Comparative spectroscopic analysis between Dille-Koppanyi test and Zwikker's test for the detection of barbiturates in illicit cases

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ABSTRACT

Barbiturates are derivative of barbituric acid which acts as central nervous system depressant. The primary detection of barbiturates is carried out by the presumptive tests viz. Dille Koppanyi test and Zwikker's test. A comparative study was done in order to determine whether there are any differences in the results of the two tests and to find out the test with more consistency. Drug samples containing Phenobarbital viz. Gardenal-30 and Gardenal-60 were used in the study. Solution mixtures of 9 different ratios were prepared in the ratios 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2 and 9:1 comprising stock solution of Phenobarbital and solvent (Methanol AR). Both the tests were performed upon the samples at different proportions on spot tile and test tubes and the absorbance values (given by UV/VIS Spectrophotometer) for all the various test results at different concentrations were compared. The study demonstrated that Dille-Koppanyi test showed better results after interpretation of the absorbance values using Independent sample 't' test and Coefficient of variation. A mean value of 2.85 was found for Zwikker's test and a mean value of 3.19 for Dille-Koppanyi test. A significant difference of p<0.01 was obtained between the results from the two tests. Further, a better consistency was found from the absorbance values of Dille-Koppanyi test as compared to the absorbance values of Zwikker's test which is shown by the values of Coefficient of variation that was found to be higher for Zwikker's test (2.00 and 5.86 for Gardenal-30 and Gardenal-60 respectively) and lower for Dille-Koppanyi test (0.78 and 2.26 for Gardenal-30 and Gardenal-60 respectively). In cases of trace amounts of drug sample being recovered from crime scene suspected to be barbiturate, primary detection can be efficiently done directly using Dille-Koppanyi test instead of Zwikker's test which will lead to a better outcome in the investigation.

Keywords: Phenobarbital,Gardenal-30,Gardenal-60,UV/VIS Spectrophotometry,Dille-Koppanyi test, Zwikker's test, Independent sample 't' test and Coefficient of variation

INTRODUCTION

Barbiturates are drugs which are derivatives of barbituric acid and act as a central nervous system depressant. They can act as a wide spectrum from mild sedation to death and reduce the activity of nerve causing muscle relaxation. Barbituric acid is the parent compound of barbiturate drugs, but it itself is not pharmacologically active. It is an organic compound

based on a pyrimidine heterocyclic skeleton. It is an odourless powder which is soluble in water. The IUPAC name is Pyrimidine-2,4,6 (1H,3H,5H)-trione . Numerous synonyms and proprietary names exist for the various barbiturates. User names include barbs, downers, christmas trees, blue heavens, blues, goof balls, blockbusters, pinks, rainbows, reds, red devils, reds and blues, sekkies, sleepers, yellow jackets, etc. Some examples of barbiturate includes phenobarbital, pentobarbital, secobarbital, allobarbital, amobarbital, aprobarbital, etc. Barbiturates come under the NDPS (Narcotic Drugs and Psychotropic Substances) Act, 1985. The Narcotic Drugs and Psychotropic Substances Act, 1985 (commonly referred to as the NDPS Act) is an act of the Parliament of India that prohibits a person to produce/manufacture/cultivate, possess, sell, purchase, transport, store, and/or consume any narcotic drug or psychotropic substance [1.2.3].

Presumptive tests are the tests which are performed as a preliminary step in the identification of a particular substance. They provide an idea whether the substance which is being tested for is possibly either present or absent. These tests help us to decide whether to proceed further and conduct analysis for the confirmation of the substance. After the presumptive testing gives a possibility of the presence of a substance, confirmatory testing is performed. Confirmatory tests are the tests which includes analysis to identify the specific substance under testing. The process of presumptive testing is simple accompanying with rapid detection. The cost required for the testing is comparatively lower than confirmatory tests.

The presumptive tests for barbiturates includes Zwikker reagent test and Dille-Koppanyi reagent test. Zwikker's test is named after the Dutch scientist Cornelis Zwikker. Reagent consists of two parts- 1) 0.5 grams of Copper (II) sulphate in 100 ml of distilled water and 2) 5% Pyridine (v/v) in Chloroform. This test turns phenobarbital, pentobarbital and secobarbital light purple. Dille-Koppanyi test was formulated in 1930s by the American biochemist James Madison Dille and the Hungarian-American pharmacologist Theodore Koppanyi. Reagent consists of two parts- 1) 0.1 gram Cobalt (II) acetate dihydrate in 100 ml Methanol is mixed with 0.2 ml glacial acetic acid and 2) 5% Isopropylamine in Methanol. This test turns phenobarbital, pentobarbital, amobarbital and secobarbital light purple [4].

