Journal of Applied Pharmacy
ISSN: 1920-4159

Mahmood et al., J Appl Pharm 2018, 10:3
DOI: 10.4172/1920-4159.1000265

Research Article Open Access

A New Colorimetric Identification of Benzodiazepines: Using Cobalt Thiocyanate as Reagent

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Abstract

A highly specific, easy to perform and cost effective color test for benzodiazepines class has been developed. This colour test produced green colour with eight benzodiazepines i.e. nitrazepam, temazepam, diazepam, bromazepam, clonazepam, estazolam, lormetazolam and alprazolam, whereas developed color was absent in other controlled or pharmaceutical substances tested during study. In this color test, one drop of concentrated hydrochloric acid was added to test substrate. Then two drops of cobalt thiocyante reagent were added in subsequently which resulted in an immediate appearance of green color. So this test can be very helpful as a presumptive screening tool for benzodiazepines testing in suspected illicit samples and pharmaceuticals. Moreover, this test can be further employed for diazepam quantitation using ultraviolet spectroscopy at 364 nm wavelength and showed linear detector response. A regression co-efficient value of 0.9996 was achieved using developed method and was effectively useful for diazepam quantitation in pharmaceutical dosage forms.

Keywords: Presumptive color testing; Benzodiazepine analysis; Benzodiazepines; Diazepam quantitation

Introduction

Benzodiazepines are psychoactive compounds having a structure which is combination of benzene and diazepine ring. The whole group of such compounds is called "Benzos" [1,2].

Benzos are frequently prescribed to treat certain ailments. Due to sedative properties, they have a high potential for abuse [3,4] especially when used with other depressants such alcohol and opiates [5,6]. Commonly prescribed benzodiazepines are Xanax (alprazolam), Librium (chlordiazepoxide), Valium (diazepam) and Ativan (lorazepam) (Figure 1).

There are few methods for the colorimetric identification of benzodiazepines in the forensic drug testing and toxicological laboratories. Two types of tests are performed for the identification of drug namely preliminary test and confirmatory test [7-9]. In case of diazepam which is abundantly abused among others benzos not many color test are available in literature. However, for diazepam and flunitrazepam, two different color reactions are available [10,11]. No single color test is available for the benzos as a whole. The object of this preliminary study was to find a single color test for benzos which could give some indication of the benzodiazepine group. Zimmermann's Reaction was applied for identification of benzos but the test does not give clear indication due to double shade (reddish purple) [12-14]. Herein a new presumptive color test for the identification of benzodiazepines has been developed. This test is rapid, easy to perform, economical and has reasonable sensitivity towards tested benzos. Another advantage of this test is that it can be used for quantitation of benzos along with its presumptive identification.

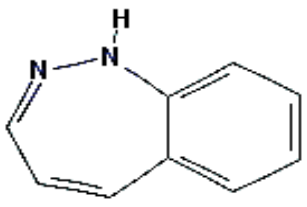

Figure 1: Benzos.

Experimental

Materials

- Cobalt thiocyanate, Chlorpheniramine maleate and Diazepam were imported from Sigma-Aldrich, spruce street St. Louis.
- Analytical grade solvents Hydrochloric acid 37%, n-Hexane 99%, chloroform 99.8%, toluene 99.6%, acetone, isopropanol as well as analytical grade chemicals Sodium hydroxide, Diphenhydramine, Quinine Sulfate, Ditheoximide, Vanillin, Caffeine, Sodium hydrogen tartrate, Calcium carbonate, Hydroxylamine, were purchased from Acros organics new jersey USA.
- Deionized water was used for reagent preparations. Absolute Ethanol was purchased from Merck Darmstadt Germany.
- Sodium Chloride, Sulfamic Acid, Starch, Lactose, and analytical grade methanol were bought from Fischer Scientific Bioreagents Fair Lawn New Jersey USA.
- Oxytetracycline, meloxicam and albendazole were taken as generous gift from Venus pharmaceuticals Lahore.
- Tablets, capsules and injections were acquired from different pharmacies (Table 1).

