

HPLC DETECTION OF SERUM AND URINARY PARAPHENYLENE DIAMINE (PPD) LEVEL AND ITS METABOLITES IN RELATION TO CARDIAC TOXICITY

Marwa Issak¹, Mona M Ali,² Sara Hamed Nasreldin Taha³, Fatma Ahmed Mahmoud⁴, Hanan Alsayed Zaghla⁵, Mahmoud Khaled Nour⁶, Samah F. Ibrahim⁷, Ahmed Samir Alsawii⁸,

^{1,3}Forensic Medicine and Clinical Toxicology, Faculty of Medicine, Cairo University, Egypt,⁷ Princess Nourah bint Abdulrahman University, Riyadh, Saudi Arabia, Saudi Arabia, ²Taif University, Saudi Arabia, ⁴Luxor International Hospital, ^{5,6,8}Critical Care Medicine Cairo University

Corresponding author: Marwa Issak: Missak1978@hotmail.com

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ABSTRACT

Hair dye poisoning is a significant emerging problem in Upper Egypt. The main component of hair dye causing toxicity is para-phenylenediamine (PPD). Ingestion of PPD could be accidental or suicidal. After oral intake, it is metabolized in the liver into N-mono-(MAPPD) and N, N'- diacetylated (DAPPD) metabolites. Tissue damage after PPD ingestions occurs due to increased free radical and oxidative stress that depletes tissue glutathione. Cardiac toxicity is a complication of PPD poisoning. It could be manifested by hypotension, different arrhythmia; besides, myocarditis and elevated cardiac biomarkers were also found. Serum and urinary levels of PPD, N-acetyl-p-phenylenediamine (MAPPD), and N--diacetyl-p-phenylenediamine (DAPPD) were measured by HPLC. A prospective cohort study was planned to determine the relationship between the serum and urinary PPD, N-acetyl-p-phenylenediamine (MAPPD) and N, on -diacetyl-p-phenylenediamine (DAPPD) levels with cardiac manifestations of the poisoned patient. Forty patients completed the diagnosis to have acute poisoning following hair dye ingestion. PPD and its metabolite concentrations did not show any significant correlation with the prevalence of cardiac toxicity and could not predict its occurrence among studied patient (p-value > .05)

Keywords: Hair dye-para-phenylenediamine-cardiac-suicidal

INTRODUCTION

Paraphenylenediamine PPD is an aromatic amine predominantly found in commercial hair dyes as well as henna. It is highly toxic as it causes severe hypersensitivity reactions. Stone hair dye (SHD) is a cheap traditional black hair dye that is widely used in Upper Egypt (Ragaa et al., 2006; Abdelraheem et al., 2014; Irfan, 2001).

Hair dyeing as well as tattoo drawing on body with Henna has been done for hundreds of years. Lawsonia inermis leaves is the plant from which black henna is extracted (El Nagdy et al., 2020)

Over many centuries, dyeing hair with henna which is derived from the leaves of Lawsonia inermis plant was traditionally used among the Egyptians. PPD is either used alone to draw black tattoo on body or

Hair dye poisoning is highly common in the Eastern world. Accidental and/or suicidal oral ingestion of commercial hair dye has been reported in India, Tunisia, Sudan, Morocco, and Egypt, especially in Upper Egypt. SHD poisoning causes higher mortality than other preparations (Sudulagunta et al., 2015; Abdel-Moeim, 2017; Kallal et al., 2005).

PPD is absorbed into the blood after oral intake into through mucosa of the

gastrointestinal tract; then, it becomes metabolized in the liver into N-mono-(MAPPD) and N, N'- diacetylated (DAPPD) metabolites (Nohenek et al., 2015). Tissue damage after PPD ingestions occurs due to increased free radical and oxidative stress that depletes tissue's glutathione (Sirinivas et al., 2010). PPD poisoning has three phases. Phase (1) starts 4-6 hours post-ingestion. It shows burning sensation in the throat and mouth, numbness, intense edema, and persistent vomiting with dehydration (Sudulagunta, 2015; Yagi et al., 1991; Abdulla & Davidson, 1996). Phase (2) starts 12 hours post-ingestion. It manifested mainly by acute tubular necrosis and renal failure (Sudulagunta et al., 2015). In Phase (3), the patient's clinical condition progresses to multiple organ system failures, and the patient dies (Abdulla & Davidson, 1996). Cardiac toxicity is a complication of PPD poisoning. It could be manifested by hypotension, different arrhythmia (Dudani et al., 2018). Also, myocarditis and elevated cardiac biomarkers were found (Melli et al., 2005). Oral ingestion of 10 mg PPD can result in extensive myocardial damage with hypotension and lethal life-threatening arrhythmias. Immediate induction of an appropriate and fast, supportive treatment, including lifesaving tracheostomy, anti-allergic therapy, and forced diuresis, could decrease PPD morbidity and mortality indices. This is because antidote is not more available (Hooff et al., 2014).

