0090-9556/98/2607-0689–700$02.00/0
DRUG METABOLISM AND DISPOSITION
Vol. 26, No. 7
Printed in U.S.A.

FENTANYL IN HAIR

Chemical Factors Involved in Accumulation and Retention of Fentanyl in Hair after External Exposure or *In Vivo* Deposition

PETER R. STOUT, DAVID J. CLAFFEY, AND JAMES A. RUTH

Department of Molecular Toxicology and Environmental Health Sciences, University of Colorado Health Sciences Center

(Received November 21, 1997; accepted March 13, 1998)

This paper is available online at http://www.dmd.org

ABSTRACT:

The levels of fentanyl extractable from mouse hair after chronic systemic administration and the suitability of externally loaded hair samples for establishing control and comparison samples were determined. Additionally, the effects of chemical modification of specific polar functionalities within the hair protein matrix on the deposition and recovery of fentanyl in hair subjected to external loading were determined. BALB/c mice entering a second phase of synchronized hair growth were treated ip with fentanyl (0.02, 0.05, or 0.10 mg/kg) on Monday, Wednesday, and Friday for 3 weeks. At that time, fentanyl concentrations in hair, as determined by GC/MS, were 0.025–0.050 ng/mg of hair. Hair samples exposed to fentanyl in phosphate buffer (ionized drug) showed no significant accumulation of drug into the hair, as determined by loss of fentanyl from the loading solution or by extraction of the hair. Hair samples exposed to nonionized fentanyl in methanolic solution (10, 50, and 100 ng/ml) showed significant accumulation of drug in the hair and significant removal of drug from the incubation solution. Fentanyl removal from solution plateaued after 24 hr, suggesting equilibration between fentanyl in solution and fentanyl in the hair. A mass balance between drug lost from the incubation solution and drug recovered from hair samples suggests that 94% of accumulated fentanyl is tightly bound to the hair matrix or resides in water-inaccessible compartments within the hair. These results suggest that fentanyl accumulation after *in vivo* administration differs, in the nature of storage, from fentanyl accumulation from external solutions and that external spiking of hair may not provide suitable control samples. Chemical modification of hair protein functionalities (reaction with diazomethane to esterify carboxylic acid groups or with acetic anhydride and pyridine to acetylate amine and hydroxyl functionalities) led to reproducible protein structure modification, as demonstrated by Fourier transform-IR and by pH titration. Hair from BALB/c mice was used. The accumulation of fentanyl was examined in hair samples exposed to fentanyl in methanol or methylene chloride solutions (10 ng/ml, 24 hr). Fentanyl was recovered from hair by 24-hr extraction in phosphate buffer, pH 6. Esterification of hair resulted in significantly less uptake of nonionized fentanyl from a methanolic solution and significantly lower recovery of drug from hair, relative to untreated hair, suggesting that carboxylic acid functionalities are necessary for the incorporation of drug. Acetylation of hair resulted in increased removal of fentanyl from methylene chloride solutions and increased recovery of fentanyl. This is consistent with the creation or expansion of a less polar compartment. Fentanyl uptake from a methanolic solution was also greater in acetylated hair. These results demonstrate that solution-accessible ionizable functionalities of hair play a significant role in the accumulation and retention of nonionized fentanyl from organic solutions.

The suitability of hair as a reliable quantitative indicator of systemic exposure to drugs of abuse remains controversial because of a number of unresolved questions. These include the undetermined chemical mechanism of systemic drug incorporation into hair and its differentiation from drug accumulation and retention in hair after external exposure. The ability to distinguish ingested drug from externally applied drug has been debated (Blank and Kidwell, 1995; Kidwell and Blank, 1996; Cone *et al.*, 1991; Baumgartner and Hill, 1992). Ultimately, a solid understanding of the chemical mechanisms of drug deposition into the hair matrix and the dynamics of drug partitioning within the hair matrix is necessary to establish the validity of using hair sampling for drug detection.

This work was supported by National Institutes of Health Grant DA09545.

Send reprint requests to: James A. Ruth, UCHSC, Campus Box C238, 4200 East Ninth Ave., Denver, CO 80262.