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Received: October 17, 2018; **Accepted:** December 11, 2018; **Published**: December 18, 2018

Citation: Mahmood Z, Muhammad S, Arshad N, Tahir MA, Qurashi MZ, et al. (2018) A New Colorimetric Identification of Benzodiazepines: Using Cobalt Thiocyanate as Reagent. J Appl Pharm 10: 265. doi: 10.4172/1920-4159.1000265

S.No	Compound	Brand Used	Manufacturer
1	Temazepam	Restoril	Novartis Pharma Limited Pakistan
2	Clonazepam	Clonatril	PolyfineChempharma (PVT) Ltd
3	Estalozolam	Esilgan	Helix Pharma (Pvt) Limited
4	Alprazolam	Xanas	Pfizer Ltd Pakistan
5	Lormetazepam	Noctamide	Bayer Pakistan (Pvt) Ltd
6	Chlordiazepoxide	Librex	Roche Pakistan Ltd
7	Lorazepam	Ativan	Wyeth Pakistan Limited
8	Bromazepam	Bromalex	Indus Pharma (Pvt)Ltd
9	Nitrazepam	Mogadon	Valeant Pharmaceutical International. Inc.
10	Ketamine	Ketasol	Indus Pharma (Pvt) Ltd
11	Naproxen	Synflex	ICI Pakistan Ltd Karachi
12	Nortryptaline	Modrin	Pharmedic Laboratories (Pvt) Ltd
13	Bupivacine	Bupicaine	Lahore Chemical & Pharmaceutical Works (Pvt) LTD
14	Morphine sulphate	Magnus MR	AGP (Pvt) Ltd.
15	Phenobarbitol	Phenobarbitone Sodium	Ameer Pharma (Pvt) Ltd Lahore
16	Atenolol	Tenormin	ICI Pakistan Limited Karachi
17	Mafenamic acid	Ponstan	Pfizer Ltd Pakistan
18	Furosemide	Laxis	Sanofi Aventis (Pakistan) Ltd
19	Hydroxyzine HCl	Atarax	Pfizer Ltd Pakistan
20	Aspirin	Disprin	Reckitt Benckiser Healthcare (UK) Ltd
21	Flurbiprofen	Ansaid	Pfizer Ltd Pakistan

Table 1: Compound obtained from Pharmacies, Brand Used and Manufacturer.

Instrumentations

- TurboVap Model#10430 (Harris oaks Blvd Suite C Chariotte NC 28269 USA) was used for solvent drying.
- Fisher digital vortex mixer model# 945416 was used for sample mixing.
- Eppendorf centrifuge model 5810 was used for phase separation.
- Gas Chromatograph-Mass Spectrometry (GC model # 7890A-Mass Spectrometer model #5975C with triple axis detector Agilent technologies).
- Fourier Transform Infrared spectrophotometer (Thermo-Fischer scientific Model # Nicolet is 10) was used for confirmation of extracted samples.
- UV-Visible spectrophotometer Evolution 300 was used for the quantitation of Diazepam.
- Analytical balance (Mettler Toledo PL 303) was used for the weighing purposes

Methods

Reagent preparation: 2% cobalt thiocyanate solution was prepared by adding 2 grams of cobalt thiocyanate powder in 100 ml Deionized water.

Extraction procedure: Extraction procedure for various types of dosage forms i.e. tablets, capsules or injectable is given in Table 2.

Color test procedure: Two glass culture tubes were taken and labelled as "Control" and "Sample". In the control tube, one drop of