The current study aimed to identify the cardiac toxicity of the orally ingested stone hair dye and its relationship to urinary levels of PPD and its metabolites.

PATIENTS & METHODS

This prospective cohort single-center study was conducted in Luxor International Hospital from January 2016 to July 2017. Acute SHD poisoning was suspected if a patient has a history of hair dye ingestion. The colored dye was retrieved from the gastric wash, and

cervicofacial edema and black urine due to myoglobinuria were present. Cases with a history of mixed poisoning as well as patients with chronic diseases, e.g., myocardial infarction, hypertension were excluded from the study.

On admission to the emergency department, a detailed medical examination is performed, followed by urine and blood sample collection, after those required investigations including creatine kinase-MB (CK-MB), creatine kinase (KC), and lactate dehydrogenase (LDH) and urinary level of PPD and its metabolites.

ECGs with baseline 12-lead were recorded with a paper speed of 25mm/s from each patient. Cardiac toxicity induced by PPD was diagnosed through ECG changes and/or increased cardiac biomarkers (Halliday, 1994).

Reagents and materials

Kinase (CK), measured by a spectrophotometric assay using LDH Assay Kit (Colorimetric) and Creatine Assay Kit (Colorimetric/Fluorometric) (ab102526) provided by abcam, Inc. Their ranges were between 0.001- 10 mm, and 1-100 mU/ml, respectively.

Creatinine kinase-MB (CK-MB) was measured by ELISA technique provided by OxisResearch™. Levels of > 9 ng/ml indicated myocardial damage.

Urinary P-phenylenediamine and the detection of its metabolites

Reagent: P-phenylenediamine (PPD) (97%), 4-amino acetanilide (MAPPD) (98%), and N,N-P-phenylenebisacetamide (DAPPD) (98+%) were purchased from Alpha Aesar, Thermofisher, Germany. Internal standard aniline (for synthesis) was purchased from the research lab. Purified water (HPLC grade) was obtained using a direct-Q gradient 8 UV system (Millipore). Dichloromethane, Acetonitril were purchased from Carlo Erba group, Inc. (HPLC grade). Hydrochloric acid, Ammonium solution 25% were from ADWIC. Potassium dihydrogen phosphate 99% was purchased

from Alpha Aesar, Thermofisher, Germany. Sodium hydroxide 97% was purchased from Alpha Aesar, Thermofisher, Germany.

Instrumentation: HPLC (high-performance liquid chromatography) using an isocratic system formed of a DionexUltiMate 3000 UHPLC; RS pump, auto-sampler, column compartment, and diode array detector. Chromatographic column reversed phase 150 mm× 4.6 mm Hypersil BDS, C18 particle size 5 μ kept at 25°C. The mobile phase was formed of acetonitrile: buffer (0.01M) potassium dihydrogen phosphate in a ratio of (20:80, v/v), pH was adjusted to 5.0 with 0.1 M sodium hydroxide. The flow rate is 1.0 mL/min. The UV detector was set to scan from 200 to 800 nm and had a discrete channel set at 240 nm (**Mohamed et al., 2014**). Retention time (Rt) for PPD, MAPPD, DAPPD was 3.96, 5.5, 8.12 min, respectively, and for the aniline (IS) 9.17 min. Data were analyzed with the Chromeleon 7 software.

Calibration procedure

Various Calibration parameters are:

1. Flow rate accuracy→
2. Injector accuracy→
3. System Precision→
4. Wavelength accuracy→
5. Detector linearity→
6. Injector linearity→
7. Gradient Performance Check→

Flow Rate Accuracy:

a) All the solvent lines were primed with Milli Q water.

b) The flow rate was set to 0.500 ml/m.

c) Outlet tubing was inserted into a 10 ml volumetric flask.

d) Stop the stopwatch when the lower meniscus reaches the 10 ml mark on the flask.

e) Record the elapsed time.

f) Time taken to collect the water was 2% of the actual value.

