Microcrystal tests for the detection of alkaloids of *Datura fastuosa* and glycosides of *Nerium odorum* and *Calatropis gigantea*

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ABSTRACT: An attempt was done to detect the presence of different toxic principles (alkaloids and glycosides) from the toxic plant specimens i.e. *Datura fastuosa, Nerium odorum* and *Calotropis gigantea* using sensitive microcrystal tests. This study involved the extraction of toxic principles from the respective plant materials using Stass Otto method and further analysis and detection of the same using different reagents. A total of 75 plant samples were collected. Microcrystal tests were performed on the slides. Crystallization products were examined microscopically. Rod shaped, needle shaped, globular shaped and square shaped crystals were observed with different reagents for the toxic principles of *D. fastuosa*, transparent rectangular crystals were obtained for *N. odorum* and needle shaped, octahedral shaped and leaf shaped crystals were exhibited for toxic principles of *C. gigantean*. The results showed a significant difference between the average positive results of crystal formation of *D. fastuosa* (13.33%), *N. odorum* (24.75%) and *C. gigantea* (15.23%). The probability of detection between *D. fastuosa* and *N. odorum was* $p < 0.01$ while *D. fastuosa and C. gigantea* was $p > 0.01$.The results of this study give a scope for further research in the similar line to explore the potential detection methods like microcrystal test which is superior to the conventional colour tests and economical to the other screening tests employed in forensic science laboratories

Keywords: Microcrystal tests, *Calatropis gigantea, Datura fastuosa, Nerium odorum*

Introduction

Microcrystal tests are presumptive tests in which a substance is identified by the formation of characteristic crystals when a certain reagent is added. Usually, such tests are conducted using a microscope (microcrystal test). An example is the acetone-chlor-haemin test for blood.

In the year 1891 Schuette studied on the principal alkaloids contained in *Atropa Belladonna*, as atropine, hyoscyamine, hyoscine, belladonnine and atropamine. These substances bear a close chemical relationship to one another, being partly isomeric and capable of being transformed into one another, e.g., hyoscyamine into atropine [1]. Atropine and hyoscyamine, both of the formula, $C_{17}H_{23}NO_3$, are capable of being resolved into the alkaloid tropine ($C_8H_{15}NO$) and tropic acid ($C_9H_{10}O_3$) by treatment with baryta-water, while hyoscine, under these conditions, is split into pseudotropine ($C_8H_{15}NO$), melting at 106° C, and tropic acid. If in these processes concentrated hydrochloric acid is employed, tropic acid ($C_9H_8O_2$) and its polymer, isatropic acid ($C_{18}H_{16}O_4$) are formed, besides tropic acid. These reactions were first established by Kraut M. in 1863 [2], Lossen in 1866[3], and subsequently studied in detail by Ladenburg and other investigators [4]. Tropine was found to be a pyridine derivative, viz.: oxy-ethyl-ethyltetrahydropyridine (C_5H_7N [C_2H_4OH]CH_3).

It is a strong, tertiary base, forming hygroscopic crystals, which melt at 62° C and boil at 229° C. They are easily soluble in alcohol and water; also soluble in ether. Heated with fuming hydrochloric acid to 180° C or acted upon by sulphuric acid and glacial acetic acid, the base tropidine, containing 1 molecule less of water, is formed, having the composition of $C_5H_6(C_2H_4)NCH_3$, that boils at 162° C. In 1880, Ladenburg recognized tropic acid to be A-phenyl-B-oxypropionic acid ($CH_2OH.CHC_6H_5.COOH$). It is soluble in alcohol and ether, to some extent in water, from which solvent it crystallizes in needles or plates, melting at from 117° to 118 °C [4]. Ladenburg, in 1889, succeeded in obtaining atropine by synthesis from its products of decomposition by evaporating a mixture of tropic acid and atropine with hydrochloric acid [5].

