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Assessing Hair Decontamination Protocols for Diazepam, Heroin, Cocaine and Δ^9-Tetrahydrocannabinol by Statistical Design of Experiments

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Abstract

Prior to toxicological analysis, hair as a matrix requires pre-treatment measures including decontamination, homogenization and extraction. Decontamination is performed to differentiate between drug present from superficial deposition and drug incorporated from systemic distribution following ingestion. There are many methods for decontamination of hair samples, mostly developed by empirically using a traditional "one factor at a time" approach, in which one independent variable at a time is changed to observe the effect on the dependent variable. The goal of the present work was to compare the efficacy of decontamination protocols using statistical "design of experiments" (DoE), which allows for analysis of multiple variables and interactions within a single experiment. Decontamination parameters included identity of aqueous and organic wash solutions, number of sequential aqueous and organic washes, order of aqueous and organic washes, and duration of each wash. DoE studies were completed to identify optimal decontamination conditions for four abused drugs with varying physiochemical properties. For this purpose, drug-free human hair was externally contaminated with diazepam, heroin, cocaine or Δ^9-tetrahydrocannabinol. Each analyte was found to have a unique set of decontamination conditions that were most effective. These included three 30-min washes with methanol followed by three with 1% sodium dodecyl sulfide for diazepam, three 30-s washes with dichloromethane followed by one with water for heroin, one 30-s wash with 1% sodium dodecyl sulfate followed by three with dichloromethane for cocaine and three 30-min washes with water followed by one with methanol for Δ^9-tetrahydrocannabinol. The results provide proof-of-principle for a DoE approach to identify effective parameters for hair decontamination for a physicochemically diverse group of drugs. The major advantage of DoE is to elucidate combinations of parameters that result in effective removal of surface contamination, a goal that would be challenging to accomplish using a one factor at a time approach.

Introduction

One of the greatest challenges and sources of uncertainty in the toxicological analysis of hair involves decontamination prior to extraction and analysis. The purpose of decontamination is to remove oils, lipids and cosmetic products and to eliminate any analyte that may be superficially associated with the surface of the hair as a result of contact with contaminated surfaces or drug vapors and residues that may exist in the environment of the individual being sampled

(1). Important considerations for the decontamination of hair samples include the choice of solvent/solution used to wash the hair, the volume of that solvent/solution used in relation to the amount of hair being decontaminated and the length of exposure of the hair to these solvents/solutions (1–5).

Removal of external contamination from hair is not necessarily a simple process. For example, a recent study reported that aqueous wash procedures, rather than removing surface contaminated drug, can actually move drug deeper into the hair structure (6). A decontamination procedure for a lipophilic drug such as Δ^9-tetrahydrocannabinol (THC) may be ineffective for polar drugs such as cocaine (COC) (7). The Society of Hair Testing has endorsed the use of an aqueous wash step followed by an organic wash (8). However, these recommendations are general, and thus a large number of analyte-specific wash procedures have emerged with a variety of effectiveness.

Studies have been conducted to compare some of the more commonly employed techniques for decontamination as complete protocols or individual variables involved in the wash protocol. For example, Stout et al. compared decontamination protocols originally reported by Cairnes et al. and Romano et al. to study their efficacy for removing cocaine (COC) from the surface of hair, finding that neither method was sufficient for removing all of the surface contamination, a result consistent with the findings of the original authors of each technique (1, 9, 10). Ropero-Miller et al. and Morris-Kukoski et al. also observed that the Cairnes et al. decontamination protocol was insufficient for removing COC from the surface of hair (11, 12). Duvivier et al. compared complete protocols for the decontamination of THC, finding that a multi-step wash with a combination of methanol and sodium dodecyl sulfate (SDS) was most effective (13). Mantinieks et al. compared the efficacy of several solvents for removing COC and methamphetamine contamination, finding again that none resulted in the complete removal of the drug (14).