Injector Accuracy:

a) The pump and detector related to the union.

b) the mobile phase was prepared to consist of a mixture of water and Methanol (70:30 v/v)

c) flow inlet rate was set of 0.5 ml/m and a run time of 1 m.

d) the column temperature was set at 25± 2°C.

e) The standard HPLC vial was filled to 2/3rd with Milli-Q water. The vial was appropriately sealed with a cap.

f) The vial was weighed, and the weight was recorded as W1 grams.

g) the vial was placed in the chromatographic system and performed

h) 50 μ l volume was injected from this vial.

i) The vial was weighed again and note the weigh after the injections as W2 grams.

The mean injected volume was 50 μ l

Wavelength Accuracy:

Procedure: Create and instrument method with a wavelength in nm and inject blank, followed by Standard preparation and note down the height or absorbance. The maximum absorbance should be ±2nm.

PDA Detector Accuracy: 3D mode was selected and set the wavelength range as 200-400nm. 20 μ l of standard preparation was injected once into the chromatographic system.

Column Oven Temperature Accuracy:

It was evaluated with a calibrated digital thermometer at 30°C and 60°C. the thermometer probe was placed in the column oven and set the column oven temperature at 30°C. after the temperature stabilized, the temperature displayed was recorded on the thermometer. Acceptance criteria: The resulting oven temperature from the thermometer display was within ±2°C of the set temperature.

Preparation of standard and quality control samples: the stock solutions were prepared using 1000 micrograms of analytical standard added to 10ml water. The preparation of a working standard solution was done by diluting the stock solution with water daily. Blank Serum

samples and blank urine were spiked to get standard calibration solutions in the range of 0.015–2 µg/mL. Quality control (QC) samples of 0.05 µg/mL (low QC), 0.25 µg/mL (medium QC), and 1 µg/mL (high QC) were also prepared. 1ml of solution contains 1mg of Paraphenylenediamine, monoacetyl PPD, diacetyl PPD. The internal standard used was aniline, and it was dissolved in water.

Injector Linearity: Standard Preparation: Accurately weigh and transfer about 60mg of the standard into a 100ml volumetric flask. Dissolve and dilute to the volume with the mobile phase. Transfer 10ml of Standard Preparation into a 100ml volumetric flask and dilute to the volume with the mobile phase

Column Oven Temperature Accuracy: It is evaluated with a calibrated digital thermometer at 30°C and 60°C. Place the thermometer probe in the column oven and set the column oven temperature at 30°C. Wait till the temperature stabilizes. Record the temperature displayed on the thermometer. Similarly, it performs the column oven temperature accuracy test at 60°C. Acceptance criteria: The resulting oven temperature from the thermometer display should be within $\pm 2^\circ\text{C}$ of the set temperature.

Sample Pretreatment: urine samples were prepared by adding 0.5 ml of urine hydrolyzed using 0.5 ml, 12N HCl for one h at 100°C, and alkalinized with conc. Ammonium hydroxide (Mohamed et al., 2014).

Sample preparation (liquid-liquid extraction): Liquid/liquid extraction (LLE) was done by adding 0.25 mL hydrolyzed urine and 1.0 mL blood to 100 µL of 100 µg/mL aniline (IS) to each of the blood and urine samples. After that, 0.25mL of conc. Ammonium hydroxide (33%) and 6.0 mL of dichloromethane were added. The tubes were then vortexed at the rate of 40 rpm for 20-minutes, then they were centrifuged at 3200 rpm for 5-minutes. The organic layer was removed and transferred to a second 10-mL polypropylene tube containing 0.5 mL of 1.0 M hydrochloric acid. The tubes were then vortex mixed for 5-minutes and centrifuged at 3200 rpm for 5-minutes. The organic layer was removed to the remaining aqueous solution, 150 µL of conc. Ammonium hydroxide and 2.0 mL of dichloromethane were added. The tubes were then centrifuged at 3200 rpm for 5-minutes. The organic layer was transferred to 5-mL glass tubes and evaporated to dryness. The dried extracts were reconstituted in the mobile phase, vortex mixed for 30-seconds and then injected into the HPLC system with an injection volume of 100µ. The flow rate is 1.0 mL/min. The UV detector was set to scan from 200 to 800 nm and had a discrete channel set at 240 nm (Mohamed et al., 2014). Retention time (Rt) for PPD, MAPPD, DAPPD was 3.96, 5.5, 8.12 min, respectively, and for the aniline (IS) 9.17 min. Data were analyzed with the Chromeleon 7 software. (Mohamed et al., 2014).