Phenobarbital is a barbituric acid derivative which acts as a central nervous system depressant. The molecular formula of Phenobarbital is $C_{12}H_{12}N_2O_3$. The molecular weight is 232.239 g/mol. The IUPAC name is 5-ethyl-5-phenyl-1,3-diazinane-2,4,6-trione. Phenobarbital binds to and activates GABA-A receptor and thus mimics the inhibitory functions of GABA. It facilitates the production of Gamma Amino Butyric Acid (GABA) which is an amino acid that helps in reducing neutron excitation [5.6]. It is widely used as sedative and anti-seizure medications. Barbiturates are abused in certain group of people for recreational purpose. It can be misused to cause overdose and lead to death [7.8]. Another important use of barbiturates is in Narco analysis. In the study, pharmaceutical drugs containing phenobarbital are taken as sample. The brand names are namely Gardenal-30, containing 30mg Phenobarbital per tablet and Gardenal-60, containing 60 mg Phenobarbital per tablet.

UV/Vis Spectrophotometry is used in order to determine the absorbance values of various solutions after the tests are conducted in order to compare the results. It is basically absorbance or reflectance spectroscopy and uses light in the visible range and adjacent UV

range. The absorbance or reflectance depends upon the colour of chemicals involved. In this electromagnetic range, the electrons undergo electronic transitions. The basic principle is that, molecules containing bonding and non-bonding electrons absorbs energy in the form of ultraviolet or visible light and thus transfers to higher anti-bonding molecular orbitals. The wavelength will be longer for those molecules for which the electrons get easily excited i.e. lower energy gap between HOMO (Highest Occupied Molecular Orbital) and LUMO (Lowest Unoccupied Molecular Orbital). There are four possible transitions viz π–π^*, n–π^*, σ–σ^*, and n–σ^* and the order of energy of transitions is σ–$\sigma^* >$ n–$\sigma^* > \pi$–$\pi^* >$ n–π^*.

The aim of the research is to compare between Zwikker's test and Dille-Koppanyi test for the presumptive detection of barbiturates and determine the test with better results. The hypothesis made were 1). There is no significant difference in the result given by Zwikker's test and Dille-Koppanyi test in the presumptive detection of barbiturates. 2). Dille-Koppanyi test gives better result than Zwikker's test in the presumptive detection of barbiturates with respect to consistency.

The absorbance values obtained after performing the two tests are compared with the help of statistical analysis through SPSS. Independent sample 't' test and Coefficient of variation are used to find out the results of the research and prove the hypotheses successfully. A difference in the test results was found and Dille Koppanyi test gave better and consistent results.

MATERIALS AND METHODS
Materials used

(i). Sample : Pharmaceutical tablets containing Phenobarbital 30 mg (Gardenal-30) and Phenobarbital 60 mg (Gardenal-60).

(ii). Chemical reagents : Zwikker's reagent – Copper (II) sulphate (98.5%, Nice Chemicals Pvt. Ltd.), Pyridine (99%, Nice Chemicals Pvt. Ltd.) and Chloroform (99%, Nice Chemicals Pvt. Ltd.). Dille-Koppanyi reagent – Cobalt (II) acetate dehydrate (27.5%, Nice Chemicals Pvt. Ltd.), glacial acetic acid (99.5%, S d Fine-Chem Limited), Methanol AR (99.5%, S d Fine-Chem Limited) and Isopropylamine (99%, S d Fine-Chem Limited). Distilled water, Methanol AR (99.5%, S d Fine-Chem Limited), test tubes, spot tile, micropipette (100-1000 μl), micropipette tips, camera and UV/Vis Spectrophotometer.

Design of the study

Zwikker's reagent and Dille-Koppanyi reagent are prepared. The stock solution of Gardenal-60 and Gardenal-30 sample are prepared by following the procedure given below.

Phenobarbital 60 mg (Gardenal-60) stock solution: The amount of Phenobarbital per tablet equals to 60 mg. 8 such tablets contains 480 mg (0.48 g) of Phenobarbital. 8 tablets of Garedenal-60 are physically crushed and mixed with 10 ml of Methanol AR. This gives the stock solution for Gardenal-60 sample which has 4.8% of Phenobarbital.

