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Research article

The concentration of three anti-seizure medications in hair: the effects of hair color, controlling for dose and age

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Abstract

Background: This paper assess the relationship between the quantity of three anti-seizure medications in hair and the color of the analyzed hair, while controlling for the effects of dose, dose duration, and patient age for 140 clinical patients undergoing anti-seizure therapy. Three drugs are assessed: carbamazepine (40 patients), valproic acid (40 patients), and phenytoin (60 patients). The relationship between hair assay results, hair color, dose, dose duration, and age is modeled using an analysis of covariance. The covariance model posits the hair assay results as the dependent variable, the hair color as the qualitative categorical independent variable, and dose, dose duration, and age as covariates. The null hypothesis assessed is that there is a no relationship between hair color and the quantity of analyte determined by hair assay such that darker colored hair will demonstrate higher concentrations of analyte than lighter colored hair.

Results: The analysis reveals that there is a significant relationship between dose and concentration for all hair color categories independent of the other covariates or the categorical independent variable.

Conclusion: There does not appear to be any relationship between carbamazepine concentration and hair color. There is a weak relationship between hair color and valproic acid concentration, which the data suggest may be mediated by age. There is a significant, moderate relationship between phenytoin concentration and hair color such that darker colored hair has greater concentration values than lighter colored hair.

Background

The use of hair assays in order to assess the presence of ingested chemical materials has been used in a wide variety of applications [1,2]. Hair has been utilized, for example, in determining the degree of exposure to heavy metals in both *post mortem* and living subjects [3]. In recent years there has been considerable focus on the recovery of illicit psychoactive substances and their metabolites from hair [4,5,6]. Others have reported on a wide variety of substances that can be recovered from

human hair samples and can be quantitatively determined. These include nicotine and cotinine [7], caffeine [8], antipsychotics and anti-anxiety medications [9,10,11,12], various antibiotics [13,14,15,16], pesticides [17], digoxin [18], and steroids [19,20]. There are several advantages to hair as a sample for analysis, but the single most important attribute is undoubtedly the long retrospective time frame to which analysis of a segment of hair corresponds. This permits an evaluation of a history of exposure that is not possible with specimens such as saliva or urine. A three-centimeter length of hair corresponds, on average, to approximately 90 days of past time or "history". For compounds that are stable when sequestered in hair this represents a very desirable property.

This enhanced temporal capacity lends itself to the possibility that hair analysis can be utilized to evaluate individual compliance for substances that are taken on a regular basis, such as anti-clotting medications, antibiotics, and anti-epileptic drugs. This can be an important diagnostic and assessment aid in medical practice and clinical evaluation of drug efficacy. Williams, for example, has advocated the use of hair analysis as a method for assessing therapeutic compliance to anti-seizure drug regimens [21]. It has been well demonstrated that the major antiepileptic medications, phenytoin (PHT), carbamazepine (CBZ), and valproic acid (VPA) are readily recovered and quantified from hair specimens [[22–29]]. Thus, assessing the presence and quantity of these substances in hair may be a reliable marker for consumption, and provide an additional source of information to the physician who has historically relied only on the patient's self-reported compliance.

Whether hair analysis can be an effective indication of patient compliance is controversial, however. It is widely accepted that the qualitative presence of these compounds can be reliably demonstrated by hair analysis. The controversial aspect is primarily based on the issue of whether the *quantitative* value of the assay can be a reliable indicator of dose. That is, is there a reliable dose/assay correlation? Tracqui et al. [30], for example, have argued that hair analysis has value for qualitative evaluation of drug use, but is "worthless" for quantitative assessment of patient compliance. Williams (1999), however, argues the opposite, and presents data for a controlled inpatient population taking CBZ which shows remarkably good consistency between dose and assay outcome. Furthermore, Williams suggests that compliance is best measured by using patients as their own control. This is done by comparing any given assay outcome to a ground state value. So hair will show a relative change in concentration. It is generally accepted that specific quantitative values related to a specific dose can not applied across study subjects due to substantive individual biovariability.