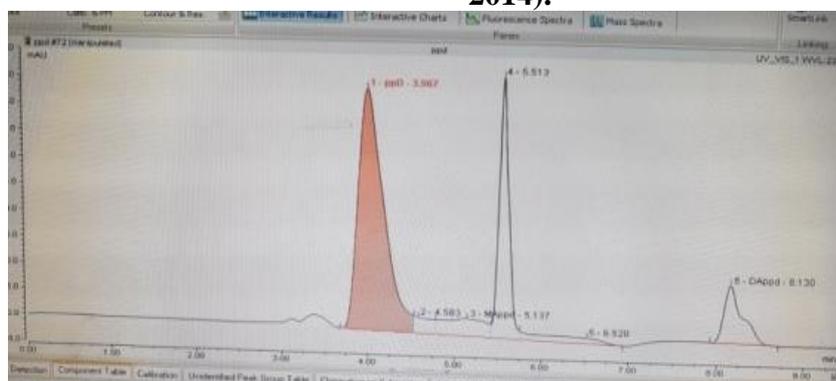


Figure (1): chromatogram of spiked urine sample containing 87.7Ug/ml of ppd,mappd,dappd as an external standard, concentrations 10ul.

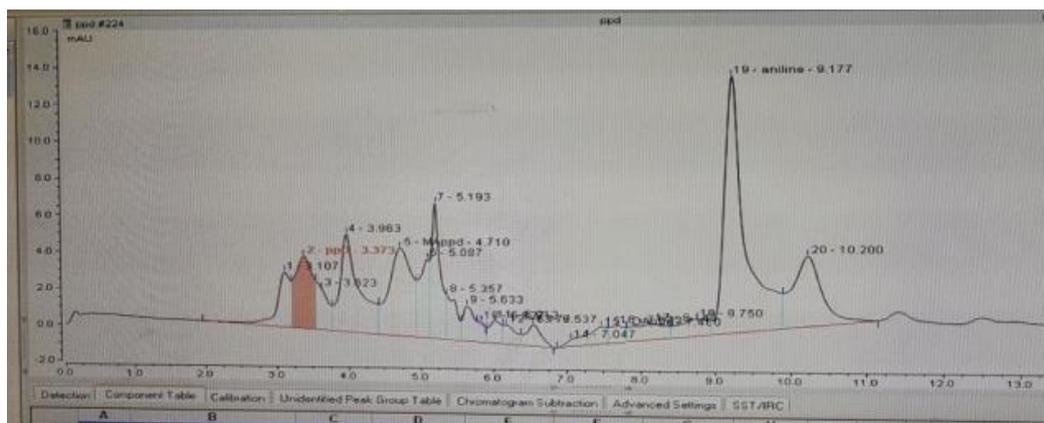


Figure (2): chromatogram of toxic urine case containin0.8939 ug/ml of PPD, 18.6315ug/ml,MAPPD,27.65ug/ml DAPPD and 20ul ANLINE as internal standard.

The validation of the analytical method was as follow:

Linearity: The presented analytical procedure proved to be linear (squared correlation coefficient $r^2 = 0.987$, $n=5$ for ppd, $r^2=0.993$ $n=5$ for MAPPD and $r^2=0.998$ $n=5$ for DAPPD).

Quality control samples: Blank samples with a series of concentrations 0.03,1, 10, 50,100,200,30,1000 ug/ml) standard PPD, MAPPD, DAPPD were prepared to investigate the linearity, LOD, and LOQ. Range 0.03-1000ug/mL. **Limit of detection (LOD) and limit of quantification (LOQ):** The LOD is the lowest amount of the analyte that can be detected. $LOD=3.3 \times St$ deviations/slope; $LOD = 0.03135$ ug/ml. **LOQ** is the lowest concentration of the analyte that can be detected. $LOQ=10 \times St$ deviation/slope $LOQ=0.095$ ug/ml

Accuracy (extraction recovery): The recovery was evaluated with three replicates of the QC sample, formed of 1ml blank added to an external standard (with three different concentrations) and 10ul of internal standard anline. Concentrations of external standard were 0.03ug/mL, 50ug/mL and 1000.ug/mL (low, medium, and high concentrations). The extraction

recoveries were calculated as the percentage of $A_{E.S}/A_{I.S}$ (ratio of the area between external and internal standard) value extracted from samples over those obtained by direct determination of the standard solutions (and internal standard) at the same concentration level. The recovery of PPD, MAPPD, and DAPPD for all the 3 QC levels were more than 80%.