Despite the potential problems associated with hair analysis, hair sampling for drug detection remains a powerful alternative for detecting the use of abused drugs for long periods of time. This reduces the necessity for testing to occur within a short time after exposure, as is required for blood and urine tests. Many drugs disappear rapidly from the urine and blood; therefore, these tests, unlike hair tests, are of limited value in determining a history of drug use.

Fentanyl is one such drug. Because of its narcotic and addictive properties, it has a high likelihood of being abused (Poklis, 1995; Schwartz *et al.*, 1994). Fentanyl is primarily used as a surgical analgesic and anesthetic agent; therefore, the primary abusers of fentanyl are reported to be medical personnel, because of their increased access (Poklis, 1995). Schwartz *et al.* (1994) were able to detect, by GC/MS, fentanyl in urine only up to 48–72 hr after administration of very large doses. Thus, a test that can detect fentanyl use for a longer period of time after dosing is necessary for effective screening of possible abuse.

Although much work on drug detection in hair has been performed, relatively little work has been done regarding the deposition of fentanyl into hair. Selavka *et al.* (1995) determined (by a GC/MS method) fentanyl concentrations in the hair of one suspect to be 0.02 ng/mg of hair. Wang *et al.* (1993) determined fentanyl concentrations (using an immunoassay method of detection) in the hair of patients after the administration of fentanyl for surgery. They determined the range of fentanyl concentrations to be 0.013–0.048 ng/mg of hair, with no significant dose-response relationship. To our knowledge, no systematic studies have examined fentanyl deposition in hair after chronic dosing.

The first objective of this study was to determine the detectability of fentanyl in hair, using a controlled animal model involving chronic fentanyl dosing. Animal models of drug deposition into hair are useful, because they allow for greater experimental control than available with humans while still allowing reasonable comparisons with *in vivo* conditions for human subjects (Gygi *et al.*, 1995). The mouse model used allowed for rigorous control of the quantity and timing of doses. This model also allowed for the control of pigmentation and, by using albino mice, drug deposition resulting from protein interactions could be distinguished from interactions with hair pigments. Additionally, other factors that affect the structure of the hair, such as diet, interindividual genetic variation, and chemical and cosmetic treatments of the hair, are more easily controlled in an animal model than in humans. Lastly, at 23 days of age, mice are entering their second period of synchronized hair growth for 24 days (Hamilton *et al.*, 1974), so the hair was uniformly growing and incorporating drug during the dosing period used in this study. Therefore, an animal model allowed for rigorous scientific control.

We also examined external loading of fentanyl into drug-free hair. Because the use of blank hair samples soaked in solutions of drug is a common practice for producing calibration standards, the suitability of this method for producing standard materials should be meticulously examined. Therefore, the second objective of this study was to examine aspects of the partitioning of externally applied drug into hair.

Incorporation of drugs, such as fentanyl, from the circulation or from external application might involve ionizable polar functional groups (such as amino and carboxylic acid groups) within the hair that could function as binding sites. The same might be true with nonionizable polar functional groups (such as hydroxyl groups). Kidwell and Blank (1996) have proposed a model for drug incorporation in which hair functions as an ion exchange membrane. They suggested that cationic species have greater affinity for hair and that this interaction is generally with carboxyl groups within the hair. To examine this, they esterified carboxylic acid groups within the hair with methanolic HCl and then examined the binding of cocaine to the hair. They indicated that the binding of cocaine would be reduced because of the treatment with acid even without esterification, and they attempted to control for this effect by soaking the hair in an aqueous buffer solution for 5 days before the binding experiments. They found that less cocaine could bind to the treated hair, suggesting that carboxylic acid groups participated in the binding of cocaine to hair.

Joseph *et al.* (1997) suggest that drugs are incorporated into hair not as an ion exchange process but as ligand binding to specific sites within the protein matrix of the hair. They contend that hair has sites that can act as cocaine receptors, because they observed cocaine binding to be reversible, stereoselective, and saturable. Additionally, they suggest that this binding site for cocaine is melanin. However, melanin might not be the principle binding site for all drugs; Ishyama *et al.* (1983) found that methamphetamine bound similarly to both light and dark hair, suggesting that drug binding might be drug specific and for some drugs might involve protein binding sites.