Compounds	Extraction solvent	Method for extraction
Alprazolam, Clonazepam, Lorazepam, Temazepam, Naproxen, Atenolol, Furosemide, Aspirin, Flurbiprofen	Acetone	Randomly selected tablet was Ground to powder and shifted to centrifuge tube. 2 ml extraction solvent was added to tablet powder and vortexed for 3 mins. Set the extraction mixture at rotator for 15 min at 50 rpm. The sample mixture was centrifuged for 4 mins @ 3500 rpm. Shifted the supernatant liquid to round bottom tube. Sample extraction procedure was repeated thrice for ensuring complete drug extraction. Solvent was evaporated on turbo evaporator @ 45°C and 15PSI air pressure to get fine drug powder. Powder was subjected to GC-MS/FTIR analysis prior to colorimetric testing.
Bromazepam, Nitrazepam, Nortryptaline, Bupivacine, Phenobarbitol	Chloroform	Same as described above
Lormetazepam	Toluene	Same as described above
Ketamine	Methylene Chloride	Ketamine injection solution was transferred to tube and 2 ml chloroform was added. Vortexed for 4 min. chloroform was pipetted out and discarded whereas aqueous layer was basifiedusing 50 mg sodium carbonate powder. 2 ml methylene chloride was added to aqueous phase and vortexed. Set the extraction mixture at rotator for 15 min at rate of 50 rpm. The sample mixture was centrifuged for@ 3500 rpm for 4 mins. Shifted the extraction solvent to a round bottom tube. Sample extraction procedure was repeated thrice for ensuring complete drug extraction. Solvent was evaporated on turbo evaporator @ 45°C and 15PSI air pressure to get drug powder. Powder was subjected to FTIR analysis prior to colorimetric testing.
Morphine sulphate	Isopropyl Alcohol and Chloroform in 1:2	Granules from morphine sulphate capsules were ground and transferred to a centrifuge tube. 0.5 ml of Ammonium Hydroxide was added and vortexed. 3 ml of extraction solvent was added. The whole solution was vortexed for 3 mins again and set on rotator for 15 mins @ 50 rpm The sample mixture was centrifuged for @ 3500 rpm for 4 mins. Shifted the extraction phase in another glass tube. Sample extraction procedure was repeated thrice for ensuring complete drug extraction. Solvent was evaporated on turbo evaporator @ 45°C and 15 PSI air pressure to get fine drug powder. Powder was subjected to FTIR analysis prior to colorimetric testing.
Hydroxyzine	Hexane	Tablet was ground to powder and shifted in centrifuge tube. 2 ml water was added to powder. Solution was vortexed and centrifuged. Liquid phase was transferred in another tube and 0.5N NaOH (ca. 3 ml) was added and vortexed. Extraction solvent was added and set on rotator for 15 mins @ 50 rpm followed by centrifugation for 4 mins @3500 rpm (hexane was in top layer). Hexane was shifted in glass tube. Sample extraction procedure was repeated thrice for ensuring complete drug extraction. Solvent was divided in two portions. HCL Fumes were passed through one portion as a result precipitates of hydroxyzine HCL were formed and collected. Second portion was evaporated on turbo evaporator @ 45°C and 15 PSI air pressure to get fine drug powder (base form). Both powders were confirmed by FTIR analysis prior to colorimetric testing.
Chloroquinine	Chloroform	Tablet was ground to fine powder and shifted to centrifuge tube. 2-3 ml of water was added. The solution was vortexed for 3 mins followed by centrifugation @ 3500 rpm for 4 mins. Supernatant liquid was pipetted out to another tube and 1 ml of 1N NaOH was added and vortexed. Then 2 ml Chloroform was added and set on rotator @ 50 rpm for 20 minutes. After centrifugation, Chloroform layer was shifted to glass tube. Sample extraction procedure was repeated thrice for ensuring complete drug extraction. Solvent was evaporated on turbo evaporator @ 45°C and 15 PSI air pressure to get fine drug powder. Powder was subjected to FTIR analysis prior to colorimetric testing.

Table 2: List of Drugs and their Extraction procedures applied.

Conc. HCl was added followed by the addition of two drops of Cobalt thiocyanate reagent whereas in the sample tube, a pinch of test powder was placed in sample tube and added one drop of Conc. HCl followed by the addition of two drops of Cobalt thiocyanate reagent. Any change or transition of color and its shifting to suitable organic solvent was noted.

Method for diazepam quantitation

Standard solution: 10 mg of Diazepam powder standard was dissolved in 10 ml of chloroform for making standard stock solution (1 mg/ml). Aliquot concentrations (250, 300, 350, 400, 450 and 500) µg/ml were taken in labelled glass tube by using micropipette. 500 µl of chloroform was taken in glass tube and marked as blank sample. Two concentration levels of 315 µg/ml and 475 µg/ml were prepared as Q_c Low and Q_c High respectively. All the tubes were completely dried in air stream using turbo evaporator. Color test was performed in "control tube" and "sample tubes" by adding 0.5 ml of conc. HCl followed by addition of 0.5 ml of Cobalt thiocyanate solution. Vortex it thoroughly then 2 ml chloroform was added for extraction of color in chloroform for quantitation. Chloroform from control tube and each calibrator was pipetted out in other tube. Each tube was extracted with chloroform three time. The tubes were dried on dry bath incubator at 60°C for solvent evaporation. Finally the tubes were reconstituted with 2 ml chloroform. The standard solutions were run on ultraviolet-visible spectrophotometer for checking of absorbance at 364 nm. Instrumental method parameters for diazepam quantitation are shown in Table 3.

Following parameters are evaluated for validation of above-mentioned quantitation method.

- Linearity
- Accuracy and precision
- Limit of detection (LOD)
- Limit of quantitation (LOQ)
- Specificity / Interference
- Solution stability

Procedure for diazepam quantitation from pharmaceutical dosage form

Step-1 (Extraction):

Tablets: One tablet was randomly selected and grounded to fine powder. Powder was shifted in eppendorf centrifuge tubes with the addition of 2 ml Chloroform as extraction medium. Sample mixture was vortexed for 3 minutes. Then sample mixture was centrifuged @4500 rpm. The supernatant was pipetted out in separate glass tube. Each tablet was extracted four times with Chloroform. Supernatant was placed on hot plate for solvent evaporation to get fine diazepam powder.

