of collateral types. Xylem vessels are spiral, annular and reticulate.
   Ground Cells: They contain starch grain.
7. *Reaction of Chemicals (Histochemical test):* Use small amount of powder drug on a glass slide and add a drop of chemical reagent and observe under mtCTOScope.
   Picric acid: Whole tissue arrangement system shows yellow colour apart from vascular bundle and few cells that are yellowish brown.
   Nitric acid (Conc.): Cork cells shows yellow colour, ground cells yellowish brown and xylem reddish brown.
   HCl (Cone.): Ground cells become yellowish brown, vascular bundle greenish brown and few scattered cells dark brown.
   Iodine: Vascular bundles turn brown; ground cells bright yellow and cork cells yellowish brown. *Ferric chloride* (Solution): Ground & cork cells become yellowish green and vascular bundle brownish yellow.
   Sulphuric acid (Cone.): Cork cells show greenish brown, ground cells yellowish brown and vascular bundles brown.
   Reaction of chemicals with crude powder drugs in test tube:
   Sulphuric acid (Cone.): Blackish brown
   Nitric acid (Cone.): Orange
   Acetic acid: Yellowish brown
   Iodine (Solution): Light brown
   Acetic acid + Sulphuric acid (Cone.): Dark brown 5%
   Ferric Chloride (Solution): Yellowish brown
   Ammonium hydroxide: Reddish brown
   Million's reagent: Light brown
   10o/c Sodium hydroxide+ a tjnge of Sulphate solution: Reddish brown
   Nitric acid (Cone.) + *Ammonium hydroxide:* Brown
   /0% Sodium hydroxide+ a drop of 5% *Lead Acetate:* Reddish brown
   Acetic cid + 2 *drops of* 5% *Ferric Chloride + Sulphuric acid* (Cone.): Brown
8. *Fluorescence Method (Observation under Ordinary light & Ultraviolet light):*
   Drug mounted in nitrocellulose prepared in amylacetate: Yellowish brown, Yellowish green *Drug mounted in IN Sodium hydroxide in methanol:* Light yellow, Greenish brown
   Drug treated in IN Sodium hydroxide in methanol, dried & mounted in nitrocellulose prepared in amylacetate: Yellowish green Green
   Drug treated with IN HCl: Dull yellow, Dull green
   Drug treated with IN HCl dried and mounted in nitrocellulose prepared in amylacetate: Light yellowish brown, Light green
   Drug treated with] N sodium hydroxide in water: Reddish brown, Dark brown
   Drug treated with] N sodium hydroxide in water, dried & mounted in nitrocellulose prepared in amylacetate: Yellowish brown, Light green
   Drug treated with 50% Nitric acid (Cone.): Orange, Dark brown
   Drug treated with 50% Sulphuric acid (Cone.): Brown Blaek
   Drug powder: Yellow Green
9. Physical test (identity, purity & assay)
   Organic matter: Not more than 2.0%
   Total ash: Not more than 9.0%
   Acid insoluble ash: Not more than 1.0% *Alcohol-soluble extract:* Not more than 8.0%
   Water-soluble extract: Not more than 12.0% *Purity:* 1 00%
   Volatile oil: Not more than 7.0% *vlw*
   pH values in:

1 % solution	6.4
10% solution	6.8

10. Chemical tests:(see test procedure below)
    Carbohydrate: Positive
    Alkaloid: Positive Sterol: Positive
    Amino acid: Positive
    Coumarin: Positive
11. Chromatography:
    Thin/ayer chromatograpl1y:Ready made Silica gel 60 plates

Solvent system:
Chloroform-Ethanol-Acetic acid
Ratio: 94:. 5 ; 1
Standard solution: 1 mg curcumin in I ml methanol
Sample: Extract I g of powdered drug with 5 ml methanol, warm slightly, filter through cotton plug and then use filtrate.
Preparation of Plate: Apply 5111 sample and same amount standard separately on a silica gel TLC plate.
Development of Plate: Use solvent mentioned above to develop the plate. When reaches to maximum, take out any dry it.
Scanning: Scan densitometrically at 366 nm wavelength and record the finger print profile.
Spots under 366 *nm:* Standard's spot display bright yellow and sample also bright yellow.
Rf value: Standard = 0.79
Sample = 0.79, 0.60 and 0.43

12. Chemical constituents: Curcuminoids (yellow colouring pigments) including curcumin, cyc1ocurcumin,5' -methoxycurcumin, demeth oxycurcumin, bis-desmethoxycurcumin, dihydrocurcumin, volatile oil i.e. d-a-phellandrene, d-sabinene, cineol, borneol, zingiberene, curione, ar-turmerone, D-sesquiphellandrene, bisacurone, curcumenone, dehydrocurdione, Q-curcumene; high contents of bis-abolene, procurcumadiol, bis-acumol, turmeronol A & B, phytosteroL stigmasterol, P-sitosterol, cholesterol, 2-hydroxymethyl anthraquinone, fatty acids. polysaccharides i.e. ukonan A, B, C & D, and inorganic constituents Ca, K, Na, Mg [1-3].
13. Assay by analytical method: By HPLC *Method* 1:
Mobile phase: Ethanol
Flow rate: 1.2 mlImin.
Column 5111
Nucleosil NH2 (Chromapack)
Detector: UV; 254 nm
Chromatogram: 3 peaks;
1) bisdesmethoxycurcumin,
2) demethoxycurcumin
3) Curcumin

Method 2:
Mobile phase: Acetonitrile-Water (55
Flow rate: :45) 1 mlImin.
Column: Hamilton PRP-l
Detector: UV/Vis.; 425 nm
Chromatogram: 3 peak;
1) bisdesmethoxycurcumin, 2) demethoxycurcumin
3) Curcumin

14. Biological method:(see test procedure below)
Antibacterial test: Positive
15. Pharmacological methods: (see test procedure below)
Anti-inflammatory test: Positive in model experiments on animals.
Phagocytic test: Positive in mice.
16. Toxicological methods: (see test procedure below)
Toxicity: Cytotoxicity positive in cell culture.

General Methods of Testing of Plant-Based Medicine:
Identification of chemical constituents by Colour Reaction: These are-classical general tests usually used for evaluation of herbal medicine/natural products/ allopathic medicine.