The third purpose of this study was to determine to what extent chemical modification of specific, polar, ionizable functionalities (carboxylic acid and amino groups) within the hair protein matrix affected the deposition and recovery of fentanyl from hair subjected to external loading. These functionalities were chemically modified to less polar, nonionizable groups, and the dynamics of external drug loading were observed. These modifications used chemical methods that did not require exposure to extreme pH conditions, as required by esterification with methanolic HCl (Kidwell and Blank, 1996), which could adversely affect the protein structure through methanolysis of amide linkages and introduce confounding effects. Modifications were independently verified by FT[1]-IR and by pH titration.

Materials and Methods

Hair Samples. For all procedures, hair samples were obtained from untreated or treated BALB/c mice by shaving with an electric shaver to within 1/100 inch of the skin. For the *in vitro* procedures and modified hair procedures, hair obtained from untreated mice was washed thoroughly in methanol to remove external oils or other contaminants that might affect subsequent experiments.

GC/MS Analysis. All samples were analyzed using a Hewlett-Packard 5890 gas chromatograph coupled to a 5972 mass-selective detector. GC separation used an HP-5 column (25 m $\times$ 0.25 mm $\times$ 0.25 μm). Head pressure was maintained at 21 kPa. An injection aliquot of 5 μl was used. The temperature was maintained at 80°C for 1 min, ramped at 30°C/min to 150°C, and then ramped at 20°C/min to a final temperature of 300°C; the purge valve was turned on at 1 min. The retention time for fentanyl under these conditions was approximately 13.0 min (fig. 1).

Electron ionization MS in the single-ion monitoring mode was used for quantitation. Fentanyl-d_5 (Radian, Austin, TX) was used as an internal standard for all samples, and the *m/z* 245/250 area ratio was used to quantify all samples with qualifier ions of *m/z* 146, 151, 189, and 194 (fig. 1). A Hewlett-Packard RTE integrator was used to determine all areas. The dwell time was set at 100 msec for each ion, and the electron multiplied voltage offset was set to 600 eV above tune. The instrument was tuned daily.

Calibration. A calibration curve was established for a 1-ml sample volume. Standards were diluted in phosphate buffer or methanol, from certified drug standards (Radian), to 0.100, 0.500, 1.0, 5.0, 10.0, 50.0, and 100.0 ng/ml. Two to four replications were used for each of the concentrations. A simple linear regression was performed using Statgraphics 6.0 software (Manugistics). The model line obtained had the equation of ratio = 0.196·concentration, with r^2 = 99.7% and r = 0.998. Lack-of-fit testing demonstrated no significant model lack of fit. The limit of quantification was approximately 0.2 ng/ml (or 0.02 ng/mg of hair), as determined by the response that was significantly distinguishable from 0 at a 95% confidence level.

***In Vivo* Procedures.** Twenty, 23-day-old, male BALB/c mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed in four groups of five mice each. Because almost all excreted fentanyl appears in the urine as norfentanyl (Poklis, 1995) and we analyzed fentanyl levels, urine contamination of the hair was not a significant problem with group housing. Mice were housed at the University of Colorado Health Sciences Center Animal Resource Center, under approved protocols. Fentanyl was obtained as fentanyl citrate (50 μg/ml USP; Abbott Labs, Chicago, IL). Mice were weighed and then given ip injections on Monday, Wednesday, and Friday for 3 weeks. Solutions used to achieve doses of 0.02, 0.05, and 0.1 mg/kg were prepared in 0.9% saline solution. These doses were selected to encompass the ED_{50} for rats of 52 μg/kg, as determined by Shingu *et al.* (1983). Control mice were given injections of saline. Mice were weighed once each week, to ensure that the mice gained weight during the study period.