Precision (relative standard deviation): for each sample, three injections were done, and the mean value was calculated.

The relative standard deviation of High (497.37ug), medium (138.32ug), and low (0.066.ug) concentrations were 1.16%, 1.23%, and 5.11%.

Statistical methods

Data were analyzed using SPSS© Statistics version 23 (IBM© Corp., Armonk, NY, USA) and MedCalc© version 15.8 (MedCalc© Software bvba, Ostend, Belgium). Qualitative data were expressed as count (%) and compared in different groups using the chi-square test, while quantitative data was tested using the student t-test. Correlations were tested using the Spearman rank correlation. P-value ≤ 0.05 was considered significant.

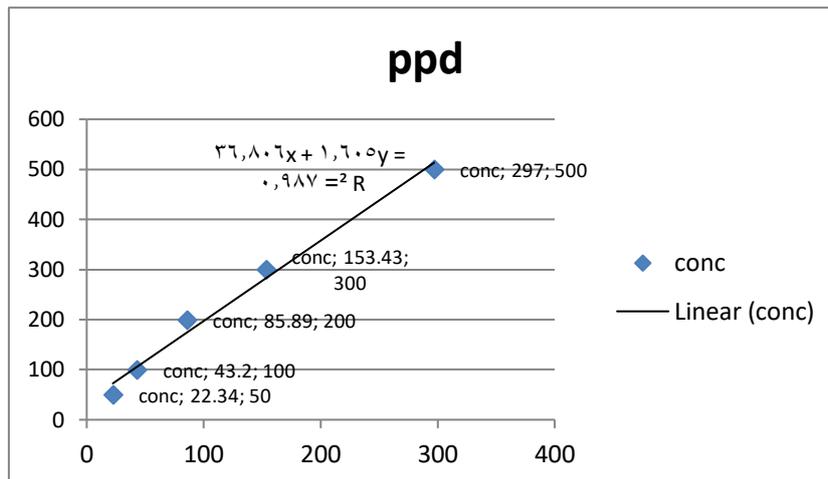


Figure (3): linear regression of the calibration curve of four concentrations of PPD

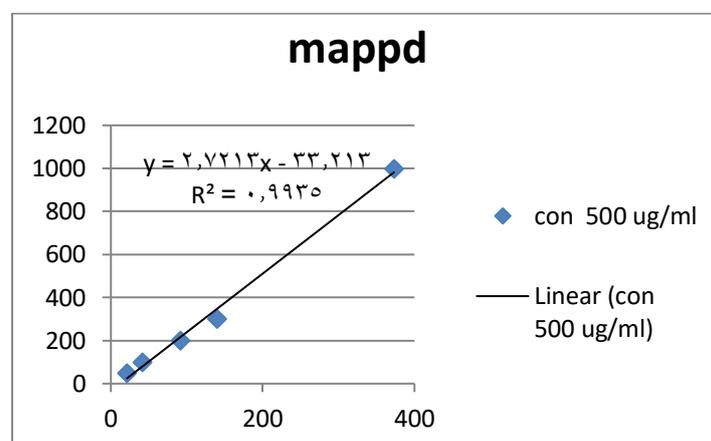


Figure (4): linear regression of the calibration curve of five concentrations of MAPPD.