Phenobarbital 30 mg (Gardenal-30) stock solution: The amount of Phenobarbital per tablet equals to 30 mg. 16 such tablets contains 480 mg (0.48 g) of Phenobarbital. 16 tablets of Garedenal-60 are physically crushed and mixed with 10 ml of Methanol AR. This gives the stock solution for Gardenal-30 sample which has 4.8% of Phenobarbital.

Preparation of solutions of different concentrations of Phenobarbital: Solution mixtures are prepared in 9 different ratios -1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2 and 9:1. The components of the solution are stock solution of Phenobarbital and solvent (Methanol AR) with a total volume of 2 ml. The same protocol is followed for both the samples.

Serial number	Ratios	Volume	Component of the solution
1	1:9	200 µl:1800 µl	Stock solution : Solvent
2	2:8	400 µl:1600 µl	Stock solution : Solvent
3	3:7	600 µl:1400 µl	Stock solution : Solvent
4	4:6	800 µl:1200 µl	Stock solution : Solvent
5	5:5	1000 µl:1000 µl	Stock solution : Solvent
6	6:4	1200 µl:800 µl	Stock solution : Solvent
7	7:3	1400 µl:600 µl	Stock solution : Solvent
8	8:2	1600 µl:400 µl	Stock solution : Solvent
9	9:1	1800 µl:200 µl	Stock solution : Solvent

Table 1 : Table showing the composition of different solutions

Procedure for the presumptive spot tests:

The same protocol is followed for both the samples. The two presumptive tests for the detection of barbiturates are performed on spot tile and test tubes.

In the spot tile, equal volume of 200 µl are taken from the 9 different solutions already prepared into 9 different cavities of a spot tile respectively which are labelled from 1 to 9. Zwikker's test and Dille-Koppanyi test are performed.

For performing the tests in the test tube, solutions of 9 different concentrations are taken respectively in 9 test tubes labelled from 1 to 9. Zwikker's test and Dille-Koppanyi test are performed.

Measuring Absorbance using UV Spectrophotometer :

UV Spectrophotometer is used to measure the absorbance values of all the coloured solutions after performing Zwikker's test and Dille-Koppanyi test for both Gardenal-60 and Gardenal-30 samples.

The parameters for UV Spectrophotometer used are as follows:

Scan: Ordinary mode

Scan speed: 120 nm/min

Lamp: Tungsten and Deuterium

Wavelength: 200 – 400 nm

Peak threshold: 0.01 – 0.50 mg/ml

Use of Statistics to prove the hypothesis:

The maximum absorbance values for each of the coloured solutions i.e. test results are taken and a table is formed. The Independent sample 't' test and Coefficient of variation are used in order to prove the two hypothesis.

RESULTS AND DISCUSSION

A comparative study in between the presumptive tests viz Zwikker's test and Dille-Koppanyi test is carried out. The two tests were compared with the help of spot test on spot tile and test tube. The absorbance values of solutions after performing the tests in test tube were measured using UV Spectrophotometer. Statistical analysis viz Independent sample 't' test and Coefficient of Variation are applied to prove the two hypothesis.

From the spot tests performed for both the samples i.e. Phenobarbital 30 mg (Gardenal-30) and Phenobarbital 60 mg (Gardenal-60), it was observed that the intensity of purple colour gradually increased ranging from light blue colour to light purple from cavity 1 to 9 in case of Zwikker's test. However, in case of Dille-Koppanyi test light purple colour was observed uniformly in all the 9 cavities in the spot tile.