One issue that is not clearly understood is the variety of factors influencing the considerable biovariability that appears between persons given identical dosages of these drugs. Clinicians general recognize that a very large number of factors can influence the drug concentration in any analytic matrix. One potential advantage of hair specimens is they may represent a relatively stable matrix over a relatively long time frame. Tracqui, even in rejecting the utility of quantitative compliance monitoring in this context, notes that a number of studies reported significant strong correlation between dose and assay outcomes for a number of different compounds, with Pearson's r ranging from .772 to .868. Another aspect of this interpretation controversy concerns whether there are systematic influences on drug concentration in hair because of color variations, differences in cosmetic treatment, and different intensities of hair hygiene. Another part of the controversy is problems related to the dependency on the accuracy self-reported patient compliance (which may or may not be accurate), and - in the case of illicit drugs - the purity and actual contents of drugs reported as consumed. The attending physicians for these patients were queried on the confidence they had on their patient's compliance, and in general they rated the compliance as good, with only a small number of missed doses. However, we recognize that there are reliable data to indicate that patient compliance to anti-seizure medication regimens may be weak, and a delimitation of this research is that there was not rigid control of patient compliance.

One of the general weaknesses characterizing much of the analysis of hair assay data has been the inadequacy of the statistical models used to assess the experimental information. Oftentimes claims or conclusions are derived from experimental data that is not supported by statistical analysis. In some cases, in fact, analyses of the data show that the conclusions reached are clearly incorrect [31]. There has been, in general, a failure in the assessment of experimental hair analysis data to use appropriate available statistical procedures, or to assess whether the critical assumptions that underlay statistical procedures are met by the data. And almost without exception, there has been no utilization of more sophisticated and sensitive statistical techniques that are available to examine multivariate phenomena. This is true even though there is a general recognition that the relationship between dose and assay are likely to mediated by a complex series of intervening events and are particularly appropriate to this analytic approach.

Materials and Methods

The analysis is based upon the concentrations obtained from scalp hair collected by cutting hair from the posterior vertex of the head. Each sample consisted of approximately 200 mg of hair. Each sample was sectioned into 2-cm segments and each segment was analyzed by FPIA and HPLC methods for CBZ and PHT and by FPIA for VPA. The number of segments for each patient varied, since it was dependent of length of hair available from each individual. Most patients have five segments and no patient provided less than two segments. The concentrations used for each case are the mean values for all available segments for that patient. HPLC values are used in the analysis of CBZ and PHT, FPIA values are used in the analysis of VPA. As well, serum assay values are provided for each patient. The serum assay typology varied, including multiple methods to assay serum for some drugs. For CBZ serum was assayed by both FPIA and GC/MS, for VPA by FPIA, and for PHT by FPIA and HPLC. Bloods were drawn from patients in the morning by their attending physician. In a few cases there were multiple bloods drawn. In such cases the mean values for the serum levels are represented in the tables. Hair color assignments were based upon observation by the attending physician, questioning of the patient and in cases of ambiguity by consultation among the field research staff, the attending physician, and the patient.

Since this is a secondary analysis of data, only a synopsis of technical procedures is presented here. Details of the instrumentation, including calibration and standardization, and elaboration of the analysis techniques are described in detail elsewhere [28] and are not repeated here. The general approach was as follows: hair samples were dissolved in a strong base (NaOH) and the dissolved material was subjected to solvent extraction. Hair samples were segmented in 2-cm sections, and 30-50 mg of hair from each segment was added to 3 ml of 1 N NaOH solution. The solution was solvent extracted with diethyl ether, centrifuged, and the supernatant evaporated. The remnant residue was dissolved in saline and subjected to analysis.

Result and Discussion

In this study we report on the relation between the concentration of three widely used anti-epileptic medications, CBZ, PHT, and VPA, in hair and the correlation between dose and concentration. In addition, we explore the relationship between concentration and hair color, between assay variability and age, and the interaction effects between these variables. We also assess the degree to which the data meet critical assumptions regarding the normality, distribution shape, and variance homogeneity. The statistical method employed is an analysis of covariance, in which each drug employs a model incorporating the concentration of the target drug in hair as the dependent variable, the hair color categorization as the independent variable, and dosage, duration of dose, and age as model covariates.