Although, as described earlier, reports do exist that compare selected hair decontamination procedures for specific drugs, to date there has been no comprehensive, statistically rigorous, side-by-side evaluation of decontamination approaches for multiple classes of drugs. The predominant approach to developing and assessing the efficacy of hair pre-treatment protocols has been to utilize a traditional "one factor at a time" approach, in which one independent variable at a time is changed to observe the effect on the dependent variable. An alternative approach to assessing pre-treatment protocols is statistical design of experiments (DoE), which involves the systematic variation of multiple independent variables (e.g., decontamination conditions) simultaneously, allowing for the variation in the dependent variable (e.g., drug removal efficiency) associated with each independent variable and combinations thereof to be observed. Considering the lack of systematic comparative analysis of decontamination protocols for a wider set of commonly abused drugs, the present study employed DoE to assess a limited set of variables in the decontamination process for diazepam (DZP), THC, COC and heroin (HER) as a proof of principle for this approach.

Materials and Methods

Chemicals and solvents

Chemicals and solvents used for LC mobile phases and sample preparation (i.e., water, methanol, 2-propanol, dichloromethane [DCM], formic acid and ammonium formate) were HPLC grade and obtained from Fisher Scientific (Hampton, NH, USA). Stock solutions for the preparation of calibration curves for DZP, HER, COC, DZP-d_5, morphine-d_3 and COC-d_3 were obtained at 1.0 mg/mL in methanol. Δ^9-THC and Δ^9-THC-d_3 were purchased as 10.0 and 1.0 mg/mL solutions in acetonitrile, respectively. Neat solid powders of DZP, HER and COC for intentional surface contamination of hair were purchased from Cayman Chemical (Ann Arbor, MI, USA). 1,4-Dithiothreitol was purchased from Sigma-Aldrich (St. Louis, MO, USA), and proteinase-K was obtained from Invitrogen (Carlsbad, CA, USA). Three kilodalton molecular weight cut off PTFE spin filters were obtained from MilliporeSigma (Burlington, MA, USA). Single donor, unprocessed, natural black color human hair (~300 g) was obtained from a commercial source.

2^4 Factorial block design and statistical analysis

The theory behind DoE is based on hypothesis testing. In a typical DOE approach, analysis of variance (ANOVA) is used to identify if there is a source of variance in a data set due to the variable treatments applied to samples rather than random error. In the present study, only factorial ANOVA is considered (i.e., no one-way ANOVA). Therefore, post-hoc testing (such as Tukey's honestly significant difference) is not useful, as every factor only has two levels and therefore there is no need to perform any adjustment for multiple comparison of multiple levels.

In 2^k factorial DoE designs, such as the one chosen for this work (Tables I and II), each factor of interest has 2 levels (i.e., high and low) for k number of factors. Therefore, there are 2^k combinations of factor levels, called "design points", with each design point

Table I. Definitions of Factors and Levels of These Factors Under Study in the 2^4 Factorial Block Design

Factor	Decontamination parameters	
A	Aqueous decontamination solvent	(+) 1% SDS (–) HPLC water
B	Organic decontamination solvent	(+) DCM (–) MeOH
C	Number of consecutive aqueous washes	(+) 3 (–) 1
D	Number of consecutive organic washes	(+) 3 (–) 1
Block 1	Wash sequence	(+) Organic before aqueous (–) Aqueous before organic
Block 2	Wash time	(+) 30 min (–) 30 s

Table II. Augmented 2^4 Factorial Block Design for Comparison of Decontamination Parameters

Block (1, 2)	Design point	A	B	C	D	AB	AC	BC	AD	BD	CD	ABD	ACD	BCD	ABC	ABCD	Block 1	Block 2
(Low, low)	1	−	−	−	−	+	+	+	+	+	+	−	−	−	−	+	Even	Even
	bc	−	+	+	−	−	−	+	+	−	−	+	+	−	−	+	Even	Even
	adb	+	+	−	+	+	−	−	+	+	−	+	−	−	−	−	Even	Even
	acd	+	−	+	+	−	+	−	+	−	+	−	+	−	−	−	Even	Even
(Low, high)	ac	+	−	+	−	−	+	−	−	+	−	+	−	+	+	+	Even	Odd
	ab	+	+	−	−	+	−	−	−	−	+	−	+	+	+	+	Even	Odd
	bcd	−	+	+	+	−	−	+	−	+	+	−	−	+	+	−	Even	Odd
	d	−	−	−	+	+	+	+	−	−	−	+	+	+	+	−	Even	Odd
(High, low)	bd	−	+	−	+	−	+	−	−	+	−	−	+	−	−	+	Odd	Even
	cd	−	−	+	+	+	−	−	−	−	+	+	−	−	−	+	Odd	Even
	a	+	−	−	−	−	−	+	−	+	+	+	+	−	−	−	Odd	Even
	abc	+	+	+	−	+	+	+	−	−	−	−	−	−	−	−	Odd	Even
(High, high)	b	−	+	−	−	−	+	−	+	−	+	+	−	+	+	−	Odd	Odd
	c	−	−	+	−	+	−	−	+	+	−	−	+	+	+	−	Odd	Odd
	ad	+	−	−	+	−	−	+	+	−	−	−	−	+	+	+	Odd	Odd
	abcd	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Odd	Odd