Injections: One injection was taken and transferred into eppendorf tubes. Chloroform was added in it. After addition of chloroform tablet was vortexed for 3 minutes and then centrifuged @4500 rpm. Chloroform was pipetted out in separate glass tube. Each injection was extracted four times with Chloroform and chloroform was collected in same tube for one injection. After completion of extraction, glass tube with chloroform was placed on hot plate for evaporation of chloroform to get diazepam.

Step-2 (Quantitation): After drying color test is performed by adding 0.5 ml of conc. HCl followed by 0.5 ml of Cobalt thiocyanate solution. Vortex it thoroughly then 2 ml chloroform is added for extraction of color in chloroform for quantitation. Then 400 ul from each tube is taken separately and 2 ml volume was made up by adding 1600 ul of chloroform for making final dilution. The final dilution was checked for their absorbance ultraviolet-visible spectrophotometer at 364 nm. Controls were always run with each batch.

Results

An immediate green color was developed with abovementioned benzodiazepines on addition of cobalt thiocyanate reagent (Figure 2-5). When ammonium thiocyanate was tried instead of cobalt thiocyanate reagent, the green color didn't appeared. Moreover oxytetracycline and diphenhydramine produced different colors (Figures 5-7). The observed green color in case of benzodiazepines or colors produced by other drugs were checked for their shiftibilty in organic solvents. It was found that green color was completely shiftable in chloroform and methylene chloride in case of diazepam while Green color developed

Method Parameter	
Quant Mode	Single wavelength
Wavelength 1	364.00 nm
Lamp	Xenon
Bandwidth	2.0 nm
Standards	6
Replicates	3
Initial curve fit	Linear
Conc. Units	µg
Conc. Dec Places	3

Table 3: Method parameter employed during Diazepam quantitation.

Figure 2: Reaction of diazepam with cobalt thiocyanate and its colour shiftibility in chloroform.

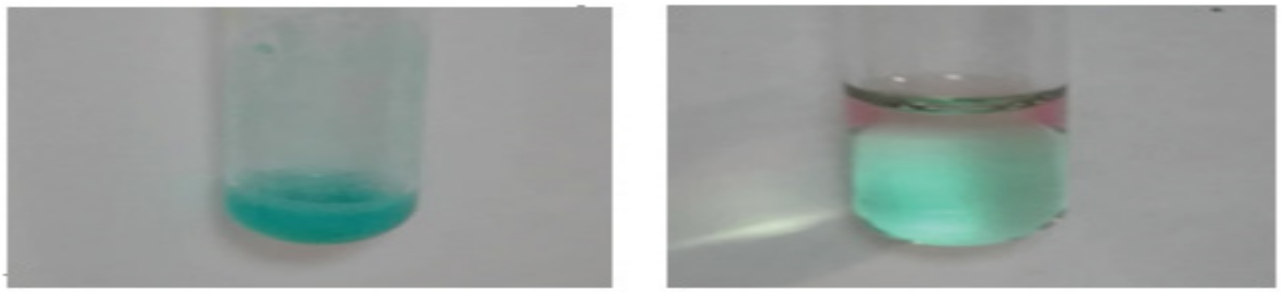

Figure 3: Reaction color of Temazepam with cobalt thiocyanate and its shiftibility in chloroform.

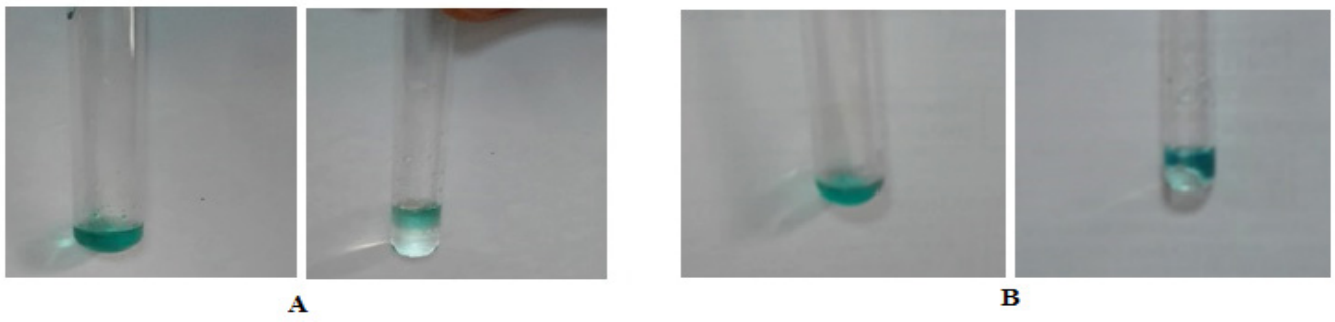

Figure 4: Reaction colour of A (Lorazepam) and B (Nitrazepam) with cobalt thiocyanate and their non-shiftible behavior in chloroform.