1. Test for Triterpenes:
Liebermann-Burchard test: Use small quantity of MeOH extract in a test tube add to it 5 ml of CHCb and 2 ml of acetic anhydride, then add two or three drops of concentrated H2SO4 carefully by gently mixing the solution. If colour change to pink indicating the presence of triterpene.
2. Test for Alkaloids:
Dragendorff test: Mix small quantity of MeOH extract in H2O, acidified it with HCI and add few drops of Dragendorff reagent. If orange precipitates appear, indicating the presence of alkaloid constituents.
3. Tests for Tannins:
(a) Lead acetate test: Dissolve powder of plants in distilled water and boil it. After boiling filter the solution and add lead acetate in the filtrate. If precipitates appear, indicating the presence of tannin.
(b) Phenazone test: To 5 ml aqueous extract of plants add 0.5 g of sodium acid phosphate first warm, then cool it and filter later. To filtrate add 2% solution of phenazone. If precipitates appear, indicating the presence of tannins.
4. Tests for Saponins:
(a) Froth test: Take small quantity of powder drug or dried extract in 1 ml water and shakes vigorously. If froth forms, indicating the presence of saponins.
(b) Add ether (cold) in small quantity of methanolic extract. After the addition of ether if precipitates are formed, indicating the presenc~ of saponins.

5. Test for Car-bohydrates:
   Molisch's test: Treat small quantity of methanolic extract with a-naphthol and concentrated sulphuric acid. If purple colour appears, indicating the presence of carbohydrates.
6. Test for Sterols:
   Salwaski test: Add few drops of H2SO4 (concentrated) directly to small quantity of MeOH extract, if a purple colour ring is formed at the upper surface of the solution, indicating the presence of sterols.

Standard Biological Methods of Testing of PlantBased Medicine:

Antibacterial Activity:

Test Organisms: l}sually antibacterial activity of pant-based medicine is standardized on *Staphylococcus aureus, Streptococcus pyogenes,*. S. *agalactiae, Corynebacterium diptheriae, Bacillus substiUs, Escherichia* coU, *salmonella typhi, Shigella boydU, Proteus* mirabilis, *pseudomonas aeruginosa* and *Klebsiella pneumonae.* The culture of organisms is maintained on stock culture agar and from the stock culture; a lqop full of the culture is inoculated to nutrient broth. This broth is incubated at 37°:tl °C for twenty-four hours. Inocula are prepared by diluting twenty-four hours old culture in saline. A dilution of 1: 100 is used usually in all the tests.

Antibacterial Assay: Prepare modified soy agar petri plates as described by Naqvi *et al.* (1987). Pour 0.1 ml of diluted culture on each plate and allow the plates to dry up for thirty minutes at 37°C. Cut few well of 6 mm diameter with the help of sterile cork borer in the inoculated agar. Fill the wells with plant extract, for control use 50% ethanol and amoxicillin as standard in a well. Incubate these plates for 24 hours at 37°C. At the end of incubation period, measures the zone of inhibition.

MEDIA:

Media used *for assay:* Tryptic Soy Agar (Difco) (Soybean-Casein Digest Agar Medium USP) *Formula per Liter:*

Bacto tryptone (Pancreatic digest of casein USP)	15 g
Bacto Soyatone (Papaic digest of soybean meal)	5g
Sodium chloride'	5g
Bacto agar	15 g
pH	7.3.z. 0.2 at 25°C

Dissolve 40 g of medium in 1000 ml of cold distilled water, and then heat the medium to dissolve completely. Later sterilize it in autoclave for 15 minutes at 15 Ibs/in2 pressure and *121°C.*

Media used *for culture maintenance:* Stock culture (Difco)
(Brain heart infusion dehydrated)
Formula per Liter:

Calf brains infusion	200g
Beef heart infusion	250 g
Bacto proteose peptone	109
Bacto dextrose	2g
Sodium chloride	5g
Disodium phosphate	.2.5 g 7.4:t0.2 at
pH	25°C

Direction: Dissolve 50 g of medium in 1000 ml of cold distilled water, and then heat to boiling to dissolve medium and sterilize it in autoclave for 15 minutes at 15 Ibs/in2 pressure and *121°C.*

Antifungal Activity:

Test organisms: Usually antifungal activity is carried out on *Aspergillus niger, Trichophyton mentagrophyta, Allescheria boydU, Microsporum canis, Pleurotus ostricatus, Drechslera rostrata, Nigrospora oryzae, Stachybotrys atra, Convularia lunata* and *Candida albicans.* The culture of organisms is maintained on Sabouraud Dextrose Agar (SDA) and usually from SDA a loop full of culture is inoculated to Sabouraud Dextrose Broth (SDB). This SDB is incubated at 37:t 1 °C for 24 hours. Inocula are prepared by diluting 24 hours old cultures in saline. A dilution of 1: 100 is used in the test, which is prepared in distilled water.

Antifungal Assay: In prepared petri plates of SDA pour 0.1 ml of diluted culture, and then dry the plates for 30 minutes at 37°C. Wells of 6 mm diameter be made with sterile cork borer in inoculated agar. Fill these wells with plant extract, 50% ethanol as control in next well and standard drug (antibiotic) in another well. Keep these plates for 24 hours at 37°C. At the end of incubation period measures the zone of inhibition (Shaikh *et al.,* 1995).

Sabouraud Dextrose Agar (SDA):
Formula glUter:

Mycological peptone	109
Glucose	40 g

Agar	15 g
pH	5.6::t 0.2 at 25°C

Directions: Add 15 g of SDA to 1 liter of distilled water to dissolve SDA completely, then heat the mixture 10 minutes to dissolve completely and autoclave it 15 minutes at 15 Ibs/in2 pressure and 121°C.

Sabouraud Liquid Medium (SLM):
Formulation g/liter:

Pancreatic digest of casein Peptic	5g
digest of fresh meat Glucose	5g
pH	20g
	5.7::t0.2 at 25°C

Directions: Dissolve 30 g of SLM in 1 liter of distilled water and heat the mixture at least 10 minutes to dissolve completely. Later sterilize the SLA medium for 15 minute in autoclave at 15 Ibslin2 pressure and 121°C.

Standard Pharmacological Methods of Testing of Plant-Based Medicine:
Normally a drug/medicine is always testified pharmacologically on the following parameters. Otherwise that drug/medicine remain doubtful for its efficacy and toxicity. These parameters are basic requirement of standardization or evaluation of drugs, therefore, UNESCO published a book on techniques used for evaluation of natural medicine [6,7].