In the year 2003, Swiatko, J studied on cocaine. The presence of cocaine in illicit drug samples is still being determined in some laboratories using spot tests and microcrystal tests. Seventeen chemical species were tested using three different spot tests (Wagner, Marquis, and cobalt thiocyanate followed by stannous chloride reactions) and two microcrystal tests (gold chloride and platinic chloride) to

determine whether the results could be differentiated from the results of these tests on cocaine. The data obtained indicated that 9 of the 17 compounds gave results similar to those from cocaine using the three spot tests, but that the results from microcrystal testing allowed for differentiation of all nine compounds from cocaine)[6]. The most definite and certain identifications that can be readily made increases of this kind are with microcrystal tests .In the year 1961 Charles C. Fulton did the study on identification of microcrystal test for certain drugs. [7].

In the toxicological work, a chromatographic band on paper is eluted with chloroform in the presence of a small volume of solution of borax and sodium sulfite. UV spectrophotometry and both colour and microcrystal tests can then be used on the eluted residue. There is a wealth of crystal tests and a particularly good one can generally be found for nearly any drug, particularly a basic one. Unfortunately the simple methanol eluate of a sprayed TLC spot contains iodide from the spray, which interferes with most of the good colour tests (the best are with concentrated H_2SO_4 reagent), and with many of the crystal tests. Potassium ion from the spray and sodium ion from the plate itself may also interfere, but the iodide interference is the most serious.

However, a considerable number of the best crystal-producing reagents already contain iodide, and these can be used with the simple methanol elute. The best tests for morphine, codeine, methadone, and many other drugs are thus available for use with TLC. Some other crystal producing reagents, not reacting with the iodine, are also available. Quinine spots can be noted by fluorescence for elution without spraying. Volatility tests can be used for methamphetamine and numerous other volatile drugs. Tests are described here for the drugs mentioned in the title: these have been used successfully on eluted residues not merely with spots of "knows" but also with spots from urine sample [8].

The best test for morphine is an old one, the simple aqueous test with K_2HgI_4. It is best obtained on a neutral solution of morphine salt or a solution only slightly acid with dilute acetic acid, and with a reagent saturated with HgI_2 (not properly Mayer's reagent, which has excess KI). It is sensitive to less than 0.05 microgram in a micro liter drop tested (with no more than an equal volume of reagent solution added), and is best with a very dilute solution just strong enough to give slight but immediate amorphous precipitation. Strangely, considering that the test goes back at least to 1885 [9].

Of 41 opium alkaloids and derivatives described by Small and Lutz, [10] eighteen are recorded as giving crystalline precipitates with chloroplatinic acid, nine with chlorauric acid, and fourteen with picric acid. In Stephenson's studies on fifty-four alkaloids, twenty-two crystalline precipitates were obtained with chlorauric acid, nineteen with chloroplatinic acid and thirteen with picric acid [11].

Elie, L., et. al (2011) has described a combined analysis of microcrystalline tests followed by LC–MS or GC–MS analysis. Microcrystalline tests are shown to be non-destructive as addition products formed were easily dissociated after the application of an appropriate solvent. Subsequent analysis of the sample was done to quantify the recovery of the drug. Examples were performed using the date rape drug γ-hydroxybutyrate (GHB) and the synthetic opioid methadone [12].

Microcrystalline test for the detection of 4-methylmethcathinone (mephedrone), benzylpiperazine (BZP) and 5,6-methylenedioxy-2-aminoindane (MDAI) using aqueous solutions of mercury chloride is also described by Elie L. et. al (2011) [12]. Each of the compounds investigated formed specific drug–reagent crystals within minutes. The uniqueness of the test was confirmed by comparison of the microcrystalline response to that of other psychoactive stimulants and a common cutting agent. The limit of detection and cut-off levels for reference standards were established to 3 g/L and 5 g/L for mephedrone, 0.5 g/L for MDAI and 0.2 g/L and 0.3 g/L for BZP, respectively. Various mixtures of standards of either mephedrone, BZP or MDAI combined with caffeine were investigated for their microcrystalline response. Results showed that simultaneous detection of drug and cutting agent was possible with the concentrations tested but were dependant on the ratio of drug to cutting agent. BZP could be detected alongside caffeine from as low as 20% (v/v), MDAI from 40% (v/v) and mephedrone from 50% (v/v) and higher. Finally, seven samples of online purchased 'legal highs' were analyzed using the developed test and the findings were compared to FTIR and GC–MS results. It was shown that 6 out of 7 samples did not contain the advertised active ingredient. Five samples consisted of BZP, caffeine and 1-[3-(trifluoromethyl) phenyl] piperazine (3-TFMPP). The microcrystalline tests carried out on these samples showed positive results for both BZP and caffeine without interference from other substances present [12].