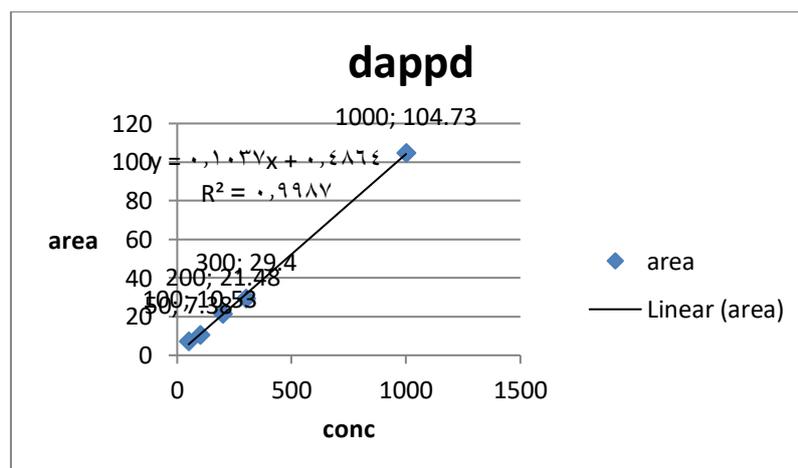


Figure (5): linear regression of the calibration curve of five concentrations of DAPPD.

RESULTS

Forty patients from the Upper Egypt region were included in the study. 75% of them were females with a mean age of 25 years. Most of the cases were suicidal poisoning (72.5%).

On admission, the mean values of heart rate, systolic and diastolic blood pressure were 129beat/min, 122 mmHg, and 72 mmHg, respectively.

Hypotension was recorded in 4 patients (10%), and ECG showed different patterns of arrhythmia in 24 patients; 7.5% of them had sinus tachycardia, 22.5% developed nonspecific ischemic changes in the form of diffuse bundle branch block, ST-segment elevation or depression, and T wave inversion, 15% had atrial and ventricular premature complexes, and 15 % developed ventricular tachycardia.

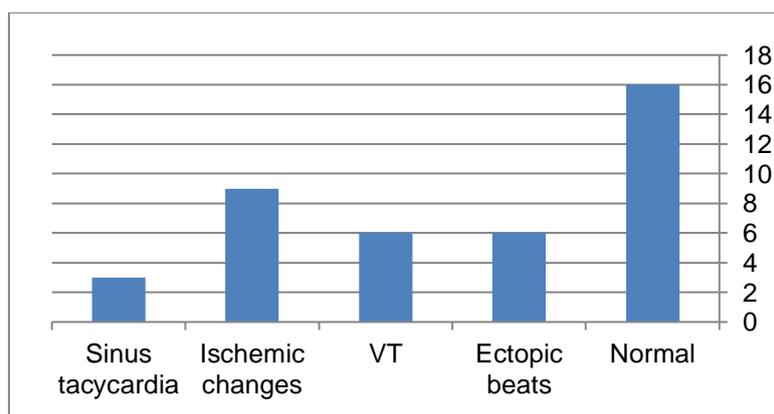


Figure (6): ECG changes on admission for studied patients

On admission, LDH and CK levels were increased with mean values 5035 and 2224U/L, respectively. CK-MB was increased in 27 patients (67.5%) with a mean value of 768 U/L. On admission, urinary PPD and its main metabolites, MAPPD and DAPPD, were detected in 38 patients (95%), where their mean values

were 231.8, 736.7, and 11776.8 μ g/ml, respectively. Forty-eight hours after treatment, urinary PPD, MAPPD, and DAPPD mean values were decreased to reach 22.8, 38.2, and 1586.8 μ g/ml, respectively.

Table (1): levels of LDH, CK, CK-MB, PPD MAPPD, and DAPPD levels on admission and 48 hours after treatment

	Mean	SD	Minimum	Maximum
LDH	5035	5990	305	21000
CK	2224	2633	40	10000
	1882	1503	1000	5178
CK-MB	768 in 29 cases	801	10	3000
	338 in 10 cases	311	31	950
PPD	231.8	468.8	0.1	2570.0
	22.8	88.6	0.1	459.0
MAPPD	736.7	1962.0	-	11700.0
	38.2	79.5	0.4	301.6
DAPPD	11776.8	22628.0	-	86135.5
	1586.8	6975.3	-	36913.0