Carbamazepine (CBZ)

The data set consists of 40 subjects (18 females, 22 males) who were on a regimen of CBZ for the control of seizures. The mean of age of this group is 30.3 years, s.d. 18.6 (median 25.0). Age skewness (.651) and kurtosis (-.506) are within normal bounds. The mean length of time (duration) of the dose regimen for this group was 16.95 months (s.d. 8.59) with a range of from 2 to 24 months. The dosage values (in mg/day) ranged from 200 to 1,000 with a mean dose of 525 mg/day (s.d. 208.47, median 450). For both duration of regimen and daily dosage the skewness and kurtosis values are within normal range (<2).

Table 1: Mean Concentration Values and Correlation Coefficients for Dose/Assay Relationship for Hair and Plasma, CBZ

Specimen	CBZ Dose (mg/day)	HPLC Hair Concentration (mcg/g)	S.D.	Correlation Coefficient (r)	FPIA Concentration (mcg/g)	S.D.	Correlation Coefficient (r)
Hair	525	18.20	6.02	0.476**	21.12	7.01	0.459**
Specimen	**CBZ Dose (mg/day)**	**GC Serum Concentration (mcg/g)**	**S.D.**	**Correlation Coefficient (r)**	**FPIA Serum Concentration (mcg/g)**	**S.D.**	**Correlation Coefficient (r)**
Serum	525	7.37	1.44	.067	7.62	1.50	.143

** significant @.01, two-tailed

Table 2: Mean Concentration of CBZ by Hair Color

Hair Color	Number of Subjects	Mean CBZ Concentration	Standard Deviation
Black	15	17.59	5.95
Brown	15	18.05	5.74
Blond	3	24.23	6.22
Gray	3	13.58	3.27
Dyed	4	19.97	7.39
Overall Mean	40	18.20	6.02

General Dose/Assay Relationship

The data for the dose assay relationship includes concentration in the plasma serum, which was determined by both FPIA and GC, and concentration in the hair, which was determined by both FPIA and HPLC. For hair assays the values are in micrograms per gram of hair, and for serum the values are in micrograms per milliliter of plasma. The findings show that there was no significant dose/assay relationship for the serum specimens with either analytic technique, and that there was a significant dose/assay relationship for hair specimens by both analytic techniques. The results are shown in Table 1.

Hair Color

It has been a source of controversy in the interpretation of hair assays whether or not hair color is a critical factor in the binding of materials in hair. It has previously reported that CBZ does not appear to have a significant "color effect" (i.e., that darker hair shows significantly higher concentrations of CBZ than light hair) [31]. We examine this finding using the current data. There are five color categorizations of hair represented among the 40 subjects. The distribution of hair color and the mean concentration of CBZ for each color are shown in Table 2. The concentration values are in micrograms/gram as measured by HPLC.

An analysis of variance for this table fails to show a significant relationship between CBZ mean value and hair color ($F = 1.374$, $p = .263$). The Levene Test for equality of error variances indicates that the homogeneity of variance assumption is met (Levene $F = .303$, $p = .874$). The K-S and Shapiro-Wilk tests for normality are consistent with a normal distribution and a normal Q-Q plot of CBZ concentration in hair shows a good approximation of normality.

Hair Color/Dose Relationship

Another factor worth consideration is the possible interaction effect between hair color and dose. The dose values and hair color categories can be a shown as a matrix array as in Table 3.

Table 3 is a crosstabulation showing the distribution of dosage values by hair color. Review of the cell distributions shows a concentration of higher dose regimens to cluster towards the lower left, indicating the possibility that persons with black hair may more likely to be on higher doses of CBZ and persons with lighter hair may be more likely to receive lower doses. The GLM covariance model is based on the mean hair assay outcomes (HPLC). The analysis of covariance (ANCOVA) allows for the comparison of quantitative dependent and qualitative independent variable while simultaneously controlling for a number of quantitative variables or covariates. In this analysis, the dependent variable is the mean concentration for the hair analysis, the independent qualitative variable is the hair color categorization, and the covariates are duration of dose (in months), dose (in mgs. per 24 hours), and patient age (in years). The outcome of this model is shown in Table 4.