Behavioural Observations: Treat the animal with different doses of different plant extract, fractions & pure compounds. Observe for a total period of 2 hours and record the behaviour according to a modified version of the procedure described by Irwin for the following behavioral activities:

Grooming: It means the movements exhibited by the animal. Use two groups of 3 animals each, for observing changes, in grooming response after administration of different extract, fractions and pure compounds. Treated group 1 with different extract, fraction and pure compounds and group 2. receives vehicle only. Treated group 1 with n-hexane, ethyl-acetate, chloroform, butanol, aqueous fractions and alcoholic extract of plant in various dose such as 96 mg/Oo4mL 24 mg/Oo4 ml 8 mg/Oo4 ml, 10 and 15 mg. 004 ml and 340 and 510 mg/Oo4 ml respectively for observing the grooming activity. ($n = 3$).

Similarly the pure compound such as lupeol, mixture of a-and f3-amyrin, f3-sitosterol and kaempferol-3, 7 -O-a-L-di-rhamnoside in a dose of 5 mg/0.5 ml, 5 mg/0.5 ml, 3 mg/0.5 ml and 5 mg/0.5 ml *i.p.* respectively administer to the animals of group 1 for observing grooniing response. Observe each group between 20-21 minutes.

Vocalization: Means various sounds exhibited by the animal. Use two groups of 3 animaVgroup for each fraction and extract. Group 1 is treat with different extract while group 2 receives vehicle only.

Use n-hexane, ethyl-acetate, chloroform, butanol, aqueous fractions and alcoholic extract of plants *i.p.* in various doses such as 96 mg/OA ml , 24 mg/OA ml, 8 mg/OA ml, 10 and 15 mg/OA ml and 340 and 570 mg/OA ml respectively, for determining the vocalization between 20 minutes to 2 hours.

Similarly use the pure compounds for example lupeol, mixture of a -and 13- amyrin, f3-sitosterol and kaempferol-3,7 o-a-L-dirhamnoside in the dose of 5 mg/0.5 ml, 5 mg/0.5 ml, 3 mg/0.5 ml and 5 mg/0.5 ml respectively for observing vocalization between 20120 minutes.

Irritability: Means feeling of restlessness; Use different fractions, extract and pure compounds in the same way as describe above for observing effects, on irritability.

Passivity: Means animal is not active; it is calm and quite. For observing passivity use different fractions, extract and pure compounds in the same way and dose regime as described above. Observe each group between 20-120 minutes.

Catatonia: It is a type of schizophrenia characterized by immobility. Adapt the same procedure and dose regime for fractions, extract and pure compounds as prescribed above for observing catatonia. Observe each group between 20-120 minutes.

Spontaneous Activity: For observing increase and decrease in spontaneous activity; use same procedure and dose regime as describe above. Observe each animal for 20 minutes.

Touch Response: Apply same procedure as described above for other activities. The dose regime for

different fractions, extract and pure compounds remain the same as describe above. Observe each animal between 20-120 minutes for confirming the increase and decrease in touch response.

Straub' Tail: Means erection *of* tail. Similarly, for obserying this activity, treat one group *of* animal with vehicle only while other group with fractions, extract and pure compounds. Observe each group for 20-120 minutes to confirm this activity.

Tremor: This reaction belongs to shaking *of* the body *of* animal. For observing tremor inject n-hexane, ethylacetate, choloroform, butanol, aqueous fraction, alcoholic extract and pure chemical compounds *i.p.* into the animals and keep under observation at least for 120 minutes.

Twitches: This activity belongs to muscular contraction. Animals receive different plant fractions, extract and pure compounds *of* the same dose regime as used for other activities. The observing time is 20-120 minutes.

Convulsion: It is temporary loss *of* consciousness with severe muscle contraction. Treat animals *of* groups 1 with different fractions, plant extract and pure compounds in the different doses. Observe each group for 20 minutes and count the number *of* convulsions animal showing.

Writhings: For observing this activity adapts the same procedure and same dose regime *of* different plants fractions, extract and pure compounds as describe above. Observe each group for 20 minutes.

Staggering Gait: Means walking abruptly; observe the animals after treatment with different plant fractions, extract and pure compounds for 20-120 minutes for staggering gait.

Righting Reflex: It is the ability to move in opposite direction during sleep. Treat the animals in the way as describe above for observing loss and gain *of* righting reflex. Observation time is between 20-120 minutes.

Body Tone: Means tightness *of* the muscles. Observe animals after introducing different plant fractions, extract and pure compounds in the dose regime as given previously for loss and gain in body tone. Observe each animal for 20-1 20 minutes.

Grip Strength: For the study *of* this activity treat again the animals with different plant fractions, extract and pure compounds according to the doses given above. Introduce a metal rod in the cages *of* the animals and observe whether they hold the rod or not. Observe each animal for 20 minutes.

Salivation: Means excess secretion *of* saliva. After introducing the fractions, extract and pure compounds in the same dose regime as given above. Observe the animals for salivation between 20-120 minutes.

Alivation: Use the same dose regime for fractions, extract and pure compounds as describe above for observing this activity. The time for observation is between 20-120 minutes.

Lacrimation: It is the secretion *of* tears from the eyes. After injecting dose *of* different fractions, extract and pure compounds as describe above. Observe the animals visually whether lacrimation is there *or* not.

Tail Erection: Means the tail becoming upright and rigid. For observing this treats the animals with plant fractions', extract and pure compounds in the dose schedule as given above. Observe each animal between 20-120 minutes, for tail erection ability.

Aggressiveness: Means restlessness and mental illness. In this study observe the animal after injecting the plant fractions, extract and pure compounds *i.p.* between 20-120 min utes, fortheir aggressiveness.

Gross Effects: This technique has been described by Dhawan & Srimal (1984) in UNESCO publication as: "After *i.p.* administration *of* the compounds to groups *of* 5 mince each *of* the animals are observed for gross behavioural effects. The animals are observed continuously for 3 hours after administration *of* the compound, then every 30 minutes for next three hours and finally after 24 hours. The proforma used for recording the observation given below in table!. CNS stimulation is judged by increase spontaneous motor activity (SMA), piloerection, exphthalmos, clonic and tonic convulsions; CNS depression is judged by reduced SMA, sedation, ptosis, crouching, catalepsy and autonomic effects like piloerection, urination, defaecation, salivation, lachrymation etc. at *12* the LDso these effects are recorded using groups *of* 5 mice and effect on the body temperature is also recorded with a telethermometer using YSI type 402 physiological probe".