Figure 5: Oxytetracycline and diphenhydramine produced different colors.

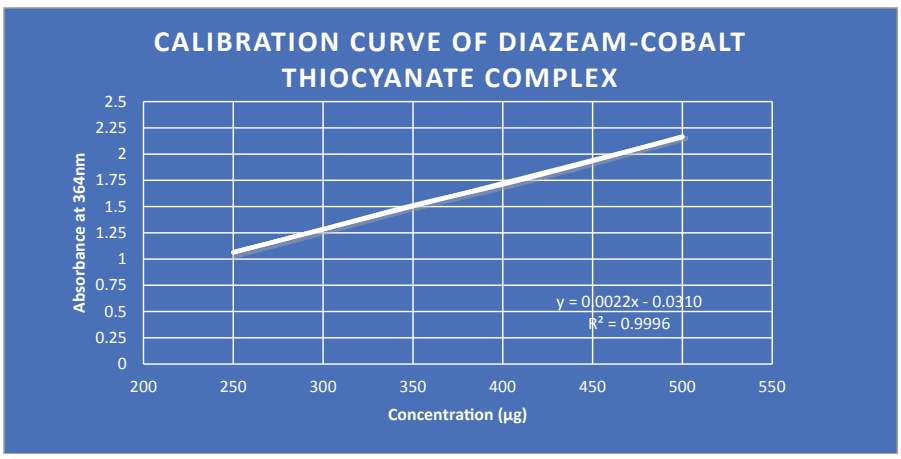

Figure 6: Calibration curve of diazeam cobalt thiocyanate complex.

by temazepam was only shiftable in chloroform (Table 4). Color produced by lorazepam and nitrazepam didn't show any shiftibilty in organic solvents (Figure 4). Colors developed by Meloxicam, naproxin were completely shiftable in methylene chloride only while colors observed in case of bupvacaine and chlordiazepoxide were shiftable in chloroform (Table 4).

Accuracy and Precision

For study accuracy of diazepam quantitation method three different conc. 250 µg/ml, 300 µg/ml and 400 µg/ml were taken and absorbance value was noted for three days.

$$\left[\text{Accuracy} = \frac{\text{Mean of calculated Concentration-Known Concentration}}{\text{Known Concentration}} \times 100\right]$$

Following are the calculations done for accuracy and precision studies.

Limit of detection

The detection limit was determined by the analysis of samples with known concentrations of analyte via visual evaluation and by establishing the minimum level at which the analyte was reliably detected.

It was found that 8 µg/ml is LOD for detection of diazepam with this method (Tables 5-8).

Limit of quantitation

25 µg/ml is LOQ for quantitation of diazepam by above mentioned method.

Stability study

Stability studies are done in two different ways.

Intraday stability study: In this study standard solution of 300 µg/ml is prepared and run on UV-Visible spectrophotometer and its Absorption is noted. This solution was then transferred to screw capped glass tube and its absorbance is noted after every 3 hour at 364 nm till 12 hours consecutively and following calculation are done to check stability.

Interday stability study: In this study standard solution of 300 µg/ml is prepared and run on UV-Visible spectrophotometer and its Absorption is noted. This solution was then transferred to screw capped glass tube and its absorbance is noted after each day for consecutive 5 days at 364 nm and following calculation are done to check stability (Tables 9-11).

Specificity/Interference

Compounds like Stearic acid, Magnesium stearate, Starch, Lactose, Talc, silica, cellulose, mannitol, caffeine and gelatin was run individually on UV-VIS to check interference at wavelength 364 nm. No compound showed any interference at 364 nm (Tables 12 and 13).