Table 4 reveals that there is a relation between mean hair concentration and dose ($F = 5.020$, $p = .032$, $eta^2 = .136$, shaded row) while the relationship between hair assay result and color is not significant ($F = .682$, $p = .609$, $eta^2 = .079$). Thus it is reasonable to conclude that differential hair assay outcomes for CBZ are not color dependent, but are significantly related to dose regimens.

Table 3: Frequency Distribution of CBZ Dosage by Hair Color

CBZ Dose (mg/day)	Hair Color					
	Black	Brown	Blond	Gray	Dyed	Total
200		2			1	3
300	1			1		2
400	5	9		1		15
500		1		1		2
600	7	1	2		1	11
800	1	2	1			4
1000	1				2	3
Total	15	15	3	3	4	40

Table 4: General Linear Model, Analysis of Covariance, Hair Assay Outcome by Color, Dose, Dose Duration, and Age, CBZ

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Eta^2
Corrected Model	428.88	7	61.27	1.995	.087	.304
Intercept	346.37	1	346.37	11.281	.002	.261
CBZDOSE	154.13	1	346.37	11.281	.002	.136
DOSEDUR	2.89	1	2.89	.094	.761	.003
AGE	.636	1	.636	.021	.886	.001
COLOR	83.78	4	20.95	.682	.609	.079
Error	982.57	32	30.71			
Total	14658.77	40				
Corrected Total	1411.45	39				

Table 5: Mean Concentration Values and Correlation Coefficients for Dose/Assay Relationship for Hair and Plasma, VPA

Specimen	VPA Mean Dose (mg/day)	FPIA Mean Concentration (mcg/g)	S.D.	Correlation Coefficient (r)
Hair	822.5	8.22	3.37	.717**
Specimen	**VPA Mean Dose (mg/day)**	**FPIA Mean Concentration (mcg/ml)**	**S.D.**	**Correlation Coefficient (r)**
Serum	822.5	60.8	20.74	.128

** significant @.01, two-tailed

Valproic Acid (VPA)

The VPA data set consists of 40 subjects (25 females, 15 males) who were on a regimen of VPA for the control of seizures. The mean of age of this group is 23.25 years, s.d. 19.15 (median 18.0). Age skewness is 1.73 and kurtosis is 3.35. The skewness is somewhat outside of normal bounds (ratio 4.6), the kurtosis (ratio 4.5) indicates a peaked distribution. Neither ratio is, however, extreme. The mean length of time (duration) of the dose regimen for this group was 16.275 months (s.d. 8.51) with a range of 23 months. The dosage values (in mg/day) ranged from 200 to 3000 mg/day with a mean dose of 822.5 mg/day (s.d. 558.6, median 600). For both duration of regimen and daily dosage the skewness and kurtosis values are within normal range (<2).

General Dose/Assay Relationship

The data for the VPA dose assay relationship includes concentration in the plasma serum, which was determined by FPIA, and concentration in the hair, which was determined by FPIA. For hair assays the values are in micrograms per gram of hair, and for serum the values are in micrograms per milliliter of plasma. The findings show that there was no significant dose/assay relation-

ship for the VPA serum specimens, and that there was a significant correlation dose/assay relationship for VPA hair specimens. The results are shown in Table 5.

Hair Color

As with CBZ, we will examine the potential for a "color effect" associated with the reported concentrations of recovered VPA. The color categorizations are identical to those used for CBZ. Table 6 reports the mean concentration of VPA by hair color category. There are no dyed specimens so four color categories are used in this analysis.

An analysis of variance for Table 6 fails to show a significant relationship between VPA mean values and hair color ($F = 2.354$, $p = .088$). The Levene Test for equality of error variances indicates that the homogeneity of variances assumptions is met (Levene $F = 2.491$, $p = .076$). The K-S test for normality is consistent with a normal distribution and a normal Q-Q plot of VPA concentration in hair shows a good approximation of normality.

Hair Color Relationship

As with CBZ, we now consider the possible interaction of hair color, dose, and recovered VPA from hair. Table 7 presents the frequency distribution of dose by hair color.

Visual inspection of the Table 7 is consistent with the impression that there may be a slight skewing of higher dosage regimens towards dark-haired subjects. We subject this data to an ANCOVA. As previously, we treat mean concentration as the dependent term, color as the independent qualitative term, and dose duration, 24-hour dose value and age as the covariates. The outcome for this model is reported in Table 8. The Levene statistic shows the variance homogeneity criteria are met, and analysis of the normality of the distribution of the mean hair values shows acceptable conformity with normality assumptions.