Effect on the spontaneous motor activity: This method is taken from USESCO publication (Dhawan & Srimal, 1984). Where it is described as:
"To study the effect of the compounds on the spontaneous motor activity (SMA) in groups of 5 mice each, photocell activity cage is used. Control score of each group (2 minutes) is recorded prior to drug treatment. A saline treated control is run concurrently graded doses of compounds are given and SMA score recorded at 30 min., 60 min. and then at hourly intervals until the effect is over. Compared to the control values any increase or decrease in the scores indicates CNS stimulation or depression respectively. Graded doses producing responses ranging between 0-100% are obtained and EDso calculated using the method of pro bit analysis (Finney, 1952)".

Effect on forced locomotor activity:
The method of Kinnard and Kiarr (19957) is employed here as (Dhawan & Srimal, 1984). "Mice are trained so that they walk on a rotating rod (Rota rod) for a period of 120 seconds. Graded doses of the compound to be tested are administered *i.p.* in groups of 5 mice each and number of animals falling in 120 seconds in a group is noted. Saline treated controls are in concurrently. The EDso calculated using the method of pro bit analysis (Finney, 1952)".

Effect on Amphetamic induced hyperactivity: Dhawan and Srimal (1984) describe this technique as:
"Control readings for SMA are recorded in groups of 5 mice each using photocell activity cage. These groups of animals are then pretreated with graded doses of the compound, 1 hour prior to the administration of d-amphetamine (5 mg/kg *i.p.)* so as to obtain responses ranging between 0-100%. In saline treated controls amphetamine produces a significant increase in the SMA. The EDso is calculated using the method of probit analysis (Finney, 1952)".

Effect on hexobarbital sleeping time: Dhawan and Srmal (1984) describe this technique in UNESCO publication as:
"Hyponosedatives and neuroleptic agents are reported to increase the barbiturate sleeping time in mice. Graded doses of the compounds under study are administered *i.p.* to groups of 5 mice each and Ihour later hexobarbitone (75 mg/kg *i.p.)* is administered. Saline treated controls are run concurrently and are also administered hexobarbitone. The sleeping time for each animal is the period for which the righting reflex is ab?ent.' The prolongation of sleeping time with respect to the control is then calculated. The significance of drug induced prolongation of sleeping time is judged using students '1' test".

Effect on condition avoidance response:
The method of Cook and Weidley (1957) is used for this activity (Dhawan & Srimal, 1984). According to this technique, "Rats are trained in the pole climbing apparatus to avoid electric shock by climbing on a wooden pole on the sound of a buzzer, when conditioned response CAR is said to have developed. On further training rats climb up the pole without even waiting for the buzzer and this is called secondary conditione<f response (SR). When animals climb up the pole after receiving an electric shock, it is called an unconditioned response (UR). Graded doses of the compound under study are administered by *i.p.* or oral route so as to obtain 0-100% blockade of these responses. Further, if the three types of responses are blocked at different dose levels of the compounds, the EDso value for the blockade of SCR, CAR and DR are calculated using the method of probit analysis (Finey 1952)".

Amphetmine toxicity test in aggregated: This technique is useful in evaluation of toxic effects of drug/medicine. It is described in UNESCO publication (Dhawan & Srimal, 1984) as: .

"Groups of 10 mice each are aggregated in identical rounded cages at 30°C and injected with amphetamine (30 mg/kg i.p.) one hour after pretreatment with graded doses of the compound under study and a reference standard compound e.g. chlorpromazine. Mortality in each group is noted after 24 hours. In the saline treated controls amphetamine produces 100% mortality within 24 hours".

Antireserpine test: To determine anti-depressive activity of medicine this technique is useful. In UNESCO publication Dhawan & Srimal (1984) describe it as:

"Groups of 5 mice each are administered graded doses of the compound under study following reserpine, in an attempt to look for reversal of reserpine induced syndrome e.g. reduced psychomotor behaviour. ptosis, crouching, hypothermia and sedation. These experimental animal models provide a possible ex peli men tal analogue of depression. Known anti

depressants e.g. imipreamine can reverse this syndrome. Other psychotropic drugs e.g. CPZ, meprobamate and phenobarbitone are ineffective. Amphetamine causes reversal, but it is transient and associated with central stimulatory *effects".*

Swimming performance test: This technique is useful in evaluation *of* adaptagenic activities *of* drug/medicine. It is described in UNESCO publication (Dhawan & Srimal, 1984) as under:

"This is used to pick up possible adaptagenic compounds which improve the swimming performance in rats e.g. Ginseng plant extracts having no CNS depressant activity and saponins are put to this test. Groups *of* 5 rats each are tested for this activity, 1 hour after pretreatment with the plant extract. Increase in the swimming performance *of* rats in terms *of* increased in the swimming activity and higher scores are taken as indicative *of* adaptogenic activity".

Anticonvulasant activity:
The method *of* Swinyard *et at.* (1952) is described by Dhawan & Srimal (1984) as: "Groups *of* 5 mice each are administered the graded doses *of* the compound under study. After one hour a current stimulus *of* 48 mA for 0.2 second is delivered through ear electrodes. In saline treated controls it produces tonic extension *of* the hind limbs. Abolition *of* this response by a compound is taken as the criterion *of* its anticonvulsant activity".

Pentylene tetrazole seizure threshold test:
In this method Swinyard *et at.* 1952 (Dhawan & Srimal, 1984) described it as: "Groups *of* 5 mice are pretreated with graded doses *of* the compound 1 hour prior to subcutaneous injection *of* pentylentetrazole (80 mg/kg). which in saline treated control group produces clonic seizures in 100% mice. Abolition *of* clonic convulsions is taken as the criterion *of* anticonvulsant activity. Convulsion *of* less then 5 seconds duration is disregarded".

Anorexigenie activity: This technique is useful in evaluation *of* toxic *effects of* drug/medicine. It described in UNESCO publication (Dhawan & Srimal,
1984) a'S:
"Groups *of* 5 mice each, individually caged, pretreated with graded doses *of* the test compound are offered milk (Sweetened & reconstituted as 25% aqueous suspension for powdered milk). Each mouse is exposed *to* 0.5 ml *of* this milk for 15 minutes. Control mice drink this quantity within 15 minutes. Any quantity *of* milk left after 15 minutes in the treated groups is taken as the measure *of* anorexigenic activity" .