Bell, S. et.al (2007) developed a simple micro fluidic device (MFD) to perform multiple color and crystal tests for controlled substance analysis. The MFD method used less sample and reagents and generated less waste than traditional spot plate methods while

performing several tests simultaneously. This methodology provided significantly more analytical information for a single sample analysis. The device was the size of a microscope slide with four analytical channels: one for microcrystal tests and three for color tests. The optimized devices were subjected to a rigorous validation study using comparative replicate analyses and several operators. Target analytes were methamphetamine, amphetamine, cocaine, and oxycodone and colour test reagents used were the Marquis, Simon, and cobalt thiocyanate. For the crystal tests, platinic chloride was used. The validation study showed the MFD's limits of detection to be in the picogrammes range. Positive tests results were observed in complex mixtures in which the controlled substance was present at concentrations of 5–10% (w/w).

The microcrystal reagents showed greater sensitivity than color test reagents when used in the device. Reagent use and waste generation using the devices was 95% less that that used and generated using the traditional methods. The device performance was also shown to be operator independent [13].

The objective of the present study was to detect the presence of different toxic principles like alkaloids and glycosides from the plant specimen i.e. *D. fastuosa, N. odorum and C. gigantea* using sensitive microcrystal tests. This study was focusing on the extraction of toxin principles from the respective plant materials and further analysis & detection of the same using different reagents. With this study an effort was made to standardize the detection methods of respective toxic principles from the plant specimen using the sensitive microcrystal tests and such generated data of method of detection could be used by the forensic scientists and toxicologists for the detection of the same toxic principles apart from using the conventional colour tests and TLC and other instrumentation.

Experimental

Sample Size

In total 75 samples of plant samples were collected i.e. Samples of seeds from 25 plants of *D. fastuosa* species and samples of roots from 25 plants of *N. odorum* and samples of leaves from 25 plants of *C. gigantea* were collected.

Criteria for the collection of sample

Only seeds of *D. fastuosa* plant, only roots of *N. odarum* and leaves of *C. gigantean* were considered. Samples from each species were collected from different plants.

Reagents used for the microcrystal tests

Frodhe's reagent:
500 g ammonium molybdate in 100 ml of distilled water.

Gerard's reagent:
2 g of mercuric chloride in 100 ml of ethanol.

Picric acid:
5 g of picric acid in 100ml of distilled water.

Lead iodide:
30 g of potassium acetate in 100 ml l of dist. water and add lead iodide until saturation.

Potassium mercuric iodide:
1.5 g of mercuric oxide and 5g of potassium iodide in 100 ml of distilled water.

Mercurous nitrite:
Mercurous nitrite in 50 ml of nitric acid till saturation.

Vanillin reagent:
50 mg of vanillin in 2 or 3 drops of concentrated sulphuric acid.

Equipments and apparatus

Binocular Microscope, beakers, glass rod, funnel, separating funnel, slides, wire guage, burner, micropipette, spatula, Petri dish, cover slip, mortar and pistil.

General Steps

- Extraction and purification of active constituent i.e., poison in matrices of interest.
- Purification of active constituent thus separated.
- Rapid screening and identification.

Stas-Otto Method

50 g of biological material was minced, mixed with plenty of rectified spirit (about 2-3 times the weight of material) in a flask and acidified with tartaric acid. The mixture was heated on the steam bath for 1-2 hours with thorough shaking at frequent intervals. The extraction was then allowed to proceed for about 24 hours with steam off. It was then filtered through a fluted filter paper. The filtrate is evaporated and the residue was again extracted with acidulated alcohol in the same way, filtered and washed several times with hot rectified spirit. The combined filtrates were evaporated in a porcelain basin on the steam bath to a syrupy consistency.