Table 6: Mean Concentration of VPA by Hair Color

Hair Color	Number of Subjects	Mean VPA Concentration	Standard Deviation
Black	17	9.65	3.39
Brown	17	7.58	3.30
Blond	3	5.80	1.36
Gray	3	6.08	2.12
Overall Mean	40	8.22	3.37

Table 7: Frequency Distribution of VPA Dosage by Hair Color

VPA Dose	Hair Color				
(mg/day)	Black	Brown	Blond	Gray	Total
200		1			1
300	2	3		1	6
400	1	3		1	5
500	3				3
600	3	3	1		7
700		1			1
800			2		2
900		1			1
1000	1	3		1	5
1500	7	1			8
3000		1			1
Total	17	17	3	3	40

Table 8 demonstrates that the relationships between dose, age, and hair color are all significant (shaded rows), while duration of dose regimen is not. Furthermore the eta^2 values, which are interpretable as a measure of the percentage of the explainable variance, show similar contributions between color and dose, and a lesser contribution due to age. Table 9 reports these contrasts using Bonferroni's criteria.

The Bonferroni criteria contrasts indicate that specific pair comparisons do not yield a significant difference, although the contrasts for black/gray and black/blond are approaching significance. Doing the pairs comparisons using Sidak criteria yields the same results. Both of these confidence interval criteria are conservative but the Bonferroni criteria are preferred on the basis that there is a relatively small number of comparison pairs.

An additional issue of importance is the potential complex interaction between dose, age, and color categorization. First, the data demonstrate a difference in mean dose value by age, although those differences are not significant for VPA (the correlation for dose and age is $r = .265$, $p = .098$). Furthermore, the covariance model can be used to generate model regression-derived mean estimates (adjusted for age and dose) that can be compared to the observed values. Both the analytic values and the estimated values are shown in Table 10.

Table 8: General Linear Model, Analysis of Covariance, Hair Assay Outcome by Color, Dose, Dose Duration, and Age, VPA.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Eta^2
Corrected Model	299.12	6	49.85	11.38	.000	.674
Intercept	10.52	1	10.52	2.40	.131	.068
VPADOSE	61.03	1	61.03	13.93	.001	.297
DOSEDUR	11.55	1	11.55	2.64	.114	.074
AGE	23.35	1	23.35	5.33	.027	.139
COLOR	51.16	3	17.05	3.892	.017	.264
Error	144.59	33	4.38			
Total	3143.34	40				
Corrected Total	443.71	39				

Table 9: Pairwise Comparisons, Hair Color Categories by Mean VPA Concentration

Hair Color (I)	Hair Color (J)	Mean Difference (I-J)	Std. Error	Sig.[a]
Black	Brown	1.811	.758	.135
	Blond	3.584	1.368	.078
	Gray	6.457	2.344	.056
Brown	Black	-1.811	.759	.135
	Blond	1.772	1.345	1.000
	Gray	4.646	2.209	.258
Blond	Black	-3.584	1.368	.078
	Brown	-1.772	1.345	1.000
	Gray	2.873	2.422	1.000
Gray	Black	-6.457	2.344	.056
	Brown	-4.646	2.209	.258
	Blond	-2.873	2.422	1.000

[a] Bonferroni adjustment for multiple comparisons

Comparison of the observed mean values and the model-estimated mean values shows that the model is relatively efficient for all color categorizations except gray hair, where the observed values are nearly twice the value of the estimated values. Since age and gray hair are collinear in the data set Table 10, it is possible that the age/color account for this departure. Regression evaluation of the color/age collinearity for either dose or mean concentration is consistent with this effect (eigenvalue, age = .228, eigenvalue, color = .081). Small eigenvalues are consistent with collinearity between the covariate factors, and thus these values are indicative of an age/color collinear condition.

To further examine this effect, we produce a final covariance model for VPA with color categorization as the independent factor and dose as the covariate. The results of this model are presented in Table 11.