Figure 1
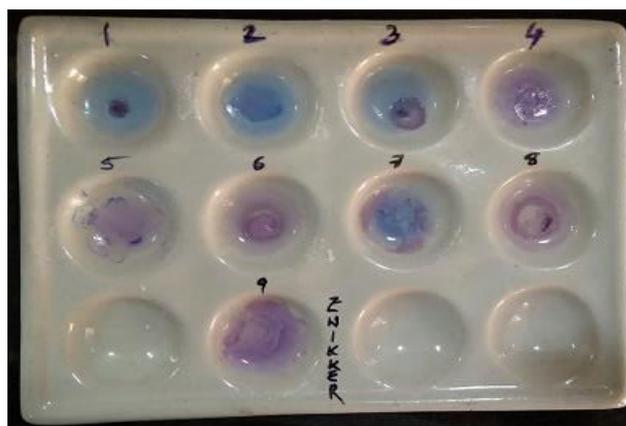
Zwikker's test on different concentration ratios of Gardenal-30 sample
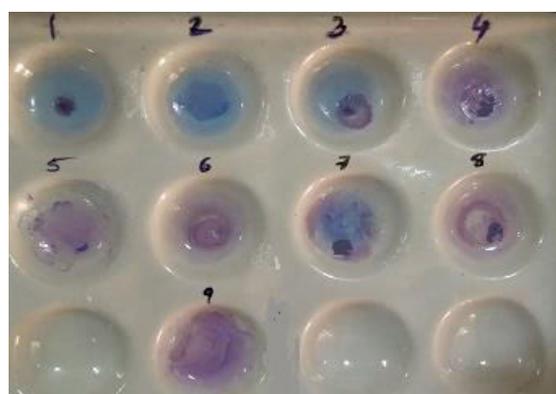
Zwikker's test on different concentration ratios of Gardenal-60 sample
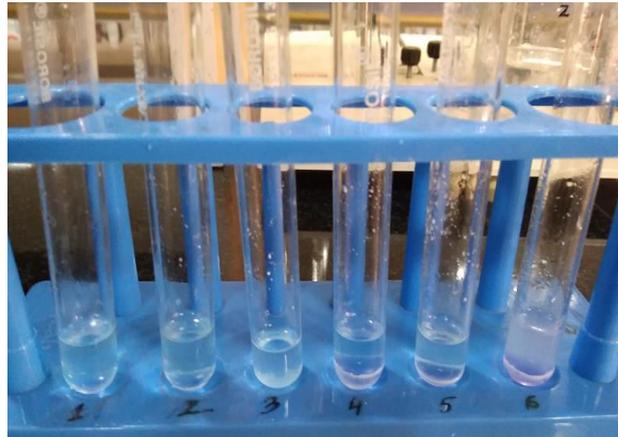
**Dille-Koppanyi test on different concentration
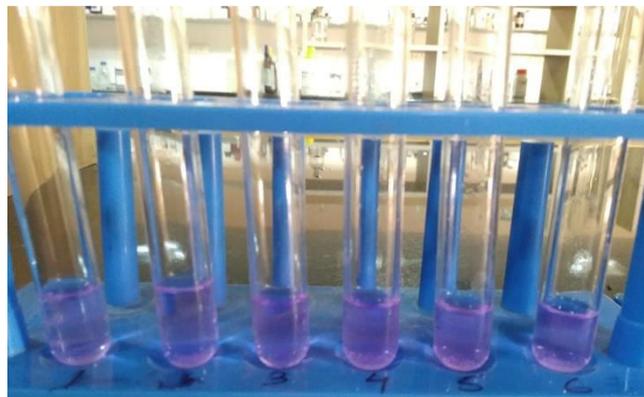ratios of Gardenal-30 sample**

**Dille-Koppanyi test on different concentration ratios
of Gardenal-60 sample**

From the presumptive tests performed on the test tubes for both the samples i.e. Phenobarbital 30 mg (Gardenal-30) and Phenobarbital 60 mg (Gardenal-60), it was observed that the intensity of purple colour gradually increased ranging from light blue colour to light purple from test tube labelled 1 to 9 in case of Zwikker's test. However, in case of Dille-Koppanyi test light purple was observed in all the test tubes labelled 1 to 9.
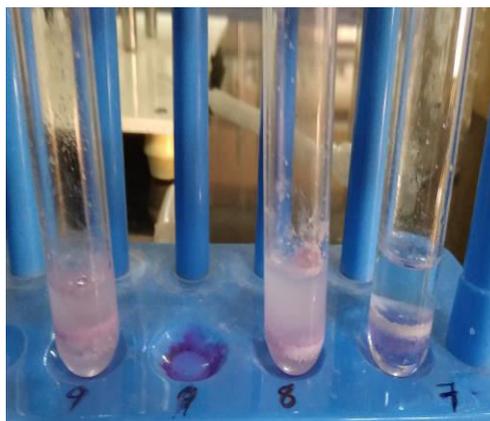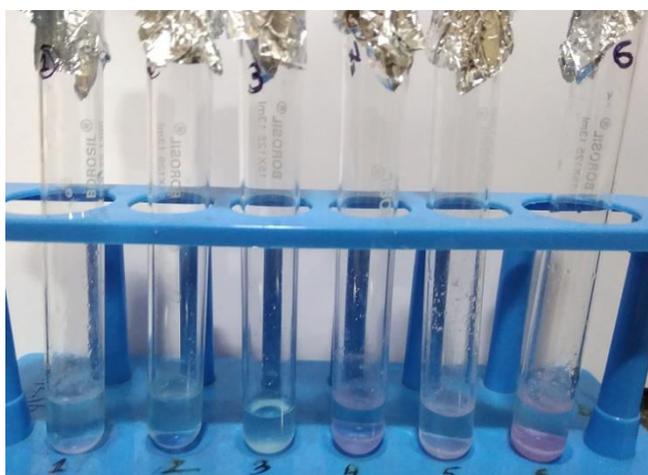
Figure 2