Spasmogenic and Spasmolytic Activity Test (Smooth Muscles Activity):
Preparation: Use rabbits *of* either sex weighing approximately 1.0 to 1.5 kg in the experiments. Make the rabbit unconscious by a blow on the back neck. Then open abdomen immediately and pulls the caecum forward to display the length *of* small intestine. Cut the intestine from animal and place it in a petri-dish or beaker containing oxygenated Tyrode's solution.

Isolated Intestine Segments Preparation: Dissect about 3-4 cm long segments *of* small intestine (jejunum or ileum) immediately from isolated intestine, and keep them alive by placing in a petridish or beaker containing oxygenated Tyrode's solution. For experiment purpose mount a piece *of* isolated smooth muscles in an organ bath *of* 70 ml capacity, filled with Tyrode's solution. Maintain 37°C temperature *of* organ bath by circulating the water throughout experiment. Bubble the perfusion solution with a mixture *of95%* oxygen and 5% carbon dioxide.

Assay Method: Equilibrate the intestine segment before starting the experiments. Record the spontaneous movements *of* intestine on oscillograph or polygraph having isotonic transducer.

For the determination *of* effects *of* plant extract on spontaneous movements *of* intestine, dissolve 0.1 g *of* crude extract in 2 or 3 ml *of* distilled water or prepare different concentration *of* crude extract for dosing. Thereafter, add dose *of* desire concentration to organ bath, after equilibration period.
Record the spasmogenic and spasmolytic effects *of* dose on the contraction and relaxation pattern *of* isolated rabbit intestine (Aqel *et at.,* 1991).

Agglutination Activity:
Dilution Preparation: Prepare different dilutions *of* plant extract by dissolving 0.1 gram crude extract in 20 ml distilled water and use dilutions 1 :2. 1 :4, 1:8 and 1: 16 for experiments. Fordilution preparation use the classical method.

Preparation of Buffer: Prepare Phosphate buffer (pH 7) by mixing 70 ml solution *of* Na2HP04 (52 gll or 5.2

g1200 ml) in 130 ml solution of anhydrous NaH2P04 (36 g/l or 3.6 g/ml).

Preparation of RBCs: Prepare RBCs (Erythrocytes) of human blood (group A+, B+, AB+, 0+, A-, B-, AB- and 0-) by simple centrifugation of the blood samples. Prepare 2% RBCs suspension in phosphate buffer (pH 7) i.e. 1 ml RBCs + 49 ml buffer.

Agglutination: For agglutination activity add1 ml of each sample to 1 ml of 2% suspension of RBCs in a small test tube, and for good results keep them in a water bath at 25°C. Run blank samples simultaneously as control. If smooth button like formation (due to the sedimentation of erythrocytes) appears at the bottom of the test tube indicating the negative activity of the sample, whereas a rough granular deposition (due to agglutination of erythrocytes) shows. a positive action. Sedimentation or granulation may appear as very weak or trace, weak, moderate or strong during experiments (Fabregas *et aZ.*, 1984).

Toxicity Tests:
Brine shrimp Bioassay Method: Prepare samples and discs of 3 different concentrations i.e. 10, 100 and 1000 Ilg/ml according to the literature. Hatch Brine shrimp *(Artemia salina)* nauplii in a specific tank prior to experiment. Transfer ten shrimps to each sample vial and then add seawater to make the volume 5 ml. Later on add dry yeast suspension as food to each vial including control. Keep the vials for 24 hours, thereafter, count the active nauplii and calculate death percentage in each dose. Analyse the data with Finney computer program in order to determine LD50 values (Meyer *et aZ.*, 1982).

Standardization Methods in Pure NaturaZ Compound There are some classical examples of natural medicine (Pant-Based medicine), which are equally useful in herbal and allopathic medicine standardization. These methods are from International Pharmacopoeia of WHO (1967).

Ascorbic acid:
Identification tests:
A. Dissolve 0.1 g in 2ml of water, add few drops of nitric acid and a few drops of silver nitrate (40 g/l), dark grey precipitates are produced.
B. Dissolve 0.04 g in 4ml of water, add 0.1 g of sodium hydrogen carbonate and about 20 mg of ferrous sulfate, shake and allow to stand a deep violet color is produced, which disappears on the addition of 5ml of sulfuric acid.

Assay method: Dissolve about 0.20 g, accurately weighed, in a mixture of 25 .ml of carbon-dioxide-free water and 25ml of sulfuric acid. Titrate the solution at once with iodine (0.1 mol/I), starch is use as indicator, added towards the end of the titration, until a persistent blue color is obtained. Each ml of iodine is equivalent to 8.806 mg of CJIS06.

Folic acid:
Identification test: The absorption spectrum of a 151lg/ml solution in sodium hydroxide (0.1 mol/I), when observed between 230 nm and 380 nm, exhibits 3 maxims at about 256 hm, 283 nm, and 365 nm. The absorbance at these wavelengths is about 0.82, 0.80 and 0.28, respectively (preferably use 2cm cells for the measurements and calculate the absorbance of 1cm layers). The ratio of the absorbance of a 1cm layer at 256 nm to that at 365 nm is between 2.80 and 3.00.

Assay method:
Standard's preparation: Dissolve 0.05 g of Folic acid in a mixture of water and ammonia (50ml and 2ml respectively) shake gently to form a complete solution, dilute it up to 100ml. add.few drops of toluene as preservative, and store in a cold place, protected from light. On the day of assay, dilute a measured portion of this stock solution with a 3% solution of potassium hydrogen phosphate to give a concentration of *10llg* of Folic acid per ml, calculated on the anhydrous bases, to obtain the standard preparation.

Procedure: Prepare 10Ilg/ml Folic acid solution. Prepare two or more sets of four suitable containers, preferably glass stopper, 50 ml centrifuge tubes, marked respectively, 1, 2, 3, 4. Add the designated volumes of the various ingredients. or multiple thereof by pipette with mixing. To all tubes of each set add 5ml of folic acid test solution. To tubes 2 and 4 add 2 ml of standard preparation. To tubes 1 and 2 add 1ml of a 0.4 % solution of potassium permanganate and to tube 3 and 4 add 1 ml of water. Allow them to stand for 2-3 min. from this point on, treat all tubes alike. Add
1ml of a 2 % solution of sodium nitrite and 1ml of 5N HCI. mix and allow it to stand 2 min. then add 1 mlof the 5% solution of ammonium sulphamate and mix with swirling, until practically all of the nitrogen dioxide has been dispelled. Add lml of a 0.1 ml of the solution of N-(l-naphthyl)-ethylenediamine hydro

chloride. mix it and allow it to stand for 10 min. then add 4 g of sodium chloride and 10 ml of *iso-butanol,* shake vigorously for 2 min. and centrifuge. Determine the absorbance of the clear supernatant layer from each tube at 550nm with a spectrophotometer, using iso-butanol as the blank. Calculate the quantity, in micro gram of folic acid in each rnl of the folic acid solution by the formula:

, 0.4 C (A1- A3) /(A2 + A3 - Al - ~)

C is the conc. in microgramlrnl of folic acid in the standard preparation

AI. A2, A3, ~ are absorbance of the solutions in the respective tubes indicated by the subscripts.