To the syrupy residue about 100 ml of rectified spirit was added very slowly with constant stirring so as to make the insoluble matter granular and not gummy. It was warmed with occasional stirring for about half an hour and filtered. This process was repeated once more and the combined alcoholic extracts were evaporated almost to dryness.

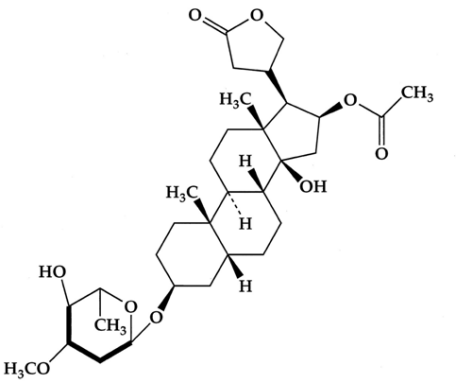
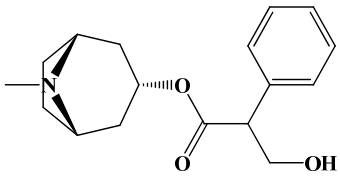
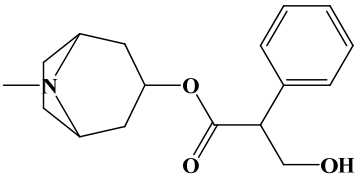
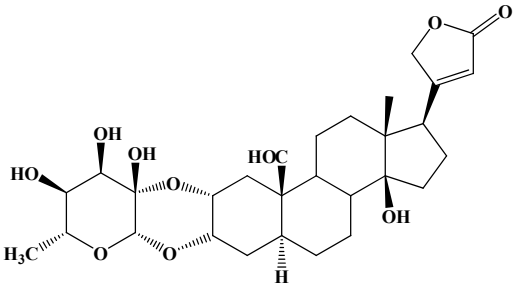
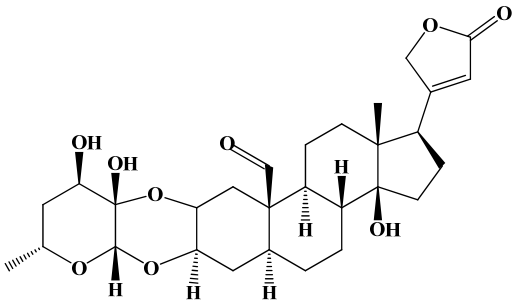
Active principles	Chemical structure
Oleandrin It is a toxic cardiac glycoside found in Oleander (*N. odorum*).	
Hyoscyamine It is pronounced hee-oh-skee-ah-meen, is a chemical compound, a tropane alkaloid. It is the levorotary isomer to atropine. It is a secondary metabolite found in *D. fastuosa*	
Atropine It is a tropane alkaloid extracted from *D. fastuosa*	
Calotoxin It is glycoside extracted from C*alatropis gigantea*	
Calactin It is a naturally occurring cardenolide which can be isolated from extracts of the aerial parts of the plant C*olatropis giganta* .	

Fig. 1: Active principles of *Datura fastuosa, Calpotropis gigantean* and *Nerium odorum*

The residue was dissolved in about 50 ml of water acidulated with dilute sulphuric acid and filtered after about an hour. The poisons were thus dissolved out by the aqueous solution which was transferred to a separating funnel and extracted with a suitable solvent (e.g. ether, chloroform) in portions of about 25 ml. This step was done so that the solvent would take up from the acid solution, alkaloids and glycosides which have escaped initial treatments for purification.

The acid aqueous solution was then rendered alkaline with a solution of ammonia, for liberating the free base from its salt. The alkaline solution is now

extracted with 25ml portions of chloroform in the same way as in the previous stage. This step was performed because the solvent would take up all the alkaloids and glycosides which were partially extracted from the acid solution. The extraction is repeated 2 or 3 times more.

The evaporated chloroform extract is purified by dissolving it in about 20 ml of water acidulated with sulphuric acid and filtering through a small filter. The filtrate is extracted with chloroform, first in acid and then in alkaline medium as in the initial stages of extraction. These extracts are evaporated to dryness for analysis.