**Zwikker's test on different concentration ratios
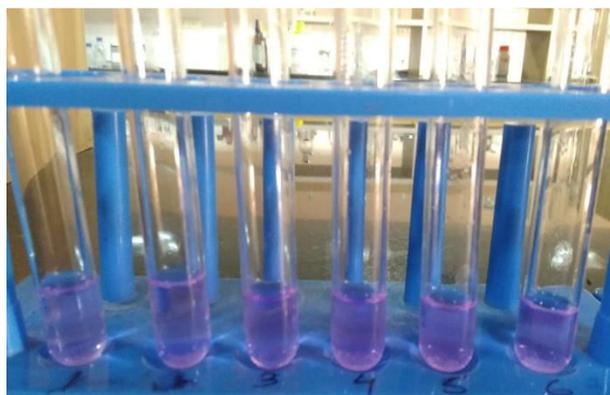of Gardenal-30 sample**

**Dille-Koppanyi test on different concentration
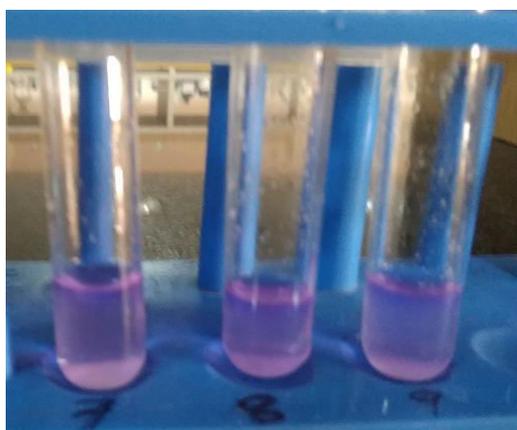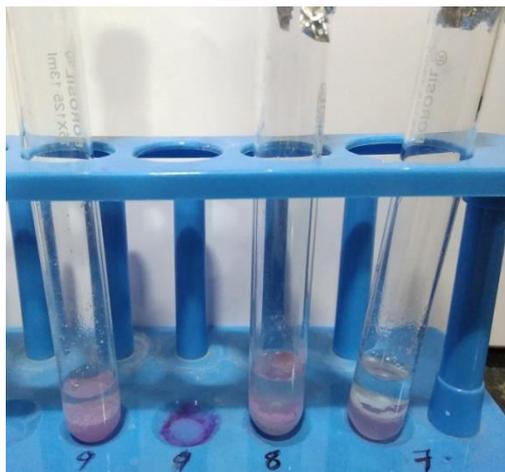ratios of Gardenal-30 sample**

Zwikker's test on different concentration ratios of Gardenal-60 sample

Dille-Koppanyi test on different concentration ratios of Gardenal-60 sample

**Dille-Koppanyi test on different concentration
ratios of Gardenal-60 sample**

   The absorbance values of all the coloured   solutions i.e. the test results obtained after performing Zwikker's test and Dille-Koppanyi test in different proportions were found out using UV Spectrophotometer. The absorbance values were then compared for both the presumptive tests. The absorbance values given by UV Spectrophotometer (according to Table 3 and 5) for both the samples i.e. Gardenal-30 and Gardenal-60 were comparatively higher and more consistent i.e. lesser variation in case of the coloured solutions obtained after Dille-Koppanyi test. The absorbance values given by UV Spectrophotometer (according to Table 2 and 4) for both the samples i.e. Gardenal-30 and Gardenal-60 were comparatively lower and less consistent i.e. more variation in case of the coloured solutions obtained after Zwikker's test.

Testing of Hypothesis

   The two hypothesis were tested with the aid of statistical methods viz Independent sample 't' test and Coefficient of Variation (represented in tabular form in Table 6, 7 and 8).