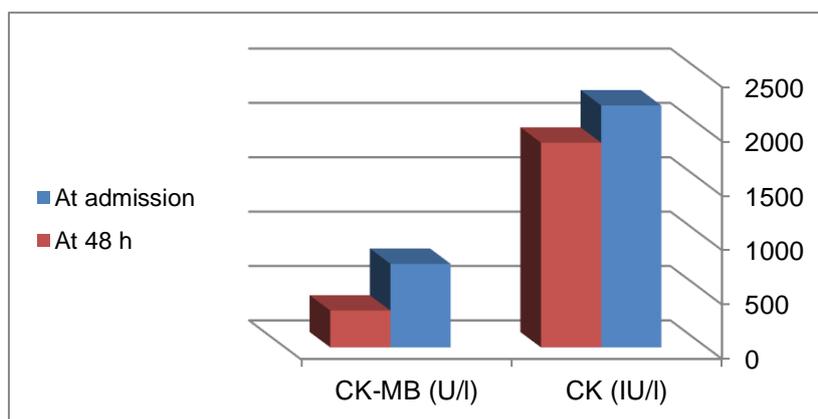


Figure (7): The mean level of CK and CK-MB at different times.

According to ECG changes and CK-MB, 27 patients had been diagnosed with PPD induced cardiac toxicity. Twenty-six patients showed multi-organ affection in variable range, where 5 cases (19.2%) have three organs affected, 7 cases (26.9%) have four organs affected, 9 cases (34.6%) have five organs affected, and 4 cases (15.4%) have 6 organs affected. Unfortunately, urinary PPD and its metabolite concentrations did not show any significant correlation with the prevalence of cardiac toxicity and could not predict its occurrence among studied patient (p -value > .05)

DISCUSSION

The total number of cases enrolled in this study was 40 out of the 30 (75%) were females, and 10 (25%) were males. This was nearly the same number of patients studied by **Mohamed KM et al. (2014)** and **Abdel-Maaboud et al. (2008)** they reported number of females in cases of poisoning in Upper Egypt was higher than that of males. This occurred due to the frequent use of cosmetics by females and the cheapness and availability of the stone dye. The studies of **AyoubFilaliet al. (2006)**, and **Ramulu et al. (2016)** showed a female preponderance with 77%, 94.7%, 89%, 93.3%. and 80.64% respectively.

In the present study, most patients were age 18- 28 years old (mean age was 25 ± 11). Many researchers agreed with our results in their studied patients as **Ramulu P et al. (2016)** and **AyoubFilaliet al.**

(2006), And **Qasim AP et al.** reported that the prevalence of self-harm by using PPD was more in the age range of 11–30 years.

Committing suicide occurs mainly due to psychological problems as well as social problems like failure in the exams. Concerning the manner of death, the intent of ingestion in our study was suicidal in 72.5%, accidental 10%, and suspected PPD poisoning in 17.5%. All cases ingested hair dye by the oral route, and all of them consumed unbranded stone hair dye. Regarding the reason of poisoning, suicidal intention was identified in the studies carried by **Mohamed KM et al. (2014)**, **Nisar Khan et al. (2015)**, **Rawat R et al. (2016)** **Jain et al. (2011)**, **Abdel-Moneim A (2017)** and **Kakkar et al. (2016)** to present 91.7%, 73.7% , 86.7%, 94.74%, 100%, 97.84%, 91.7% and 95.7% respectively. **Rawat R et al. (2016)** reported that the psychological evaluation was normal in all these patients. This indicates that most suicidal attempts were impulsively precipitated by either scolding from parents, family quarrels, or socio-economic failure.

Hair dye is a potent cardiotoxin, and its ingestion leads to myocardial damage. Owing to myocardial damage, there is a release of cardiac-specific markers such as Trop T, Trop I, and CPK-MB. In this study, 60% of patients had ECG changes, 75% of patients show elevated serum level of CPK-MB level up to 3000 U/l. Our results were in accordance with the results of **Rawat R et al. (2016)**, and **Ramulu P**

et al. (2016) concluded that cardiac arrhythmias were documented in 40%, 11.53%, and 38%, respectively.

The cause of myocardial damage might state by **Senthilkumaran et al.**, who noted that the incidence was bound to patients' susceptibility to PPD, apart from the amount of dye ingested. However, oral ingestion of PPD in doses >10 g caused myocardial infarction, especially with the consumption of unbranded stone hair dyes. In our study, we reported one case of myocardial infarction, which also recorded by **Brahmi et al. (2006)** and **Jatav et al. (2008)** reported a case of myocarditis with myocardial infarction caused by consumption of PPD where they confirmed it by angiography, that showed septo-apical hypokinesia due to spasm of the left anterior descending coronary artery.