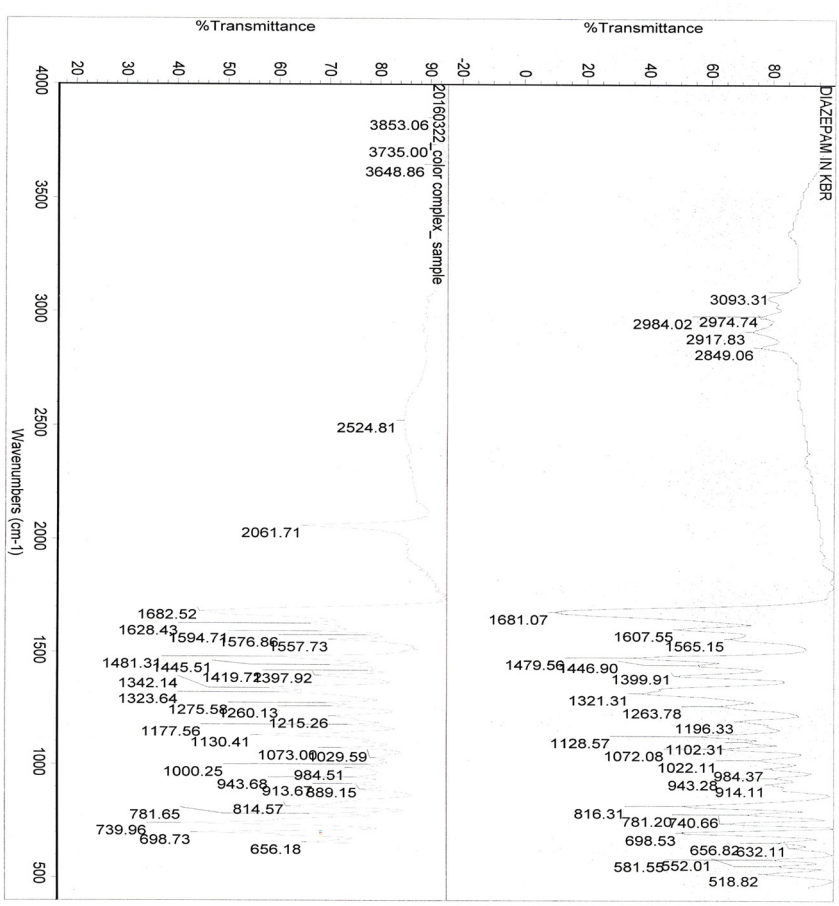

Figure 7: FTIR spectra of Color complex and Diazepam.

S.No	Drug Name	Color Observation	S.no	Drug Name	Color Observation
1	Temazepam	Green color	**26**	Sodium Hydrogen Tatarate	No Color
2	Diazepam	Green color	**27**	Sulfamic Acid	No Color
3	Estalozolam	Green color	**28**	Calcium Carbonate	No Color
4	Alprazolam	Green color	**29**	Hydroxyl amine	Purple colour
5	Lormetazepam	Green color	**30**	Nortryptaline	blue color
6	Chlordiazepoxide	Blue colour	**31**	Bupivacine	Blue color of drug
7	Lorazepam	Green color	**32**	Cannabis Resin	No Color
8	Clonazepam	Green color	**33**	Opium	No Color
9	Bromazepam	Green color	**34**	Glusose	No Color
10	Nitrazepam	Green Color	**35**	Starch	No Color
11	Albandazole	Bright blue drug ppts.	**36**	Lactose	No Color
12	Meloxicam	Blue Color	**37**	Morphine sulphate	No Color
13	Oxytetracycline	Dark greenish yellow color	**38**	Phenobarbitol	No Color
14	Diphenhydramine	Blue color	**39**	Atenolol	No Color
15	NaCl	No Color	**40**	Ephedrine	No Color
16	Pyrimethamine	No Color	**41**	Chlorphenaramine maleate	No Color
17	Methyl Phenidate	No Color	**42**	Flurbiprofen	No Color
18	Quinine	No Color	**43**	Methamphetamine	No Color
19	Dithiooxamide	No Color	**44**	Mafenamic acid	No Color
20	Chloroquinine	No Color	**45**	Silica	No Color
21	Vaniline	No Color	**46**	Furosemide	No Color
22	Caffeine	No Color	**47**	Cobalt Chloride	No Color
23	Ketamine	No Color	**48**	Hydroxyzine HCl	No Color
24	Diclofenac Sodium	No Color	**49**	Aspirin	No Color
25	Naproxen	Blue color	**50**	Stearic Acid	No Color

Table 4: List of different tested compounds and their reaction colour.

Compound	Suitable solvent for shifting of Colour
Chlordiazepoxide	Chloroform
Diazepam	Chloroform, Methylene Chloride
Temazepam	Chloroform
Meloxicam	Methylene Chloride
Naproxen	Methylene Chloride
Bupivacaine	Chloroform

Table 5: Benzodiazepine color and suitable solvent for its shifting.

Calibratior (µg/ml)	Absorbance (A°) at 364 nm
250	1.064
300	1.285
350	1.512
400	1.711
450	1.935
500	2.168

Table 6: Calibration Curve for Diazepam Quantitation.