Nicotinic acid:

Identification tests:

1. Triturate a small quantity with twice its weight of dinitrochlorobenzene, gently heat about 0.01 g of the mixture in the test tube until it melts, continue the heating for a few second longer, cool and add 3rnl of ethanolic potassium hydroxide, a deep red to deep wine red color is produced.
2. Dissolve about 0.05 gm in 10 rnl of O.IN sodium hydroxide, add 1.5 rnl of dilute acetic acid and 3rnl of copper sulphate solution, a blue precipitate gradually forms.
3. Heat a small quantity with twice its weight of sodium carbonate, pyridine, recognize by its odour, is produced.

Assay method: Dissolve 0.3 g accurately weighed in 50ml of freshly boiled and cooled water and titrate with *0.1 N* sodium hydroxide using phenolphthalein as indicator. Each rnl of *0.1 N* sodium hydroxide is equivalent to 0.01231 g of nicotinic acid.

Salicylic acid:

Identification test: Dissolve 0.14 g in Iml of sodium hydroxide and add 5 ml of water than add lrnl of ferric ammonium sulfate, mix thoroughly and allow it to stand for a minute a deep violet colour is produced.

Assay method: Dissolve 0.3 g of sample of salicylic acid in 15 ml neutralized ethanol, add 20ml of water and titrate with *0.1 N* sodium hydroxide, using phenol red as a indicator. Each rnl of *0.1 N* sodium hydroxide is equivalent to 0.01381 g of salicylic acid.

Aminophylline: It is a stable combination of Theophyline and Ethylenediamine..

Identification tests:

1. Dissolve 1 gm in 10 ml of water and neutralize with dilute HCI, a white precipitate is formed, filter it and wash with water, dry at 105°C. From this precipitate take 10 mg in a porcelain dish, add lrnl HCl and 0.5 rnl hydrogen peroxide, evaporates to dryness on a water-bath. Add one drop of ammonia the residue acquires a purple colour, which is destroyed by the addition of a few drops of sodium hydroxide.
2. Another method is dissolve 0.05 g in 1 rnl of water and adds 2 drops of copper (IT) sulfate a deep violet colour is produced.
3. Warm 0.05g with 2rnl of sodium hydroxide (80g/1) and 2 drops of chloroform, an isocyanide, perceptible by its characteristic odour is produced.

Assay for Theophylline: Place about 0.25g accurately weighed in a 250rnl conical flask, add 50rnl of water and 8 ml of ammonia (1 00g/1) and gently warm the mixture on a water bath until complete solution is affected. Add 20rnl of silver nitrate *O.lmoVl),* mix, heat to boiling and boil for 20min. cool to between 5°C and 10°C for 20 min. filter through a filtering crucible under reduced pressure and wash the precipitate 3 times with 10 ml portions of water. Acidify the combined filtrate and wash with nitric acid (1000 g/l), add an excess of 3 rnl of the acid. Cool, add 2rnl of ferric ammonium sulphate (45 g/l), and titrate the excess of silver nitrate with ammonium thiocyanate (O.lmoVl). Each rnl of silver nitrate (0.1 mol/l) is equivalent to 18.02 mg of aminophylline.

For Ethylenediamine: Dissolve 0.5 g, accurately weighed, in 30 ml of water and titrate with HCI, using bromocresol green/ethanol as indicator. Repeat the operation with out the substance being examined and make any necessary corrections. Each ml of hydrochloric acid is equivalent to 3.005 mg of ethylenediamine.

Ampicillinum:

Identification test: To a test tube add 2mg of sample, 2mg of chr~motropic acid sodium salt and 2 ml of sulfuric acid. Place the test tube in a suitable bath at 150°C and starts a stopwatch at the moment of immersion. The test tube is shaken every half min. and the colour of the contents is noted, after 2 min. the ampicillin containing solution turns purple.

Suspend 0.01 g in 1 ml of water and add 2 ml of a mixture of potassium cupric tartrate and 6 ml of water, a magenta-violet colour is immediately produced.

Table
Proforma for recording the CNS activity *

Code: 0 = Not done
- = No effect; + = mild effect; ++ = strong effect
↓ = mild depression, ↑ mild stimulation , ↓↓ strong depression
↑↑ = strong stimulation

OBSERVATIONS	EFFECTS							
	Up to 3 hr.	3 ½ h.	4 h	4 ½ h	5 h	5 ½ h	6 h	24 h
Gross activity								
1. SMA								
2. Respiration								
3. Writhing								
4. Ataxia								
5. Tremor								
6. Convulsions (Clonic/tonic)								
Posture & tone								
7. Body								
8. Limbs								
9. Tail (Straub)								
10. Catalepsy								
11. Inclined plane test								
Eyes								
12. Pupils								
13. Ptosis								
14. Reactivity to sound/touch								
Reflexes								
15. Pinnal								
16. Corneal								
17. Righting								
18. Piloerection								
19. Cyanosis								
20. Salivation								
21. Lachrymtion								
22. Diarrhoea								
23. Analgesia								
24. Body temperature								
25. Mortality								
26. Any other effect								
27. Any other Significant effect								

LD_{50} mg/kg CNG effects (Tick one) – No effect – Stimulant – Depressant
Date Signature

Assay method-1:
For total Penicillin: Dissolve 0.5 g sample in 100 ml of carbon-dioxide-free water and adjust the pH electrometrically to 9.60 at 25°C with *0.1N* sodium hydroxide remove the electrode assembly and wash with carbon-dioxide-free water. To the combined solution and washing add 50 ml of *0.1N* sodium hydroxide. heat on a water bath for thirty min. with precaution against the absorption of carbon dioxide. cool rapidly to 25°C. Replace the electrode assembly and titrate to pH 9.60 with *0.1N* HCl, maintaining the temperature at 25°C through the titration. Repeat the

operation with out the ampicillin, the difference between the titration represents the amount *of* sodium hydroxide required. Each ml *of 0.1 N* sodium hydroxide is equivalent to 0.03494 g *of* total penicillin calculated as ampicillin.