After 3 weeks, mice were sacrificed by cervical dislocation and hair was shaved from the backs of the mice. Hairs were 5 mm in length or less and were left intact for all extractions. Aliquots of 10 mg of hair were washed for 30 sec with methanol, and the hair was mixed with 1 ml of phosphate buffer. After

[1] Abbreviations used are: FT, Fourier transform; TMS, trimethylsilyl.

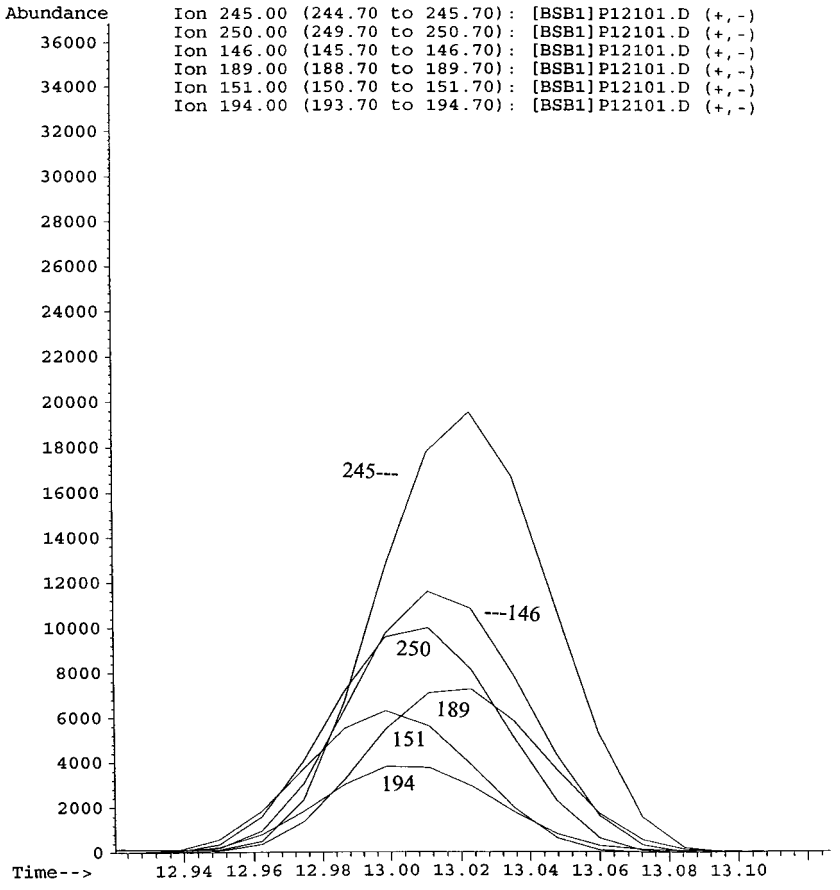

FIG. 1. *Sample ion chromatograph showing ions monitored in the single-ion monitoring mode.*

This sample was from a solution control. The concentration was determined to be 9.5 ng/ml by using fentanyl-d_5 (*m/z* 250, 151, and 194) as the internal standard and taking the 245/250 ratio.

incubation overnight at 45°C, the solution was then decanted, internal standard was added (5 ng/ml), and fentanyl was isolated by solid-phase extraction.

Solid-phase extraction was accomplished using solid-phase, ion exchange extraction columns (Clean Screen ZSDAU020; United Chemical Technologies, Bristol, PA). The procedure followed was the procedure recommended by the manufacturers. Briefly, columns were conditioned sequentially with water, methanol, and phosphate buffer, pH 6. The samples were then applied to the columns. After sequential washing with water, 1 M acetic acid, and methanol, the analytes were eluted with a methylene chloride/isopropanol/ammonia (78:20:2) solution. This was evaporated to dryness, and the samples were reconstituted in 50 μl of ethyl acetate. The samples were then analyzed by GC/MS.

External Loading of Nonmodified Hair. *pH Titration of Hair.* Ten milligrams of drug-free albino hair were suspended in 10 ml of distilled deionized water. The pH values were monitored with an Accumet glass pH probe and meter (Fisher Scientific, Pittsburgh, PA) as equivalents of NaOH or HCl were added. The titration curve was constructed by expressing all acid and base additions in micromole-equivalents of NaOH per milligram of hair. Two titrations of separate aliquots of hair were performed, to determine the reproducibility of the titrations.