Linearity Parameters	
Range	250 – 500 µg/ml
Slope	0.0044
Intercept	0.0310
Co-relation coefficient	0.9996
Solution Stability	6 days
Accuracy	99.50 +/- 0.54
Mean Recovery(% Raw Material)	99.60 +/- 0.44
LOD	10 µg/ml
LOQ	25 µg/ml

Table 7: Linearity Parameters.

Calibrator (µg/ml)	Day	Mean Conc. Recovered (µg)	Standard Deviation	Accuracy	Precision
250	1	247.83	0.55%	99.1%	0.22%
	2				
	3				
300	1	295.16	0.50	98.39%	0.17%
	2				
	3				
400	1	399.55	0.84	99.88%	0.21 %

Table 8: Calibration Curve for Diazepam Quantitation.

Day	Accuracy	Precision
1	**Accuracy at 250 µg/ml** = (247.83-250)/250 x 100 = - 0.868 % or 99.1%	**Precision at 250 µg/ml** = (0.55/247.83) x 100 = 0.22%
2	**Accuracy at 300 µg/ml** = (295.16-300)/300 x 100 = -1.61% or 98.39 %	**Precision at 300 µg/ml** =(0.50/295.16) x 100 = 0.17 %
3	**Accuracy at 400 µg/ml** = (399.55-400)/400 x 100 = - 0.125 % or 99.88 %	**Precision at 400 µg/ml** = (0.84/399.55) x 100 = 0.21 %

Table 9: Accuracy & Precision Determination for Diazepam-cobalt thiocyanate complex Quantitation.

Calibrator Concentration	Absorbance	Standard deviation of Response	LOD =3.3 x Std. deviation of response/ Slope of response = (3.3 * 0.014) / 0.0057
250 µg/ml	1.431	0.014653024	8.48332966

Table 10: Calculation for limit of detection.

Calibrator Concentration	Absorbance	Standard deviation of response	LOD =10 * std. deviation of response/ slope of response = (10 * 0.014) / 0.0057
250 µg/ml	1.431	0.014653024	25.70705958
250 µg/ml	1.454		
250 µg/ml	1.47		
250 µg/ml	1.445		
250 µg/ml	1.435		
250 µg/ml	1.465		
250 µg/ml	1.439		
250 µg/ml	1.459		
250 µg/ml	1.469		
250 µg/ml	1.439		

Table 11: Calculation for determining LOQ.

Time (hr)	Quantity (300 µg/ml)	Absoption (A°)	Concentration recovered (300 µg/ml)	%age concentration	Quantity deficient	%age error	Average	Standard deviation	%RSD
1 HR	Solution 1	1.25	299.76	99.92	0.24	0.08	99.84	0.08	0.08
	Solution 2	1.25	299.52	99.84	0.48	0.16			
	Solution 3	1.25	299.29	99.76	0.71	0.24			
3 HR	Solution 1	1.25	298.57	99.52	1.43	0.48	99.60	0.08	0.08
	Solution 2	1.25	299.05	99.68	0.95	0.32			
	Solution 3	1.25	298.81	99.60	1.19	0.40			
6 HR	Solution 1	1.25	298.10	99.37	1.90	0.63	99.37	0.08	0.08
	Solution 2	1.25	298.33	99.44	1.67	0.56			
	Solution 3	1.25	297.86	99.29	2.14	0.71			
9 HR	Solution 1	1.24	296.67	98.89	3.33	1.11	99.02	0.12	0.12
	Solution 2	1.24	297.14	99.05	2.86	0.95			
	Solution 3	1.24	297.38	99.13	2.62	0.87			
12 HR	Solution 1	1.24	295.95	98.65	4.05	1.35	98.52	0.12	0.12
	Solution 2	1.24	295.24	98.41	4.76	1.59			
	Solution 3	1.24	295.48	98.49	4.52	1.51			

Table 12A: Intraday stability study.

Average of recovered quantity	Accuracy within day (12 hours) = 100X calculated conc-Known conc/ known conc	Precision within day (12 hours) =(SD/quantity calculated)*100
299.52	99.84	0.03
298.81	99.60	0.03
298.10	99.37	0.03
297.06	99.02	0.04
295.56	98.52	0.04

Table 12B: Intraday stability study.