representing a unique grouping of treatment factors which make up the list of design points to be studied (15). The factors are denoted with upper-case letters (A, B, C, D, etc.). Each factor has a "high" and a "low" level. The 2^k notation refers to the number of combinations of factor levels, with k being the number of factors under study. The DoE approach thus allows evaluation of multiple combinations of pre-treatment parameters in a single experiment. If the factor is listed in the design point, then that factor is held at the high level; if it is not listed, then it is at the low level. As an example, design point "bc" has the high level of factor B and factor C applied to it, while factor A and factor D are at the low level (Table II).

In the augmented design, the interaction effects are determined by considering the product of the individual factors. Using design point "bc" as the example again, the interaction effect for AB is determined by understanding the product of A*B. In this case, A is low and B is high. When a negative is multiplied by a positive, the result is negative, which is indicated in the AB interaction column for design point "bc". Some interaction effects are not observable. In the augmented design, no two columns are the exact same or exact opposite of one another, which means the factors are linearly independent and a linear model, such as ANOVA, may be used to analyze the variance in the resultant data set (15). However, there are four columns that defy this rule; columns for AD, BCD, ABC and block. These columns are said to be completely confounded with each other, and thus the variance in the dataset due to these factors cannot be understood with this design. These confounding factors are also called the defining contrasts and are chosen within the block design.

When a 2^k design is broken into 2^m blocks, the blocks are of the size $2^k/2^m$ or 2^{k-m}, where m is the number of blocking factors. Each blocking factor has two levels. Each blocking factor has a corresponding defining contrast, with which it is completely confounded. These defining contrasts are usually chosen to be high-level interactions, that is, three- and four-factor interactions. Once selected, design points are designated as odd or even, depending on the number of letters in common with the chosen defining contrast, distinguishing their level within each block (15).

In the specific design chosen for this work, ABC was chosen as the defining contrast for block 1, BCD was chosen as the defining contrast for block 2 and, as a result, AD was completely confounded with the interaction effect between block 1 and 2. A 2^4 factorial block design was implemented in this study to understand how the parameters listed in Table I affect the recovery of drug intentionally applied to hair under different decontamination protocols. These parameters were selected based on common parameters used in published, validated techniques. The design selected to study these parameters limits the number of conditions that can be tested within each parameter; these can be extended with additional studies.

The application of these parameters to hair samples was dictated by the design matrix (Table II). For this study, 30 mg aliquots of hair were externally contaminated with 1 mg of neat drug powder for each replicate ($n = 3$) of each design point when analyzing for DZP, HER and COC. The hair and neat drug were added to steel milling jars and shaken in a Mini-BeadBeater 24 ball mill (Biospec; Bartlesville, OK, USA), without milling beads, for 30 s at 3,200 rpm to homogeneously coat the hair with drug. Contaminated hair strands were then removed from the milling jar with forceps and transferred to a fresh container for subsequent procedures. The application of THC was performed by pipetting 1 mL of a 1 mg/mL solution of drug in acetonitrile onto a 30 mg aliquot of hair. Following vortexing, solvent was evaporated in an Eppendorf Vacufuge Plus (Hauppauge, NY, USA) under the aqueous vacuum conditions at 45°C for 45 min. Samples were decontaminated by adding 1 mL of the decontamination solvent/solution and agitating for 1 min on an orbital shaker, as specified in the design matrix (Table II). The hair samples were then dried overnight.