Loading of Hair at Different pH Values. To examine the effect of hair matrix ionization on the external loading of fentanyl into hair, hair was loaded after addition of varying concentrations of NaOH to the methanolic solution. Ten milligrams of hair were suspended in 10 ml of methanol, and 10 ng/ml fentanyl was added as fentanyl citrate. Sodium hydroxide was added to achieve concentrations of 0, 0.001, 0.005, and 0.008 μM. Samples were then continuously stirred for 24 hr. Solution aliquots, methanol washes, and hair extracts were prepared and analyzed as described above.

External Loading Experiments. Drug-free BALB/c mouse hair was obtained, and 10-mg samples were incubated in 10 ml of methanol made basic by the addition of 0.001 μM NaOH. Fentanyl was added to achieve concentrations of 10, 50, and 100 ng/ml. Control samples, without hair, were concurrently analyzed to quantitate fentanyl loss resulting from the basic conditions of the solution. Samples were continuously stirred for the entire time period. At each time point (4, 20, 44, and 68 hr), 1-ml aliquots of the methanolic solutions were removed. These were evaporated to dryness and reconstituted in 50 μl of ethyl acetate.

Hair was separated from solution at each time point, allowed to dry overnight, weighed, and vortex-mixed with methanol for 30 sec. Because fentanyl is an amide drug that is hydrolyzed under the harsh acidic or basic conditions of typical hair extractions, various milder conditions have been suggested for fentanyl extraction. Dilute HCl was used by Selavka *et al.* (1995), methanol was used by Wang *et al.* (1993), and Sorensen buffer, pH 7.6, was used by Uhl (1997). We chose a modified method using phosphate buffer (1 ml, 100 mM, pH 6), in which samples were incubated overnight at 45°C. The solution was then decanted, mixed with internal standard (5 ng/ml), and prepared as described above for the *in vivo* hair samples.

Additional hair samples were soaked in phosphate buffer with 10, 50, or 100 ng/ml fentanyl for 44 hr. Aliquots of the phosphate buffer solution were prepared using the same solid-phase extraction columns mentioned above, and the hair was treated in the same manner as previously described.

Modified Hair Experiments. *Chemical Modification of Hair.* Esterification of solution-accessible carboxylic acid groups within the hair to methyl esters was accomplished by reaction with diazomethane in ether. Diazomethane was produced by reacting 1 g of Diazald (Aldrich, Milwaukee, WI) with 1 g of KOH in a diazomethane generator (Aldrich). Hair (200 mg) was presoaked in ether to reduce the potential formation of explosive diazomethane crystals. This hair was then combined with the diazomethane/ether mixture and allowed to react overnight. The excess diazomethane and ether were allowed to evaporate.

Solution-accessible amino and hydroxyl groups within the hair were acetylated to amides and acetyl esters, respectively, by reaction with acetic anhydride and pyridine. Hair (200 mg) was combined with 2 ml of acetic anhydride (Mallinckrodt, Paris, KY) and 100 μl of pyridine (Aldrich) at 70°C for 2 hr. The excess reagent was decanted, and the hair was washed several times with water. Dilute HCl was added to form a salt with pyridine (to facilitate its removal), and the hair was washed extensively with distilled deionized water.

All solution-accessible polar functionalities within the hair were modified to TMS derivatives by reaction with bis(trimethylsilyl)trifluoroacetamide/1% trimethylchlorosilane (Sigma-Aldrich, St. Louis, MO). Two hundred milligrams of hair, under nitrogen, were combined with 2 ml of bis(trimethylsilyl)trifluoroacetamide/1% trimethylchlorosilane and allowed to react at 70°C for 2 hr. Excess solution was decanted, and the hair was dried under a stream of nitrogen.

Characterization of Modified Hair. To characterize changes in the chemistry of the hair samples after each chemical modification, titration curves were constructed by suspending 10 mg of hair in 10 ml of distilled deionized water. A glass pH probe (Accumet Basic; Fisher Scientific) was used to monitor the pH as aliquots of NaOH and HCl were added and allowed to equilibrate. Two separate samples of each type of modified hair were titrated, to ensure the reproducibility of the results.