Sl No. (Different concentrations)	Wavelength (nm)	Absorbance (A)
1	212	2.8661
2	215	3.0000
3	215	2.9658
4	213	2.8038
5	212	2.8948
6	213	2.9115
7	213	2.8726
8	213	2.8854
9	212	2.8661

Table 2 : Table showing absorbance values after Zwikker's test for Gardenal-30 sample

Hypothesis 1 is interpreted with respect to Independent sample 't' test. We reject the null hypothesis i.e. there is a significant difference ($p < 0.01$) in the result given by Zwikker's test and Dille-Koppanyi test in the presumptive detection of barbiturates. Hypothesis 2 is interpreted with respect to Coefficient of Variation. The hypothesis is accepted from the fact that the coefficient of variation value for Dille-Koppanyi test is found to be lesser (0.78 and 2.26 for Gardenal-30 and Gardenal-60 respectively) than the coefficient of variation value for Zwikker's test (2.00 and 5.86 for Gardenal-30 and Gardenal-60 respectively). It means that Dille-Koppanyi test gives more consistent and better result than given by Zwikker's test, which is reflected by lower coefficient of variation in case of Dille-Koppanyi test.

Table 3: Table showing absorbance values after Dille-Koppanyi test for Gardenal-30 sample

Sl No. (Different concentrations)	Wavelength (nm)	Absorbance (A)
1	261	3.0000
2	221	2.9376
3	222	3.0000
4	268	2.9611
5	269	3.0000
6	274	3.0000
7	222	3.0000
8	221	3.0000
9	272	3.0000

Table 4 : Table showing absorbance values after Zwikker's test for Gardenal-60 sample

Sl No. (Different concentrations)	Wavelength (nm)	Absorbance (A)
1	215	2.9002
2	212	2.8693
3	211	2.6402
4	214	2.8611
5	214	3.0000
6	208	2.4970
7	213	2.8794
8	213	2.9586
9	210	2.6962

Table 5 : Table showing absorbance values after Dille-Koppanyi test for Gardenal-60 sample

Sl No. (Different concentrations)	Wavelength (nm)	Absorbance (A)
1	235	3.4237
2	231	3.4078
3	234	3.3458
4	233	3.4202
5	242	3.2358
6	244	3.3768
7	242	3.3072
8	244	3.4895
9	246	3.4377

Table 6 : Table showing the parameters for Independent sample 't' test and Coefficient of Variation

| Sample	Name of the test		Total
	Zwikker	Dille-Koppanyi	
Gardenal-30	9	9	18
Gardenal-60	9	9	18
Total	18	18	36

Table 7 : Table showing the significant difference in absorbance of barbiturates (Gardenal-30 and Gardenal-60 Tablets) with respect to Zwikker's test and Dille-Koppanyi test

| Variable	Name of the test	N	Mean	Standard Deviation	t	Sig.
Absorbance of Barbiturates	Zwikker	18	2.85	0.13	-5.73	0.00*
	Dille-Koppanyi	18	3.19	0.21		

Table 8 : Table showing the coefficient of variation for both Zwikker's test and Dille-Koppanyi test

| Test	Sample	Mean	Standard Deviation	Coefficient of Variation
Zwikker	Gardenal-30	2.90	0.06	2.00
	Gardenal-60	2.81	0.16	5.86
Dille-Koppanyi	Gardenal-30	2.99	0.02	0.78
	Gardenal-60	3.38	0.08	2.26