Time (hr)	Quantity (300 ug/ml)	absoption (A°)	concentration recovered (300 mg/ml)	%age concentration	Quantity deficiet	%or	Average	Standard deviation	%RSD
1 day	Solution 1	1.22	291.90	97.30	8.10	3.24	97.41	0.12	0.12
	Solution 2	1.22	292.62	97.54	7.38	2.95			
	Solution 3	1.22	292.14	97.38	7.86	3.14			
2 day	Solution 1	1.21	289.76	96.59	10.24	3.41	96.72	0.12	0.13
	Solution 2	1.22	290.48	96.83	9.52	3.17			
	Solution 3	1.21	290.24	96.75	9.76	3.25			
3 day	Solution 1	1.21	289.05	96.35	10.95	3.65	96.35	0.16	0.16
	Solution 2	1.21	288.57	96.19	11.43	3.81			
	Solution 3	1.21	289.52	96.51	10.48	3.49			
4 day	Solution 1	1.20	287.14	95.71	12.86	4.29	95.61	0.18	0.19
	Solution 2	1.20	286.19	95.40	13.81	4.60			
	Solution 3	1.20	287.14	95.71	12.86	4.29			
5 day	Solution 1	1.20	286.19	95.40	13.81	4.60	95.13	0.28	0.29
	Solution 2	1.19	284.52	94.84	15.48	5.16			
	Solution 3	1.19	285.48	95.16	14.52	4.84			

Table 13A: Interday stability study.

Day	Average	Accuracy within day (5 days)	Precision within day (5 Days)=(SD/quantity calculated)*100
1	292.22	97.41	0.04
2	290.16	96.72	0.04
3	289.05	96.35	0.05
4	286.83	95.61	0.06
5	285.40	95.13	0.10

Table 13B: Interday stability study.

Discussion

Reaction of hydrochloric on benzodiazepines resulted in the formation of benzophenone [15] which immediately reacted with added cobalt thiocyanate, producing a green color because thiocyanate share negative charge approximately equally between sulfur and nitrogen. As a consequence act as nucleophiles at either sulfur or nitrogen which describes the mechanistic behavior evolving in this reaction. The complexes of cobalt thiocyanate formed in aqueous media were found to consist of two species-$[Co(SCS)]^{+}$ and $[Co(NCS)_6]^{-4}$ with the number of possible water molecules in the coordination spheres undetermined. The complex $[Co(NCS)]^{+}$ is present in aqueous solutions containing an excess of cobalt, while an excess of thiocyanate produces [Co(NCS) a]$^{-2}$ [16,17]. Previous Studies have shown that many metal thiocyanates can be extracted from aqueous solutions by means of non-polar organic solvents. Absorptancy curves have been run on solutions of cobalt thiocyanate in non-aqueous solvents. Such extractions have been utilized extensively in the isolation of thiocyanate complexes of such metals like iron, uranium, and molybdenum. Moreover, shift of green color to organic solvent can be supported by the observation of Young and Hall where blue color of thiocyante was soluble in ether. Their importance to the present discussion lies in the emphasis they add to the solubility phenomena attributable to the thiocyanate group effect.

FTIR analysis of formed Diazepam-cobalt thiocyanate complex was performed. C=N of diazepam showed a substantial shift from 1607.65 cm^{-1} to 1628.43 cm^{-1} which confirm the reaction occurred between diazepam and cobalt thiocyante and support proposed complexes structure. Whereas C=O group (1681 cm^{-1}), C-Cl Stretching (781), Ar. C – H Bending (740 cm^{-1}), Ar. C - C Stretching 1442.8 do not participate in this chemical reaction.

Probably one molecule of cobalt thiocyanate reacted with 2 molcules of diazepam forming dimer. FTIR complex also showed the appearance of band at 2061.71 cm^{-1}

Diazepam showed a significant absorption in ultraviolet region after the formation of complexes with cobalt thiocyanate reagent in acidic medium which were shiftable in the organic layer. During study, no interference was observed with pharmaceutical excipients and confirms the sensitivity and specificity of developed method.

Previously reported tests lack their propensity to produce a single convinced color for different benzodiazepines to imagine them as a definite class. An earlier method described using formaldehyde-sulfuric acid gives yellow colour with bromazepam while it gives orange color on reacting with diazepam, estazolam lorazepam, lormetazolam, nitrazepam. Similarly, method using alkaline DMSO produced different color for different benzodiazepines [10]. Another drawback of that test was its high sensitivity towards moisture. Zimmermann's reaction was also applied for the identification of benzos but the test does not give clear indication due to double shade (reddish purple color) [12-14]. whereas our described method have a unique feature to produce a single color (green) and it was found much yonder towards such limitations as reagent was prepared using deionized water.

Conclusion

A new presumptive color test for the identification of eight benzodiazepines has been developed which were tested during study. This test is rapid, easy to perform, economical and has reasonable sensitivity towards benzodiazepines. It can be used for the quantitation of benzodiazepines like diazepam using UV-visible spectroscopy. Experimental data showed that detector response showed a linear behavior with increasing analyte concentration which resulted in reliable quantitation limits.

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