A randomized list of design points was used to determine the order in which samples were prepared and analyzed, with three replicate samples for each design point. Quantitation of the drug detected in each sample allowed for subsequent statistical analysis by ANOVA F-Test and Tukey's HSD to determine which, if any, parameters or combination(s) of parameters resulted in significant differences in the recovery of drug from the washes.

Extraction and purification of analytes from hair

Drug that may have been incorporated deeper into the hair matrix was determined following extraction by enzymatic hydrolysis of the hair proteins following decontamination. Enzymatic degradation of the hair matrix was conducted by first incubating decontaminated hair with 12 mg/mL aqueous 1,4-dithiothreitol for 2 h at 37°C,

followed by 2 mg/mL aqueous proteinase K for 24 h (16). For cleanup of hair extracts, Agilent Technologies (Santa Clara, CA, USA) Bond Elut Certify mixed mode SPE cartridges were used following a method characterized for hair samples by Miller et al. (17).

Analysis of washes and extracts

Extracted samples and wash solutions were analyzed by high-performance liquid chromatography followed by triple quadrupole mass spectrometry. In brief, a 2 μL injection was performed onto a reversed-phase HPLC column (Agilent Technologies 1.8 μm Zorbax Eclipse Plus C_{18} rapid resolution HD column (2.1 × 150 mm; 1.8 μm) for separation of the analytes. The aqueous mobile phase used was water with 0.1% formic acid and 5 mM ammonium formate. The organic phase was methanol with 0.1% formic acid. The separation was conducted using a gradient solvent system at 0.3 mL/min flow rate. A multiple reaction monitoring (MRM) method was employed for the identification of analytes using an Agilent 6,470 triple quadrupole mass spectrometer operated in positive electrospray ionization mode. The peaks were confirmed by two MRM transitions corresponding to the integrated chromatographic peaks, one as a quantifier and the other as a qualifier. These transitions were identified by Agilent's MassHunter Optimizer software and are listed in Table III. Drug recovery from wash solutions was calculated by summing the amount of drug detected in the sequential washes and dividing by the difference in the amount of drug left in the milling jar after transfer from the initial mass of drug initially applied to the hair.

Results

Figure 1 shows the plots of the percent recovery of each analyte following decontamination for each design point. In general, a limited number of design points for each analyte were found to produce the highest total recovery of drug in the wash solutions. The parameters that were applied to the samples within these treatment groups were tabulated to draw conclusions about the most effective decontamination protocols for each analyte. The similarities between design points with the highest recoveries were used to make a consensus statement about which of the two levels of each parameter tested was more effective for removing each of the analytes from the surface of hair.

ANOVA F-Test results for the decontamination of DZP (Supplementary Data Table 1) indicated that significant (i.e., P-value < α-level, 0.05) individual and combinatorial factors included aqueous decontamination solvent/solution (A), the combinatorial effect of aqueous solvent/solution and the organic decontamination solvent (AB); the combinatorial effect of aqueous solvent/solution and number of consecutive aqueous washes (AC); the combinatorial effect of organic solvent and number of organic washes (BD); the combinatorial effect of aqueous solvent/solution, organic solvent and number of consecutive aqueous washes (ACD); the combinatorial effect of aqueous solvent/solution, organic decontamination solvent and number of consecutive organic washes (ABD) and the combinatorial effect of all main factors (ABCD).

Recoveries of DZP in wash solutions ranged from 40 ± 15% to 94 ± 18%, with the highest recoveries noted for design points c, ad and abcd (Figure 1A). Decontamination parameters for these optimal design points are summarized in Table IV. Blocking factors 1 and 2 were the same for all of the samples in the treatment groups associated with these design points; employing the organic wash before the aqueous wash and decontaminating for 30 min during each wash step. Design points ad and abcd had in common the use of 1% sodium dodecyl sulfate (SDS) as the aqueous decontamination solution. Additionally, three sequential organic washes were applied to samples for design points ad and abcd. Design points c and ad had in common the use of methanol as the organic decontamination solvent. Design points c and abcd both utilized three consecutive washes with the aqueous decontamination solvent/solution.