FT-IR is a standard tool for the investigation of protein structure and was used to assess gross changes in the structure or conformation of hair proteins after chemical modifications (Bandekar, 1992; Arrondo *et al.*, 1993). Primary and secondary protein structures can be determined by examining the spectral patterns in the amide I region (1720–1550 cm^{-1}). This spectral band corresponds to the C═O stretch, with some contribution from the C—N stretch and the C—C—N deformation (Bandekar, 1992), and is sensitive to the local environment of those bonds within the protein. Analyzing these data by performing FT procedures and obtaining second derivatives to resolve absorption peaks is a standard procedure (Arrondo *et al.*, 1993). Therefore, this method was used to independently verify the reduction or loss of specific functional groups.

FT-IR spectra were obtained for samples of untreated hair and samples of hair after all chemical modifications using a Nicolet Magna-IR 550 spectrometer. Approximately 0.65 mg of hair was annealed into 0.300 g of KBr (Aldrich). Second-derivative normalized spectra in the amide I region were obtained using OmniC analysis software (Nicolet, Madison, WI).

External Loading Experiments with Modified Hair. Aliquots (10 mg) of drug-free BALB/c mouse hair were incubated in 10 ml of methanol with 0.001 μM NaOH, so that fentanyl would be present as the free base. Identical samples were incubated in methylene chloride with free-base fentanyl extracted into the loading solution. Fentanyl was added to achieve a concentration of 10 ng/ml. Samples were continuously stirred for 24 hr, after which 1-ml aliquots of solution were removed. These were evaporated and reconstituted in 50 μl of ethyl acetate. This allowed determination of fentanyl removed from the incubation solution. Controls without hair were used to determine drug stability in solution.

To determine extractable fentanyl contents, hair was separated from the solution after 24 hr and allowed to dry overnight. The sample was then weighed, to account for any hair lost in the transfer, and vortex-mixed with methanol (2 ml) for 30 sec. This wash was decanted and mixed with 5 ng/ml internal standard, the solvent was evaporated, and the sample was reconstituted in 50 μl of ethyl acetate for analysis. After the hair samples were allowed to dry, phosphate buffer (1 ml, 100 mM, pH 6) was added and the samples were incubated overnight at 45°C. The solution was decanted, 5 ng/ml internal standard was added, and fentanyl was isolated by solid-phase extraction, as described previously.

Statistical Analysis. All data from these procedures were analyzed by analysis of variance, followed by least-significant difference, multiple-range testing, using Statgraphics 6.0 software. Significance was assumed for all interactions at $\alpha = 0.01$.

Results

***In Vivo* Results.** The aqueous extractable fentanyl levels (mean ± SE, $N = 5$) are presented in fig. 2. The extractable fentanyl concentrations of the dosed groups were significantly different from those of the control animals, although no significant differences were observed among dosed groups. The extractable fentanyl levels appeared to plateau with increasing doses. Additionally, no fentanyl was detectable in the methanol washes of the mouse hair, suggesting that group housing of the mice did not result in significant contamination of the hair.

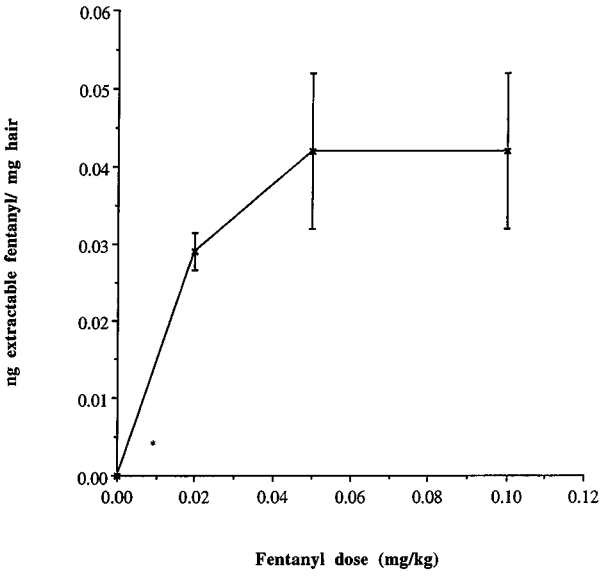

FIG. 2. *Fentanyl extractable, by soaking in phosphate buffer for 24 hr at 45°C, from hair of mice dosed at various doses (mean ± SE,* N = *5).*