Figure 3 (UV Spectrums of few solutions obtained after Zwikker's test and Dille-Koppanyi test)
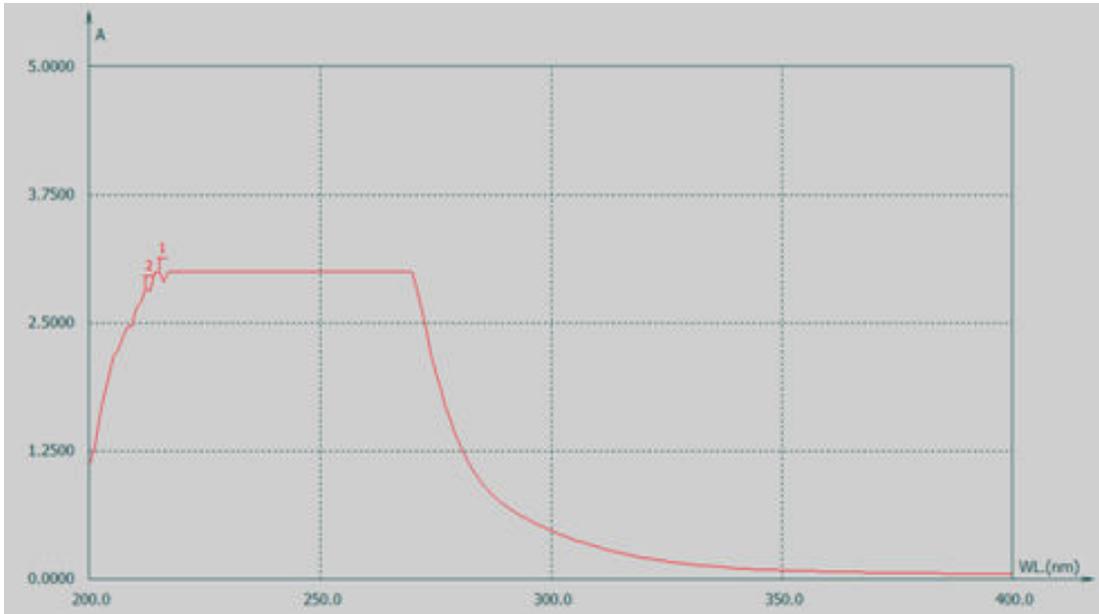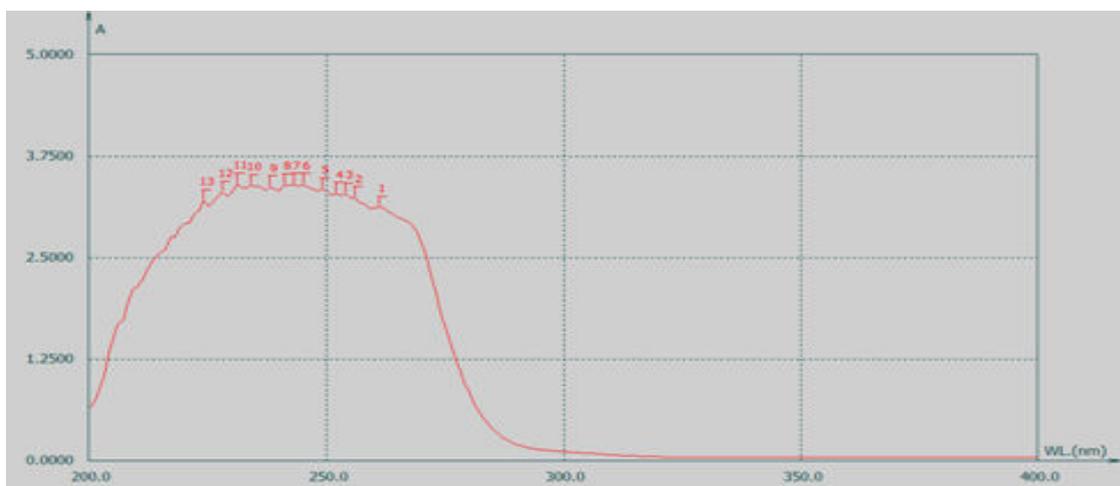