ANOVA F-Test results for the decontamination of HER (Supplementary Data Table 2) indicated that all individual factors and combinations of factors resulted in P-values less than the α-level (0.05) and thus were significant. Recoveries of HER in wash solutions ranged from 12 ± 3% to 124 ± 18%, with design points ab, bd and cd demonstrating the highest total recoveries of HER (Figure 1B). Decontamination parameters for these design points are summarized in Table V. Common variables for design points bd and cd included HPLC water as the aqueous decontamination solvent, three consecutive organic washes before the aqueous wash and decontamination for 30 s during each wash step. Commonalities between design points ab and bd included DCM as the decontamination solvent and only one aqueous wash applied to the hair.

ANOVA F-Test results for the decontamination of COC (Supplementary Data Table 3) indicated that all individual factors and combinations of factors resulted in P-values less than the α-level (0.05), and therefore had a significant effect. Recoveries of COC in wash solutions ranged from 61 ± 9% to 119 ± 1%, with the highest recoveries observed for design points bd, abd, acd and abcd (Figure 1C). Decontamination parameters for these design points are summarized in Table VI. Each of these design points employed three consecutive organic washes. Design points bd, abd and acd each used a single aqueous decontamination wash for 30 s. The aqueous decontamination solution applied to samples in treatment groups abd, acd and abcd was 1% SDS. The organic decontamination solvent applied to samples in treatment groups bd, abd and abcd was DCM. Design points abd and acd represented treatment with the aqueous decontamination solvent/solution first, whereas bd and abcd utilized organic solvent first.

ANOVA F-Test results for decontamination of THC (Supplementary Data Table 4) indicated that individual factors A and B, along with all combinations of factors, resulted in P-values less than the α-level (0.05). Recoveries of THC in wash solutions ranged from 22 ± 9% to 83 ± 10%, with highest recoveries detected for design points bc, ac, d, cd and b (Figure 1D). Decontamination parameters for these design points are summarized in Table VII. Design points bc, d, cd and b utilized HPLC water as the aqueous decontamination

Table III. MRM Transitions Used for Identification and Quantitation of Analytes via HPLC–MS

Analyte	MRM transitions	
	Precursor (*m/z*)	Products (*m/z*)
Cocaine	304	18282
Cocaine-d_3	307	18585
Diazepam	285	222193
Diazepam-d_5	290	198154
Heroin	370	211165
Morphine-d_3	289	12661
Δ^9-THC	315	193123
Δ^9-THC-d_3	318	19693