The 2-factor model (dose and color) presented in Table 11 are consistent with the conjecture that age and color demonstrate some interactive or synergistic effect, since color alone fails to attain significance.

Table 10: Mean Concentration Values and Model-Estimated Mean Values, VPA

Hair Color (N)	Mean Concentration VPA (S.D.)	Estimated Means[1], VPA (S.E.)
Black (17)	9.65 (3.39)	9.74 (.572)
Brown (17)	7.58 (3.30)	7.93 (.525)
Blond (3)	5.81 (1.36)	6.15 (1.24)
Gray (3)	6.08 (2.12)	3.64 (2.09)

[1]Evaluated at model covariates: VPA dose (mg/24hr) = 822.5, Age = 23.25

It is reasonable to surmise that the color effect for VPA is, at best, weak and mostly likely collinear with age. However, there is an effect for color that is operating as a stronger effect than discerned in the analysis of CBZ. This effect appears to be dependent on the stringency of the significance criteria used in the analysis, and the degree to which the analytic model disaggregates the hypothesized variable relationships. Selecting less conservative criteria will produce a weak, generalized significant color/assay relationship, but application of either more conservative methods to assess pair-relationships (e.g. Bonferroni criteria), or use of more elaborate model specifications (e.g., step-wise ANCOVA) fail to sustain significance. One of the more serious constraints on the VPA analysis presented here is the very limited number of persons with gray hair, and the number of light-haired persons in general (only 6 of the 40 subjects were not classified as either black or brown hair). A more confident analysis would require a sample with larger numbers

Phenytoin (PHT)

The PHT data set consists of 60 subjects (26 females, 34 males). The mean of age of this group is 35.85 years, s.d. 16.45 (median 35.0). Age skewness (.197) and kurtosis (-.844) are within normal bounds (skewness ratio = 0.64, kurtosis ratio = -.844). The mean length of time (duration) of the dose regimen for this group was 16.52 months (s.d. 8.88) with a range of from 2 to 24 months. The dosage values (in mg/day) ranged from 100 to 400 with a mean dose of 243.3 mg/day (s.d. 103.12, median 250). For both duration of regimen and daily dosage the skewness and kurtosis values are within normal range (<2).

General Dose/Assay Relationship

The data for the dose assay relationship includes concentration in the plasma serum, which was determined by HPLC, and concentration in the hair, which was determined by both FPIA and HPLC. For hair assays the values are in micrograms per gram of hair, and for serum the values are in micrograms per milliliter of plasma.

We note that, in contrast to both CBZ and VPA, Table 12 reveals that there is a significant bivariate dose/assay correlation for the serum specimens, and for the hair specimens by both analytic techniques.

Table 11: General Linear Model, Analysis of Covariance, Hair Assay Outcome by Color and Dose VPA

Source	Type III Sum fo Squares	df	Mean Square	F	Sig.	Eta^2
Corrected Model	263.71	4	65.928	12.82	.000	.594
Intercept	185.62	1	185.62	36.09	.000	.508
VPADOSE	190.93	1	190.93	37.13	.000	.515
COLOR	35.61	3	11.87	2.31	.093	.165
Error	179.99	35	5.14			
Total	3143.34	40				
Corrected Total	443.71	39				

Table 12: Mean Concentration Values and Correlation Coefficients for Dose/Assay Relationship for Hair and Plasma, PHT

Specimen	PHT Mean Dose (mg/day)	HPLC Hair PHT Mean Concentration [sd] (mcg/g)	Correlation Coefficient (r)	FPIA Hair Mean PHT Concentration [sd] (mcg/g)	Correlation Coefficient (r)
Hair	243.3	13.71 [7.32]	.882**	13.67 [7.20]	.883**
Specimen	PHT Mean Dose (mg/day)	HPLC Serum Mean PHT Concentration [sd] (mcg/ml)	Correlation Coefficient (r)	FPIA Serum Mean PHT Concentration [sd] (mcg/ml)	Correlation Coefficient (r)
Serum	243.3	14.00 [3.40]	.779**	14.2 [3.50]	.743**

** significant @.01, two-tailed

Hair Color

Table 13 reports the number of subjects for each of four hair color categorizations, the mean concentration of PHT for that color category, and the related standard deviation. Visual examination of the table shows that both black and brown color had notably higher concentrations than either blond-haired or gray-haired subjects.