UV Spectrum for Zwikker's test on solution 2 of Gardenal-30 sample
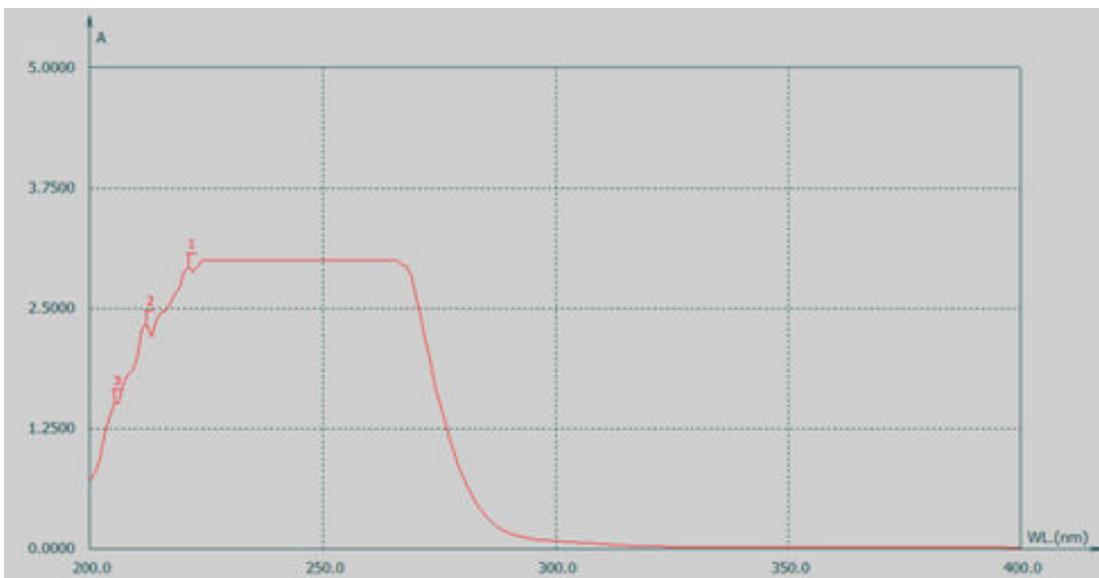
UV Spectrum for Dille-Koppanyi test on solution 2 of Gardenal-30 sample
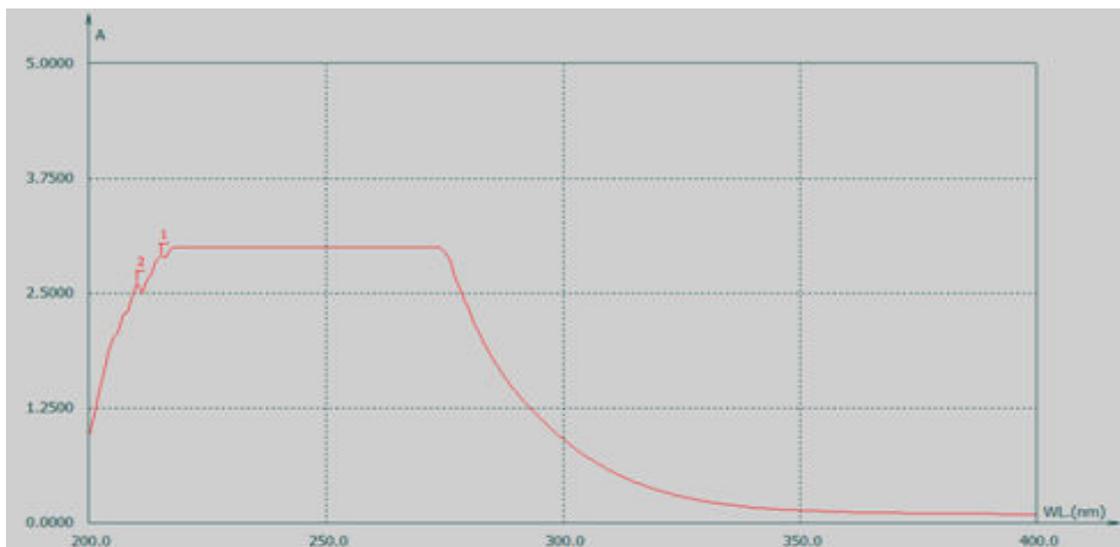
UV Spectrum for Zwikker's test on solution 2 of Gardenal-60 sample

UV Spectrum for Dille-Koppanyi test on solution 2 of Gardenal-60 sample

CONCLUSION

The presumptive tests most widely used for detection of Barbiturates are Zwikker's test and Dille-Koppanyi test. A comparative study in between the two tests has been carried out to determine whether there are any differences shown in the test results. Further, a statistical analysis was made to determine which test gives better result with regard to consistency.

An analysis was made based upon the results of Zwikker's test and Dille-Koppanyi test on the spot tile and also in the test tubes. It was found that there is an overall uniform light purple colour in all the cavities on the spot tile and test tubes containing varying amount of Phenobarbital for Dille-Koppanyi test. However, for Zwikker's test, the light purple colour was not uniform in the cavities on the spot tile and the test tubes for different solutions of Phenobarbital. The colour intensity ranged from light blue to light purple.

Further, the absorbance values for the coloured solutions i.e. test results obtained after the two tests were compared. It was found that the absorbance values were higher and more consistent with lesser variation for Dille-Koppanyi test whereas

the absorbance values were lower and less consistent with more variation for Zwikker's test.

The two hypothesis were proved with the help of statistical analysis viz Independent sample 't' test and Coefficient of Variation. Hypothesis 1 was rejected and it was proved that there is a significant difference in the result given by Zwikker's test and Dille-Koppanyi test in the presumptive detection of barbiturates. Hypothesis 2 was accepted and it was proved that Dille-Koppanyi test gives more consistent and better result than given by Zwikker's test.

Barbiturate drugs are encountered in various cases including possession, transportation, manufacture and trading of barbiturate and its products. Preliminary testing indicates whether the drug is a barbiturate or not a barbiturate and further provides an idea to proceed with the confirmatory test. As found from the study, Dille-Koppanyi test shows better consistency with regard to test results. Therefore, in cases where only a trace amount of the substance is available and presumptive testing of barbiturates are required to be performed, Dille Koppanyi test can be directly performed instead of Zwikker's test to get a better and profound result. It will ensure better result of trace quantity of evidence and lead to better outcome in the investigation of the illicit case. Further, this shall also contribute to the concept of green chemistry.

ACKNOWLEDGEMENTS

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