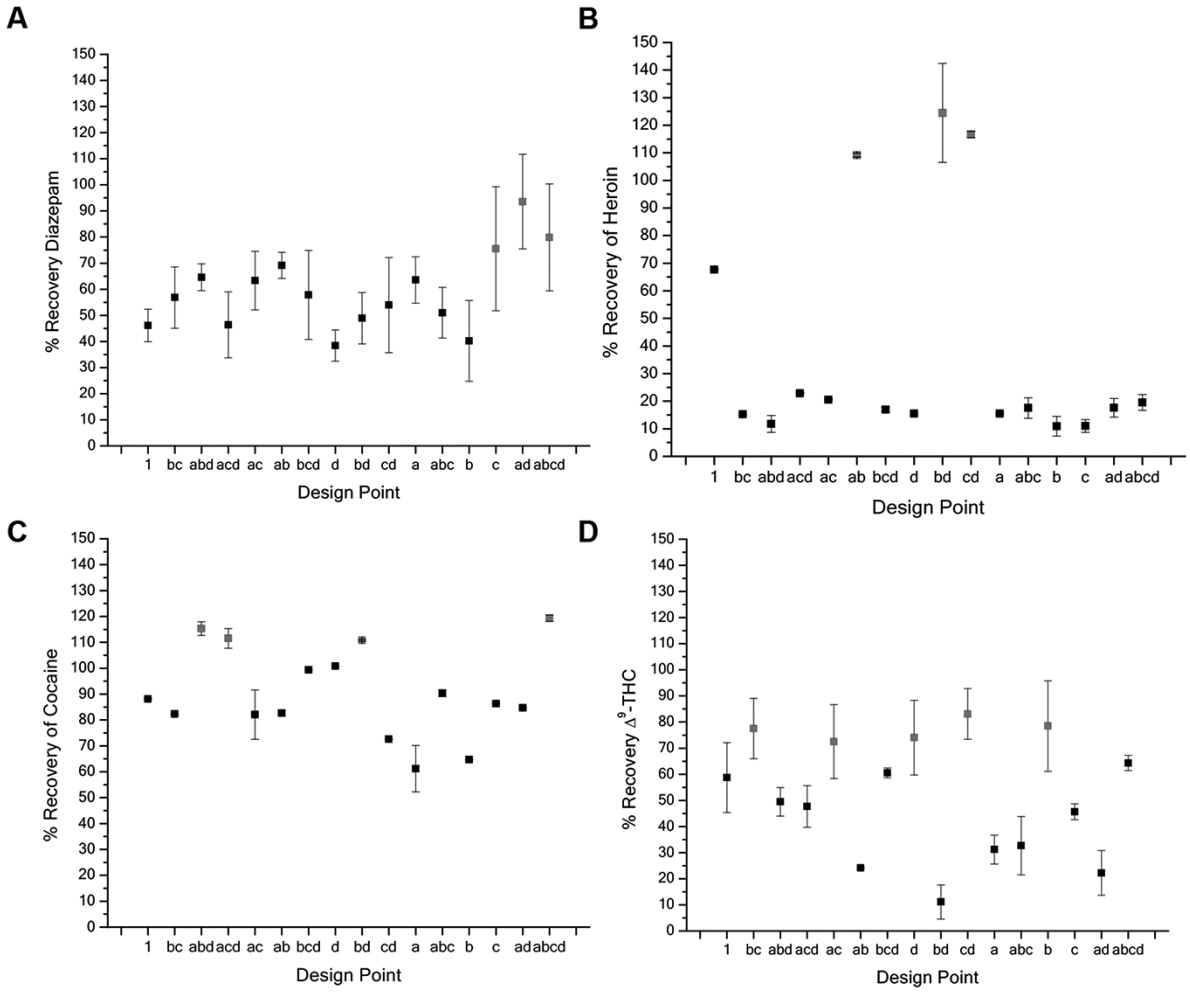

Figure 1. Plot of decontamination efficiency (% recovery) of (A) DZP, (B) HER, (C) COC and (D) THC detected in the wash solutions for each design point ($n = 3$). The *y*-axis is the percent recovery of the drug; the *x*-axis is the design point. Points highlighted in red represent design points with the highest observed recovery. Optimal parameters associated with each design point are summarized in Tables IV–VII.

Table IV. List of Parameters for the Design Points Resulting in the Highest Recovery of DZP

Wash parameter	Design point		
	c	ad	abcd
A: Aqueous decontamination solvent	HPLC water	1% SDS	1% SDS
B: Organic decontamination solvent	MeOH	MeOH	DCM
C: Number of aqueous washes	3	1	3
D: Number of organic washes	1	3	3
Block 1: Sequence of washes	Organic first	Organic first	Organic first
Block 2: Wash time	30 min	30 min	30 min
Drug recovered in washes	76 ± 24%	94 ± 18%	80 ± 20%

solvent, and design points bc, ac and d included application of the aqueous wash prior to the organic. Design points bc, ac and cd had in common the use of three consecutive aqueous washes, while design points bc, ac and b employed a single organic wash. A 30 min wash was utilized for design points ac, d and b.

Consensus parameters that resulted in the highest recovery for each analyte are summarized in Table VIII. These data indicate that the optimal combination of decontamination parameters was different for each individual drug.

Discussion

There are numerous methods that are commonly referenced for decontamination of hair samples prior to toxicological analysis. For example, Wang and Cone reported a decontamination technique that called for submersion of the hair samples in methanol for ~1 min (18). A method in which hair was vortexed in DCM three times for 5 min each was reported by Kintz (19). Another method reported initially by Cairnes et al. included first vigorously agitating hair in n-propanol for 15 min at 37°C, then in 0.01 M phosphate-buffered

Table V. List of Parameters for the Design Points Resulting in the Highest Recovery of HER

Wash parameter	Design point		
	ab	bd	cd
A: Aqueous decontamination solvent	1% SDS	HPLC water	HPLC water
B: Organic decontamination solvent	DCM	DCM	MeOH
C: Number of aqueous washes	1	1	3
D: Number of organic washes	1	3	3
Block 1: Sequence of washes	Aqueous first	Organic first	Organic first
Block 2: Wash time	30 min	30 s	30 s
Drug recovered in washes	109 ± 1%	124 ± 18%	116 ± 1%