Assay method-2:
Dissolve 0.12 g, accurately weighed, in sufficient water to produce 500ml. Transfer 10 ml *of* this solution to a 100ml volumetric flask, add 10 ml *of* buffer borate, pH 9, 1 ml *of* acetic anhydride/dioxin, allow to stand for 5 min. at room temperature and dilute to volume with water.

Transfer two 2 ml aliquots *of* this solution in to separate tubes. To one tube add 10 ml *of* imidazole/mercuric chloride, mix, stopper the tube, and place in a water-bath at 60°C for exactly 25 min. cool the tube rapidly to 20°C (solution A).

To the second tube add 10 ml *of* water and mix (solution B).

Immediately measure the absorbance *of* a lcm layer at the maximum at about 325 nm, against a solvent A and water for solution B.

From the difference between the absorbance *of* solution A and that *of* solution B, calculate the amount *of* ampicillin in the substance being tested by comparison with ampicillin, similarly and concurrently examined. In an adequate calibrated spectrophotometer the absorbance *of* the reference solution should be 0.29 .:t 0.02.

Acacia:
Identification:

1. To 5 ml *of* a 2 percent solution add 1 ml *of* lead sub-acetate test solution, a flocculent, white precipitate is produced.
2. Dissolve 0.25 g, in powder, in 5 ml *of* water by shaking, adds 0.5 ml *of* hydrogen peroxide solution (10 vol.) and 0.5 ml *of* guaiacum tincture. Shake and allow it to stand for a few minutes; a deep blue or bluish-green colour develops.

Amphetamine Sulphate Tablets:
Identification: Triturate a quantity *of* the finely powdered tablets, equivalent *to* about O.IOg *of* amphetamine sulphate with ten ml *of* water for thirty minutes, and filter, take 5 ml *of* filtrate in 5ml *of* water, add 5ml *of* sodium hydroxide (test solution), cool to about 15°, add Iml *of* mixture *of* benzyl chloride and 2 volumes *of* ether, insert the stopper and shake well for 3 min, filter the precipitate, wash with 10 ml *of* cold water, recrystallize from ethanol 50%, the crystals so obtained are dried at 105°, having a melting point *of* 133°.

Assay method: Weigh and powder 25 tablets. Boil an accurately weighed quaintly *of* the powder, equivalent to about O.Olg *of* Amphetamine sulfate, with 2 ml *of* dilute hydrochloric acid. Cool, transfer in to a separator, add 3 ml *of* sodium hydroxide test solution and extract with 4 successive quantities, each *of* 10 ml, *of* chloroform R. Filter the chloroform extract through a pledged *of* cotton covered with a layer *of* anhydrous sodium sulfate R into 5 ml *of* glacial acetic acid R. Remove the chloroform by evaporation on a water-bath, cool, add 5 ml *of* dioxan R, 2-3 drops *of* acetous crystal violet test solution and titrate with *0.01 N* acetous perchloric acid to the shade *of* colour which represent the true end point or determine the end point potentiometrically, repeat the, operation without sample, the difference between the titrations represent the amount *of* 0,0 IN acetous perchloric acid required. potentiometric titration may be carried out by using a glass electrode, the junction should have a reasonably low electrical resistance and there should be a minimum *of* transfer *of* liquid from one side *to* the other. Care should be taken *to* prevent leakage *of* potassium chloride from the calomel electrode into the titration liquid, for example, by using a sintered glass plate. Each ml *of* O.OIN acetous perchloric acid is equivalent *to* 0.001842 g *of* (C9 H13 Nh, H2SO4. Calculate the average weight *of* amphetamine sulphate in the tablets;

Theobromine sodium:
Identification tests:

A. Dissolve about 1 g in 20 ml *of* warm water and neutralize with dilute acetic acid, a white crystalline precipitate *of* theobromine is formed.
B. Acidify a solution in water with dilute sulfuric acid and filter, the filtrate yields the reactions characteristic *of* acetates.
C. The residue left after ignition yields the reactions characteristic *of* sodium.

Assay method:
For additi.onal sodium: Dissolve about 0.5 g. accurately weighed, in 75 ml *of* hot water. add 20 ml *of 0.1 N* sulfuric acid, or a sufficient quantity *to* give

an acid reaction to phenol red, boil to remove carbon dioxide, cool and titrate the excess of *0.1 N* sulfuric acid with 0.1 N sodium hydroxide, using phenol red as an indicator. Each ml of *0.1 N* sulfuric acid is equivalent to 0.002299 g of additional Na.

For theobromine: To the solution from the previous assay add about 20 ml of *0.1 N* silver nitrate and titrate the liberated nitric acid with *O.lN* sodium hydroxide, using phenol red as an indicator. Each ml of *0.1 N* sodium hydroxide is equivalent to 0.01801 g of theobromine.

Cocaine:

1dentification tests:

A. To I ml of a 1 % solution add lor 2 drops of a 3% solution of chromium trioxide in water, a yellow precipitate is produced which dissolves on shaking the solution. On the addition of further drops of the solution of chromium trioxide or of hydrochloride acid, a permanent precipitate is produced.

B. Heat about 0.1 g in powder with 1 ml of sulfuric acid for 5 min. at 100°C and mix it with 2 ml of water carefully, the aromat~c odour of methyl benzoate is perceptible, when solution is cooled and allow to stand some for some hours, crystals of benzoic acid separate.

C. Dissolve 0.05 g in 1.5 ml of water add 8.5 ml of alum and 5 ml of potassium permanganate, stirs briskly for several .seconds, characteristic rectangular violet plates are formed.

D. Dissolve 0.1 g in 2 ml of water, add 4 drops of dilute ammonia, extract the alkaloid with 10 ml of ether and remove the ether by evaporation, melting-temperature of the crystalline residue, after drying in a desiccator, about *9TC.*

Assay method: Take about 0.5 g sample, carry out non-aqueous titration as describe previously. Each ml of *O.lN* acetous perchloric acid is equivalent to 0.03398 g of cocaine hydrochloride.

Codeine:

Identification test:

A. Dissolve 0.005 g in 1 ml of sulfuric acid, add 1 drop of ferric chloride and warm on a water bath, a bluish-violet colour is produced which is changed to red on the addition of 1 drop of dilute nitric acid.