Dosed mice had significantly more extractable fentanyl in their hair than did nondosed mice. *, below the limit of quantification.

pH Titration Results. Titration results are presented in fig. 3. The initial pH of the hair suspension was approximately 4.45. Upon titration with NaOH, a reproducible buffering effect was seen at approximately pH 7.4, as demonstrated by the plateauing in the curve at pH 7.4. It is apparent from this plot that enough NaOH needed to be added to the solution to overcome the buffering capacity of the hair and make the solution basic enough that fentanyl was present as its free base. This was achieved at an NaOH concentration of 0.001 μM NaOH (represented in fig. 3 as 5 μmol-equivalents of NaOH/mg of hair). This concentration was used in all of the loading experiments. This concentration of NaOH produced a solution pH of approximately 9.5.

Results of Loading at Different pH Values. Fig. 4 shows the loss of fentanyl from the loading solution as a difference from the solution controls (loading solution with no hair present). A significant pH effect was observed in the amount of fentanyl lost from solution, relative to controls, with significantly more fentanyl being lost from solution as the concentration of NaOH was increased.

Fig. 5 presents the detectable fentanyl levels in the 30-sec methanol washes of hair loaded at various concentrations of NaOH. The detectable fentanyl levels in the methanol washes of the 0.008 μM samples only were significantly greater than 0; this is consistent with the results seen in fig. 8.

In fig. 6, a significant effect of pH on the fentanyl extractable from the hair by phosphate buffer extraction was also observed. The amount of fentanyl recovered from the hair after loading increased with increasing concentrations of NaOH up to 0.005 μM NaOH. No significant difference between the 0.005 μM and 0.008 μM series was observed.

External Loading Experiment Results for Nonmodified Hair. Fig. 7 illustrates the concentration of fentanyl remaining in the meth-