Table VI. List of Parameters for the Design Points Resulting in the Highest Recovery of COC

Wash parameter	Design point			
	bd	abd	acd	abcd
A: Aqueous decontamination solvent	HPLC water	1% SDS	1% SDS	1% SDS
B: Organic decontamination solvent	DCM	DCM	MeOH	DCM
C: Number of aqueous washes	1	1	1	3
D: Number of organic washes	3	3	3	3
Block 1: Sequence of washes	Organic first	Aqueous first	Aqueous first	Organic first
Block 2: Wash time	30 s	30 s	30 s	30 min
Drug recovered in washes	111 ± 1%	115 ± 3%	112 ± 4%	119 ± 1%

Table VII. List of Parameters for the Design Points Resulting in the Highest Recovery of THC

Wash parameter	Design point				
	bc	ac	d	cd	b
A: Aqueous decontamination solvent	HPLC Water	1% SDS	HPLC Water	HPLC Water	HPLC Water
B: Organic decontamination solvent	DCM	MeOH	MeOH	MeOH	DCM
C: Number of aqueous washes	3	3	1	3	1
D: Number of organic washes	1	1	3	3	1
Block 1: Sequence of washes	Aqueous first	Aqueous first	Aqueous first	Organic first	Organic first
Block 2: Wash time	30 s	30 min	30 min	30 s	30 min
Drug recovered in washes	77 ± 11%	72 ± 14%	74 ± 14%	83 ± 9%	78 ± 17%

Table VIII. Summary of Consensus Parameters Resulting in the Highest Mean Recovery of Analytes

Wash parameter	DZP	THC	COC	HER
A: Aqueous decontamination solvent	1% SDS	HPLC water	1% SDS	HPLC water
B: Organic decontamination solvent	MeOH	MeOH	DCM	DCM
C: Number of aqueous washes	3	3	1	1
D: Number of organic washes	3	1	3	3
Block 1: Sequence of washes	Organic first	Aqueous first	Aqueous first	Organic first
Block 2: Wash time	30 min	30 min	30 s	30 s

saline containing 0.01% bovine serum albumin, pH 6, twice for 30 min each, and finally with the same buffer twice for 60 min each (9). A more complex method, reported by Baumgartner and Hill, included staining with methylene blue to assess the porosity of the hair sample, followed by washing with ethanol for 15 min at 37°C with shaking. More porous samples were then washed with a mixture of ethanol and water. Non-porous and slightly porous hair were washed with 0.01 M phosphate-buffered saline, pH 5.6; 30-min washes were conducted in sequence with the aqueous solution until the concentration of drug in the washes reached a plateau (20).

Stout et al. compared the methods reported by Carines et al. and Baumgartner and Hill to determine which was most effective at removing COC from the surface of hair that was intentionally contaminated and then exposed to a synthetic sweat solution (1). Neither method was found to be successful for complete removal of drug applied to the hair, a result consistent with a number of other studies aiming to remove COC external contamination (6, 9, 10, 12, 14, 18). These workers also studied the effects of different decontamination methods on hair morphology by means of scanning electron microscopy, finding that each method could be associated with some level of damage to hair structures (2). Observed

damage included lifting of the cuticle cells, changes to the cell membrane complex between cuticle cells, and substantial degradation of the cuticle, which in some cases resulted in complete loss of cuticle cells. Finally, Cuypers et al. reported that the use of certain solvents for the purpose of decontamination may in fact encourage the migration of the drug into deeper hair structures, a result questioning the currently held approaches for decontamination procedures (6).

While much investigation into the decontamination of COC from hair has been conducted, there are fewer reports examining other common drugs of abuse. Tsanaclis et al. examined the use of a combination of drug and metabolite levels with drug present in wash solutions to assess decontamination of THC, finding that 2 washes of less than 1 min each with methanol were sufficient (7). Duvivier et al. tested a series of washes for the removal of THC from the surface of hair, finding that the most effective methods were multi-step processes with methanol and SDS (13). Mantinieks et al. studied the decontamination of hair intentionally contaminated with methamphetamine, finding that, compared to heptane, DCM, acetonitrile and 2-propanol, methanol was the most effective organic decontamination solvent for this compound (14).