B. Yields the characteristic reactions of phosphates.

Assay method: Dissolve about 0.15 g of sample in 5 ml of ethanol, add 20 ml of water and 5 drops of methyl red and titrate with *O.lN* HCI. Each ml of *O.lN* HCI is equivalent to 0.02994 g of Codeine.

Caffeine:

1dentification tests:

A. To about 0.01 g contained in a porcelain dish, add 1 ml of HCI and 10 drops of hydrogen peroxide, evaporate to dryness on a water bath, when the dish is inverted over a vessel containing a few drops of dilute ammonia, the residue acquires a purple color, which is destroyed by solution of fixed alkalis.

B. A cold saturated solution in water gives, with tannic acid a white precipitate, which is soluble in excess of the reagent.

C. A cold saturated solution in water gives no precipitate with iodine, but on the addition of dilute HCI, a brown precipitate is formed which re:dissolves on the addition of a slight excess of sodium hydroxide.

Assay method: Carry, out the non-aqueous titration method as describe previously, using about 0.2 g sample dissolved in 10 ml of acetic anhydride and 20 ml of benzene. Each ml of *0.1 N* acetous perchloric acid is equivalent to 0.01942 of caffeine.

Colchicine:

1dentification tests:

A. Dissolve about 0.05 g in 1 ml of ethanol, add 1 drop of ferric chloride, a garnet-red colour is immediately produced.

B. Mix about 0.001 g with 2 drops of sulfuric acid, a lemon-yellow colour is produced, on the addition of a drop of nitric acid, the colour changes to greenish-blue, rapidly becoming reddish and finally yellow or almost colourless, upon then adding an excess of sodium hydroxide, the colour is changed to red.

C. Boil 0.1 g with 0.5 ml of dilute HCI and 10 ml of water under reflux for one hour. Add 40 ml of boiling water and filter. Cool the filtrate and extract with 20 ml of chloroform. Filter the chloroform extract and evaporate to dryness on a water-bath. Dissolve the residue in 0.5 ml of dioxin and precipitate by adding 10 ml of ether, melting-temperature of the colchicines thus obtained, about 176°C.

D. Dissolve about 0.01 g in 2 ml of dilute HCI a deep-yellow colour is produced. Heat to boiling, a dark olive-green color is produced. Cool and shake with about 2 ml of chloroform, the latter assumes a red colour.

Assay method for Colchicine tablets: Weigh the powder of 20 tablets. Stir an accurately weighed quantity of the powder, equivalent to about 0.001 g of colchicines, with 1 ml of *0.1 N* HCI in a 300. ml porcelain dish. Add purified kieselguhr in small portion and mix until the. mass appears uniform and almost dry. Place a small pledged of glass wool at the bottom of a suitable chromatographic tube (with diameter about 10 mm and length about 600mm) and transfer the mixture quantitatively to the tube. Pack the powder by carefully tapping the tube on a table-top. Elute the colchicines with 50 ml of chloroform. Evaporate the chloroform extract on a water-bath, dissolve the residue in ethanol and sufficient ethanol to produce 100 ml. Determine the absorbance of 1 em layer of the resulting solution at 351 nm and calculate the amount of colchicines, by using colchicines as a reference substance. Calculate the average weight of colchicines in the tablet.

Morphine HCl
Identification tests:

A. Dissolve 0.05 gin 5 ml of water and add 1 drop of ferric chloride, a blue colour is produced. This colour is destroyed by acids, ethanol or by heating.
B. Dissolve 0.02 g in a mixture of 1 volume of dilute sulfuric acid with19 volumes of water and add 0.5 ml of a saturated aqueQus solution of potassium iodate, an amber color is produced. Shake with chloroform, the latter a~,sumes a violet colour.
e. Mix 0.20 g with 0.10 g of anhydrous sodium acetate and 1 ml of acetic anhydride and boil under a reflux condenser for 5 min. cool and add 15 ml of 30% solution of sodium carbonate carefully. Scratch to induce crystallization, wash with water and re-crystallizes from 2ml of ethanol and 3 ml of water, dry at 105°e. The diacetylmorphine thus obtained has a melting temperature of a bo utI 7 3 ◦ e.

Assay method: Carry out the method for non-aqueous titration. using about 0.4 g accurately weighed. Each ml of *0.1 N* acetous perchloric acid is equivalent to 0.03218 g of morphine HCI.

Pyridoxine Hydrochloride;
Identification: Place 1 ml of 0.01 % solution in water in each of two test tubes. To each test tube add 2 ml of a 20% solution of sodium acetate. To the first tube add 1 ml of water and to the second tube add 1 ml of a 4% solution of boric acid and mix. Cool both tubes to about 20°C, and rapidly add to each tube 1 ml of a 0.5% solution of 2,6-dichloroquinone chlorimide in ethanol, in the first tube a blue colour is produced, rapidly fading and becoming red in a few minutes, but in the second tube no colour is produced.

Assay method: Carry out the non-aqueous titration method as above in the assay method of amphetamine, using about 0.3 g of sample. Each ml of *0.1 N* acetous perchloric acid is equivalent to 0.02056 g of pyridoxine hydrochloride.

Ephedrine Hydrochloride:
Identification tests:

A. Dissolve 0.01 gin 1 ml of water and add 0.1 ml of copper sulfate, followed by 2 ml of sodium hydroxide, the liquid becomes violet in colour. Add 1 ml of ether and shake, the ethereal layer is purple and the aqueous layer is blue.
B. Dissolve 0.2 g in 5 ml of water, add 2 ml of sodium hydroxide, shake with 4 successive quantities, each of 15 ml of ether, wash the mixed ethereal solutions with 5 ml of water and allow the ether to evaporate just to dryness on a warm water-bath. Dissolve the residue in 30 ml of chloroform, cover the dish and set aside for twelve hours, crystals of ephedrine hydrochloride separate from the liquid, which after drying yield the reactions characteristic of chlorides.
e.To 1 ml of a 5% solution made alkaline with sodium hydroxide, add a few drops of potassium permanganate, and heat, benzaldehyde and methylamine vapours are evolved, whieh are alkaline to litmus paper.

Assay method: Carry out the method for non-aqueous titration as described in the amphetamine. Using about 0.4 g accurately weighed. Each ml of *0.1 N* acetous perchloric acid is equivalent to 0.02017 g of Ephedrine HC\.

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