Micro-crystal test

Different slides were marked for different alkaloids and glycosides. Two drops of respective alcoholic alkaloidal/glycosidal residue were placed on the slide. Respective reagents were added to the alkaloidal/glycosidal sample. Slides were kept for 15 min for crystallization to occur. The crystallization products were examined under microscope.

Table 1: Percentage of crystal formation in experiments with different reagents for different extracts

Sample No.	Name of reagent	R1	% R1	R2	% R2	R3	% R3	R4	% R4	R5	% R5	R6	% R6	R7	% R7	Total no. of positive result	Average percentage of positive results
1	Extract of Calatropis	10	40	0	0	16	64	0	0	10	40	17	68	0	0	53	30%
2	Extract of Datura	0	0	14	56	12	48	0	0	10	40	12	48	0	0	48	27.14%
3	Extract of Nerium	0	0	0	0	0	0		0	0	0	0	0	19	76	19	10%

Table 2: Difference between percentages of positive results

Sample No	Name of the reagent	Average percentage of positive results	Difference b/w%age	Difference b/w%age	Mean	Standard deviation
1	Extract of Calotropis	30	3.81(1-2)	26.66(1-3)	15.23	16.15
2	Extract of Datura	27.14	3.81(2-1)	22.85(2-3)	13.33	13.46
3	Extract of Nerium	10	26.66(3-1)	22.85(3-2)	24.75	2.64

Table 3: Mean, standard deviation, t-value and p-value of difference between percentage of positive results

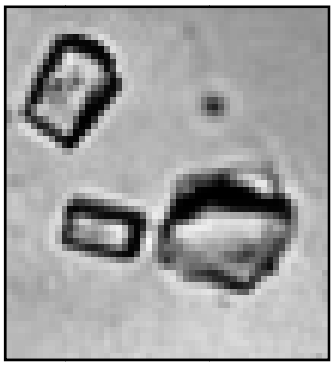
Plant extracts experimented	Mean	Standard deviation	t-value	p-value
1. (a) Extract of *C.gigantea/D.fastuosa* (b) Extract of *D. fastuosa/N. odorum*	15.23	16.15	0.46	>.01
2. (a) Extract of *D. fastuosa/C. gigantea* (b) Extract of *D. fastuosa/N.odorum*	13.33	13.46	4.14	<.01

Results

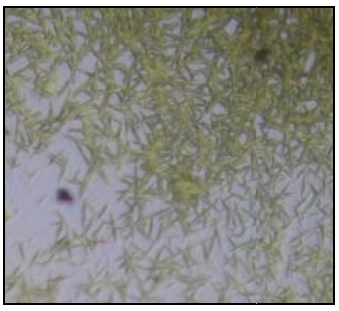
Extract of *D. fasuosa*:

- With Gerard's reagent: Rod shaped crystals were appeared showing positive result in 14 out of 25 trials i.e. 56% .
- With Picric acid : Needle shaped crystals were appeared showing positive result in 12 Out of 25 trials i.e. 48%
- With Lead iodide, it showed no positive result
- With Potassium mercuric iodide, square shaped crystals were appeared showing positive result in 10.out of 25 trials i.e. 40%
- With mercurous nitrite iodide, lobular shaped crystals were appeared showing positive result in 12.out of 25 trials i.e. 48%

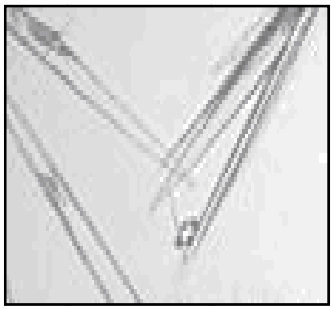
(a) Octahedral shaped crystals (*C. gigantea*)



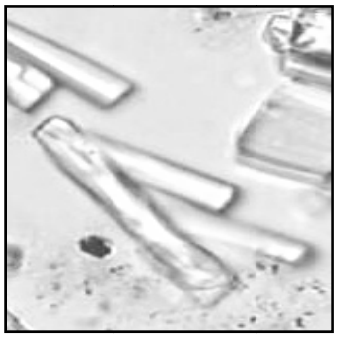
(b) Square shaped crystals (*D. fastuosa*)