The reported applications of DoE to optimizing conditions for forensic hair analysis are currently quite limited. Mueller et al. compared a number of pre-treatment parameters in the analysis of ethyl glucuronide in hair using a Plackett–Burman experimental design (21). However, this design did not consider combinatorial effects of the factors under study on the dependent variable. Alladio et al. used a two-factor full factorial design to investigate and optimize the extraction of ethyl glucuronide from hair (22). Restolho et al. employed a DoE approach to optimize the use of ionic liquids to remove surface contamination of opiates and cannabinoids from hair (23, 24).

In the present study, a DoE matrix combined with ANOVA F-test analysis was employed to systematically assess the effects of two levels of four different independent decontamination parameters on recovery of four physicochemically diverse drugs from externally contaminated hair. Unlike previous studies, the DoE approach allowed for the determination of multiple significant factors impacting the decontamination of each analyte in a single experiment. In addition, DoE allowed the determination of the effects of combinations of decontamination factors (e.g., solvents, wash times, order of washes, etc.), a capability that facilitates the study of the impact of changing multiple variables within the experiment. The present report is the first in which multiple parameters in hair decontamination were simultaneously compared for a variety of drugs. Results of the ANOVA F-Test for COC and HER showed that all of the individual sources (i.e., A, B, C and D) were significant. In contrast, for DZP and THC, only one or two of the individual sources, respectively, were significant. Perhaps more importantly, higher-level interactions (e.g., AB, ABC, etc.) were significant for all four drugs, indicating that combinations of the variables had a significant effect on the level of drug detected in the wash solutions.

There are several previous reports that compared wash protocols following the Society of Hair Testing guidelines (13, 14, 25). These studies concluded that methanol was the most effective organic solvent at removing surface contamination, while 1% SDS was the most effective aqueous decontamination solution for the removal of a variety of analytes. Some previous observations are in contrast to findings reported herein. For example, in the present study, DCM was found to be more effective than methanol at removing COC from the hair surface. Other work has shown that, for removal of THC contamination, SDS was the most effective aqueous solvent/solution, while methanol, DCM and chloroform were effective organic solvents (13). In contrast, in the present study it was found that water and methanol were the most effective solvents for removing THC from hair. Some of these inconsistencies may be attributable to differences in experimental approach, including intentional contamination via cannabis smoke and the use of synthetic sweat applied to the hair following surface contamination with the drug. Alternatively, it may not be valid to directly compare results from hair decontamination studies using a "one factor at a time" approach to those generated using a multivariate technique such as DoE.

An important result of the present study is that only a limited number of treatments for each drug were found to be most effective at decontaminating the surface of hair. For example, effective decontamination of HER was associated with only a small number of design points, with much poorer results demonstrated for the other combinations. Conclusions about the most effective decontamination protocols for each analyte can be drawn from the commonalities between the treatment groups (i.e., design points), as summarized for the present study in Table VIII, most notably that a minimum of four consecutive washes were necessary for the decontamination of the analytes. One might be tempted to rationalize these optimal conditions based on drug solubility, polarity, ionization parameters and the penetrability of the hair sample, all of which have been hypothesized to influence the efficacy of wash parameters. However, it must be noted that the mechanisms of surface binding of drugs to hair components are poorly understood, making predictions about optimal decontamination conditions difficult at best. Furthermore, the major advantage of the DoE approach as applied herein was to elucidate combinations of parameters that would result in effective removal of surface contamination, a goal that would be challenging to accomplish a priori based on consideration of physicochemical characteristics alone.

Conclusions

The present study demonstrated proof of principle for the successful use of a DoE approach to identify effective combinations of experimental parameters for hair decontamination for a physicochemically diverse group of drugs. Each of the analytes under study was found, within a limited set, to have a unique set of decontamination conditions that were most effective. Additional studies will be conducted to test the applicability of the DoE approach and the optimized decontamination methods identified in the present study with authentic hair specimens, specifically hair with different colors and porosities. It is also valuable to conduct DoE studies that include additional solvents/solutions for decontamination and further optimization of wash time.

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Supplementary data

Supplementary data is available at *Journal of Analytical Toxicology* online.

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