A univariate ANOVA on the data in Table 13 reveal that there is a significant difference by hair color ($F = 10.2$, $p = .000$). However, the Levene Test indicates that the homogeneity of variance assumption is not met. An alternative analysis using Tamhane's procedure, allowing an assessment of unequal variances comparisons, also indicates significance, but allows for post-hoc contrasts for each specific color group. The individual comparisons are shown in Table 14.

Table 13: Mean Concentration of PHT by Hair Color

Hair Color	Number of Subjects	Mean PHT Concentration	Standard Deviation
Black	24	16.35	5.79
Brown	18	16.76	7.68
Blond	8	7.92	2.92
Gray	10	6.55	4.82
Overall Mean	60	13.71	7.32

Table 14: Tamhane Post-Hoc Comparison of Color Groupings, PHT

Color Group Contrast	Significant?	p Value
Black/Brown	No	1.000
Black/Blond	Yes	.000
Black/Gray	Yes	.000
Brown/Blond	Yes	.002
Brown/Gray	Yes	.001
Blond/Gray	No	0.978

Table 15: Frequency Distribution of PHT Dosage by Hair Color

PHT Dose (mg/day)	Hair Color			
	Black	Brown	Blond	Gray
100	4	2	2	6
200	4	6	4	2
300	12	4	2	2
400	4	6	0	0
Total	24	18	8	10

Table 16: General Linear Model, Analysis of Covariance, Hair Assay Outcome by Dose, Dose Duration, Age, and Hair Color, PHT

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Eta^2
Corrected Model	2750.79	6	458.46	59.70	.000	.871
Intercept	78.61	1	78.61	10.24	.002	.162
AGE	48.64	1	48.64	6.34	.015	.107
DOSE	1593.67	1	1593.67	207.54	.000	.797
DOSEDUR	11.04	1	11.04	1.44	.236	.026
COLOR	227.13	3	75.71	9.86	.000	.358
Error	406.98	53	7.68			
Total	14441.1	60				
Corrected Total	3157.77	59				

The ANOVA indicates that that there are significant differences in concentration of PHT by hair color. The overall mean concentration values appear to cluster, with the black/brown hair contrast showing no significant difference, and blond/gray contrast showing no significant difference. However, contrast of either blond or gray hair to black or brown hair does show significant difference. The overall distribution of PHT in the hair assays appears to meet the normality assumptions well.

Hair Color/Dose Relationship

In order to further assess the nature of the hypothesized color effect, it is important to assess whether or not there is an interaction effect of color and dose. Is the effect a result of dosing values are higher for dark-haired subjects? Table 15 reports the relative frequency of daily dose regimens by hair color group.

Table 15 indicates that the higher dose values appear to cluster to some degree with black and brown-haired subjects. In order to assess this an ANCOVA model including dose, dose duration, age, and hair color is assessed. Results of that model are shown in Table 16.

Table 16 indicates that age, dose, and color are significant factors in the model (values shaded gray are significant) while dose duration is not. This is identical to the model outcome for VPA. Table 17 shows the Bonferroni criteria for all pairwise combinations, their means differences, and their significance outcomes.

Table 17: Pairwise Comparisons, Hair Color and Concentrations, PHT

Hair Color (I)	Hair Color (J)	Mean Difference (I-J)	Std. Error	Sig.[a]
Black	Brown	.168	.865	1.000
	Blond	3.287	1.245	.065
	Gray	5.902*	1.265	.000
Brown	Black	-.168	.865	1.000
	Blond	3.119	1.292	.116
	Gray	5.734*	1.327	.000
Blond	Black	-3.287	1.245	.065
	Brown	-3.119	1.292	.116
	Gray	2.615	1.680	.754
Gray	Black	-5.902*	1.265	.000
	Brown	-5.732*	1.327	.000
	Blond	-2.615	1.680	.754

[a] Bonferroni adjustment for multiple comparisons * mean difference is significant at he .05 level

As with VPA, the pairwise contrasts show that the black and gray and brown and gray contrasts are significant, while the remaining pairs do not have significant mean differences (the same results is obtained using Sidak criteria). It suggests, as with VPA, that there may be a color/age/dose confounding action involving either collinear or confounding relationships between hair color, dose, and age. In order to assess this we first consider the observed means and the estimates mean values from the ANCOVA model. This is presented in Table 18.