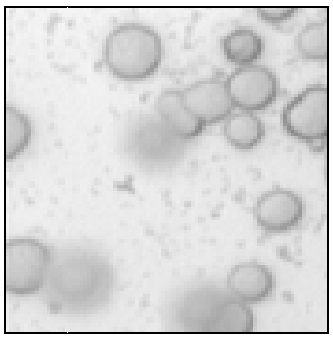
(c) Needle shaped crystals *(C. gigantea)*



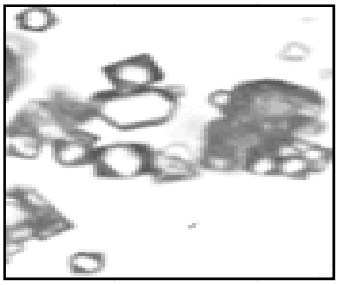
(d) Needle shaped crystal (*D. fastuosa*)



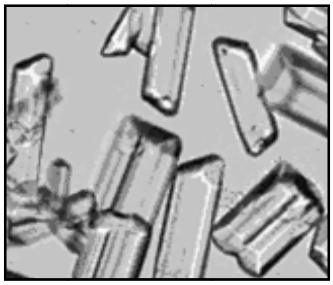
(e) Rod shaped crystals (*C. Gigantea)*



(f) Globular shaped crystals (*D. fastuosa*)



(g) Hexagonal shaped crystals *(C. gigantea)*



(h) Rectangular shaped crystals *(N. odorum)*

Fig 2: Microcrystls formed after reaction of different alkaloids & glycosides with different reagents

Extract of *C.* gigantean:

- With Frodhe's reagent, rod shaped crystals were appeared showing positive result in 10 out of 25 trials i.e. 40%
- With Picric acid, needle shaped crystals were appeared showing positive result. in 16 out of 25 trials i.e. 64% results
- With Lead iodide, it showed no positive result
- With Potassium mercuric iodide, octahedral shaped crystals were appeared showing positive result in 10.out of 25 trials i.e. 40%
- With mercurous nitrite iodide, leaf shaped crystals were appeared showing positive result in 17out of 25 trials i.e. 68%

Extract of *N. odorum*:

- With Picric acid, lead iodide, Potassium mercuric iodide and mercurous nitrite iodide, no positive result.
- With vanillin, transparent rectangular shaped crystals appeared showing positive results in 19 out of 25 i.e. 76%.

The results showed a significant difference between the average positive results of crystal formation of *D. fastuosa* (13.33%), *N. odorum* (24.75%) *C. gigantean* (15.23%) and the probability of detection between *D. fastuosa* and *N. odorum was* $p < 0.01$, *D. fastuosa and C. gigantea* was $p > 0.01$

Conclusion

Although most of the forensic laboratories are employing preliminary screening test like colour test and TLC and also confirmatory test using instrumentation ,there is a need for the rapid and economical screening methods like microcrystal tests for toxic principles of forensic significance i.e. the alkaloids & glycosides of plant species like *D. fastuosa*, *C. gigantean* and *N. Odorum*. Presently, toxic principle identification is based on derivative formation of a special kind of precipitates which are obtained and have reproducible and readily recognizable crystal habits; characteristic of the particular toxin. Experience has shown that the product resulting from the reaction of toxin and reagent is distinctive and readily formed. Usually, in fact, mere inspection under the ordinary microscope is quite satisfactory - when the crystals are truly characteristic - but the polarizing microscope is often useful, and data of optical crystallography can be used, not only on crystalline derivatives, but also on the original salt. The present study has given promising results using microcrystal test for the detection of alkaloids and glycosides of *D. fastuosa*, *C. gigantea,* and *N. Odarum* using different reagents. The present study give a scope for further research in the similar line to explore the potential detection methods like microcrystal test which is superior to conventional colour test use in forensic science laboratory for the detection. This is also helpful for forensic toxicologist and forensic scientist under researchers to adopt such methods.

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