About 10% of our studied patients developed shock due to myocarditis, which was a fatal complication. Vasopressors and inotropes are needed in 10% and 7.5%, respectively. **Kallel et al. (2005)** recorded that shock occurred in 26.3% of patients, which necessitated vasopressors. It is explained by myocardial rhabdomyolysis and hypovolemia or result differed from that recorded by **Kallel et al. (2005)** that might due to his study was retrospective and only on 19 patients.

In our study, the mean urinary level of PPD, MAPPD, and DAPPD showed a gradual decrease level until 48 hours from admission. We recorded that the major metabolite excreted via the urine was diacetyl-PPD which, in accordance with **Mohamed KM et al.**, urine samples were hydrolyzed using hydrochloric acid before extraction due to the presence of N, N-diacetyl-PPD (the main metabolite) which is present in high concentration levels in urine samples. The urine of five volunteers who had used a commercial hair dye preparation containing 1.1–1.6 g of PPD was examined. The major metabolite detected by this technique is N, N-diacetyl-PPD. The diacetyl derivative of the PPD was found to be excreted in the urine for

42h after dyeing, the average amount being 0.14 mg/ml per person.

Hueber-Becker et al. reported that the total excretion was $0.54\% \pm 0.25\%$ of the applied dose. The majority (86%) of absorbed radioactivity was excreted within 24h after application. **Nohynek et al** and **Goetz et al.** revealed that the majority of 80-95% of the PPD metabolites excreted via the urine consisted of monoacetyl-PPD and diacetyl-PPD.

In contrast to our study, we agreed that the major metabolite excreted via the urine was diacetyl-PPD. And we agreed with SCCP that after 48 hours, most PPD and its metabolites were eliminated. We disagreed with them in amounts of excreted PPD and its metabolites as their studies all after topical hair dye application. After PPD ingestion, the primary metabolism will be more influenced by NAT2, which is in the guts and liver. We couldn't determine the percentage of the PPD, and its metabolites excreted via the urine, as there was no definite data about the amount ingested.

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الكشف عن مادة بارافينيلين ديامين (صبغة الشعر) ومشتقاته في الدم و البول باستخدام تقنية اتش بي السى و علاقتها بالاعراض السمية على القلب

مروة اسحق¹, منى محمد على², ساره حامد نصر الدين طه³, فاطمه احمد³, حنان السيد زغله⁴, محمود خالد نور⁵, سماح فنحى ابراهيم⁶, احمد سمير الصارى⁷
^{1,3} قسم الطب الشرعى و السموم الاكلينيكيه, كلية الطب, جامعة الفاهره مصر, ² جامعة الاميره نورا الرياض المملكة العربيه السعوديه, ⁷ جامعة الطائف المملكة العربيه السعوديه, ⁴ مستشفى الاقصر العام, ^{5,6,8} قسم طب الحالات الحرجه جامعة القاهره

يعتبر التسمم عن طريق تناول صبغة الشعر من اكثر اسباب التسمم في صعيد مصر. و يعد المكون الاساسى لصبغة الشعر هى مادة البارافينيلين ديامين. ان تلك المادة يحدث اما عن طريق الخطأ او كوسيله للانتحار خاصة فى الاناث. بعد تناول تلك ماده فأنها تدخل الى الكبد و تتحول الى مشتقات مادة البارافينيلين ديامين و تبدأ فى تدمير خلايا الجسم محدثة عدة اضرار منها فى الكبد و الرئه و الكلى و التأثير على القلب والجهاز الدورى. ينتج عن سمية هذه المادة على القلب حدوث هبوط شديد فى ضغط الدم, انواع مختلفه من اضطرابات ضربات القلب, كما يحدث ايضا التهاب فى عضلة القلب و قد يودى الى رفع نسبة انزيمات القلب فى الدم. فى هذه الدراسه تم قياس نسبة البارافينيلين ديامين و مشتقاته فى الدم و البول و ايجاد علاقه بينها وبين تغيرات رسم القلب, نسبة انزيمات القلب فى الدم. وقد تبين من هذه الدراسه ان تناول مادة البارافينيلين ديامين تودى الى مضاعفات فى القلب و الجهاز الدورى ولكن لا توجد علاقه مباشره بين نسبة البارافينيلين ديامين و مشتقاته فى الدم و البول و بين مضاعفات القلب والجهاز الدورى.