Table 18: Mean Concentration Values and Model-Estimated Mean Values, PHT

Hair Color (N)	Mean Concentration PHT (S.D.)	Estimated Means[1], PHT (S.E.)
Black (24)	16.35 (5.79)	15.184 (0.58)
Brown (18)	16.76 (7.68)	15.02 (0.67)
Blond (8)	7.91 (2.92)	11.96 (1.11)
Gray (10)	6.55 (4.82)	9.23 (1.09)

[1]Evaluated at model covariates: PHT dose (mg/24hr) = 243.3, Age = 35.85

Table 18 reveals that the model-estimated means for dark hair (black and brown) are lower than the observed values, and the estimates for light colored hair are greater. This is a departure from the VPA pattern, and is consistent across category with a color/concentration positive relationship.

We can assess this effect by examining the ANCOVA model retaining dose and hair color as the sole covariates (replicating the procedure applied to VPA) and examining the surviving significant relationship. The results of this model are shown in Table 19.

Consistent with the approach applied to VPA, we delete age from the model in Table 19. Age is removed from the model in this case to see if it diminishes the color effect, as was the case VPA. Comparison of Tables 17 and 18 show that the eta^2 is diminished, but is still significant and makes a substantial contribution to the explained variance. Dose still has, however, the higher eta^2 value. Further pairwise comparisons show that there are significant mean value contrasts between the light hair categories (gray and blond) and the dark hair categories (black and brown). There are, however, no significant contrasts within those groups (i.e., no significant mean differences between black and brown hair or blond and gray hair).

Conclusions

This assessment of the recovery of three compounds, CBZ, VPA, and PHT, from hair samples identifies a variable effect of hair color on assay concentration by drug type. The generalized hypothesis assessed is that darker coloration of hair is associated with a greater concentration of analyte in the hair. For CBZ, hair color does not appear to exert a significant effect on the quantity of the compound recovered from hair. For VPA there appears to be a very weak color effect, but the number of light-haired subjects attenuates the strength of the conclusion. For all drugs there appears to be a significant positive relationship between dose and hair concentration. We also suggest that for VPA there appears to be effects of collinearity between age and hair color, and possibly dose and hair color. Both of these phenomena could produce confounding effects in trying to describe the dose/assay relationship. The limitations of the data are that the subjects are a clinical, non-random, population and the range of dosages across all color grouping was not uniform, nor was dosage duration. Furthermore, the number of light hair subjects was, for certain drugs, very small.

Table 19: General Linear Model, Analysis of Covariance, Hair Assay Outcome by Dose and Hair Color, PHT

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Eta^2
Corrected Model	2648.08	4	662.02	71.44	.000	.839
Intercept	2.25	1	2.25	.243	.624	.004
DOSE	1532.31	1	1532.31	165.35	.000	.750
COLOR	191.68	3	63.89	6.895	.001	.273
Error	509.69	55	9.27			
Total	14441.1	60				
Corrected Total	3157.77	59				

Additional material

Hair Color, Dose, Dose Duration, HPLC and FPIA Concentration per Segment, HPLC and FPIA Serum Concentration, Carbamazepine

Raw patient data for carbamazepine analysis of hair and serum.
CBZ Data Table.doc
[http://www.biomedcentral.com/content/supplementary/1472-6904-1-2-s1.doc]

Hair Color, Dose, Dose Duration, HPLC and FPIA Concentration per Segment, HPLC and FPIA Serum Concentration, Valproic Acid

Raw patient data for valproic acid analysis of hair and serum.
VPA Data Table.doc
[http://www.biomedcentral.com/content/supplementary/1472-6904-1-2-s2.doc]

Hair Color, Dose, Dose Duration, HPLC and FPIA Concentration per Segment, HPLC and FPIA Serum Concentration, Phenytoin

Raw patient data for phenytoin analysis of hair and serum.
PHT Data Table.doc
[http://www.biomedcentral.com/content/supplementary/1472-6904-1-2-s3.doc]

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