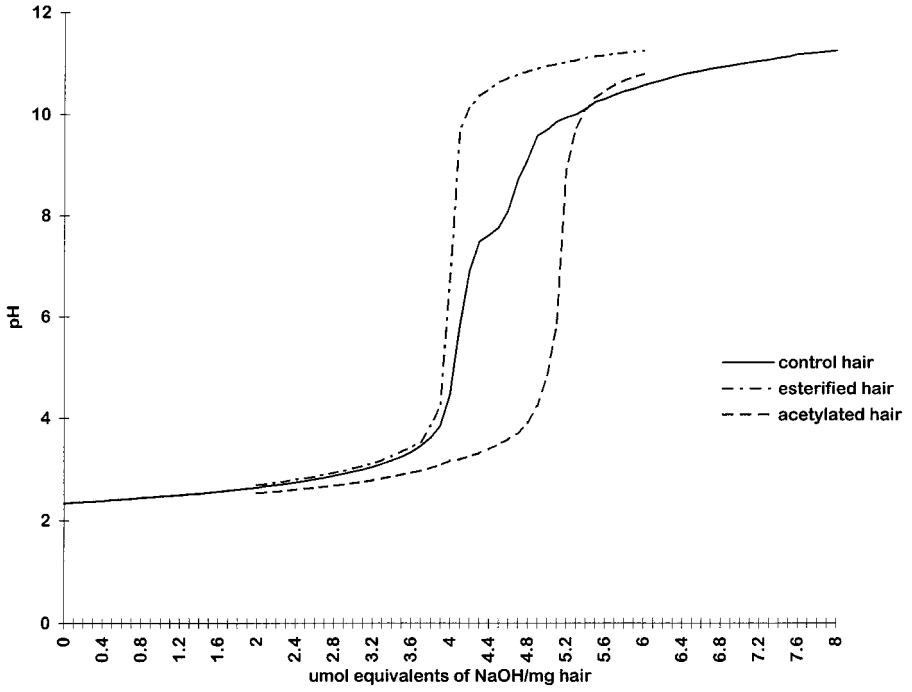

FIG. 3. *pH titration curves for normal and modified hair.*

Two replicates of titration of hair are shown. Hair was titrated from its initial pH when suspended in water with NaOH or HCl. The initial pH of the solution after equilibration of the hair with the water was 4.5. A reproducible buffering effect was evident at approximately pH 7. The 0.001 μM concentration of NaOH used in subsequent loading experiments is represented on this graph as 5 μmol-equivalents of NaOH/mg of hair; this concentration of NaOH resulted in a pH of 9.5. Acetylated hair appears more acidic, as marked by the shift in the titration curve to the right of the curve for control hair. This is consistent with a loss of amino groups that could act as bases. Esterified hair appears more basic, as marked by the shift in its curve to the left of the curve for control hair. Again, the results are consistent with a loss of ionizable carboxylic acid groups. Both modifications result in the loss of the buffering capacity evident in the control hair at pH 7.6.

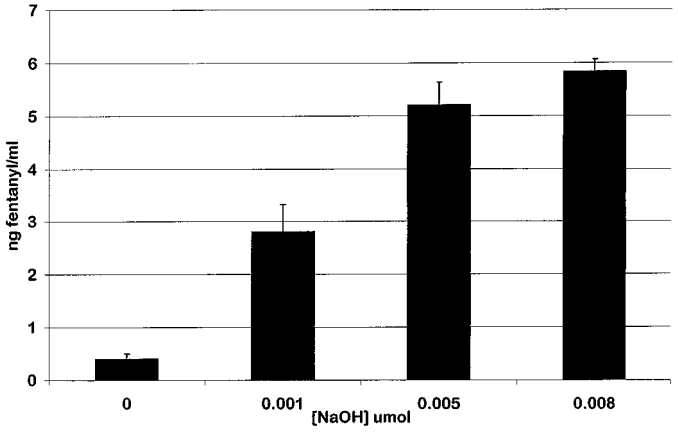

FIG. 4. *Fentanyl remaining in solutions used to load hair at various concentrations of NaOH in methanol (mean ± SE,* N = *3).*

The plot represents the differences between solution controls (with identical solution conditions but with no hair) and sample loading solutions after 24 hr. The pH effect was significant at the $\alpha = 0.01$ level.

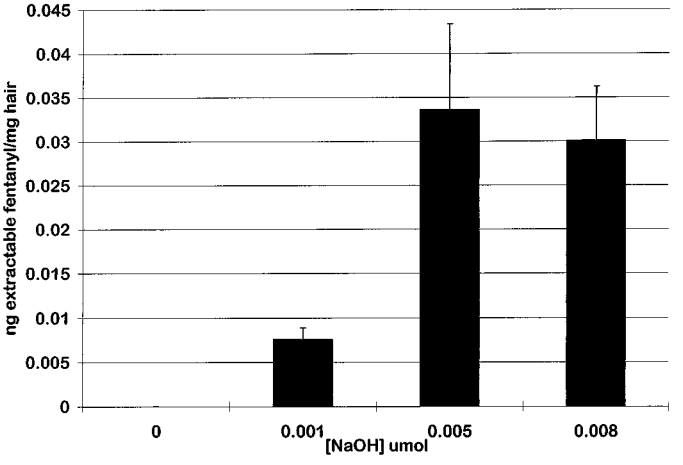

FIG. 5. *Fentanyl recovered in methanol washes of hair exposed to fentanyl in methanolic solutions containing various concentrations of NaOH (mean ± SE,* N = *3).*

The plot represents the amount of fentanyl detected in the 30-sec methanol washes of hair after loading in methanolic solutions with varying concentrations of NaOH. Only the 0.008 μM samples were significantly greater than 0 at the $\alpha = 0.01$ level.

anolic solution used for the external loading experiments at each of the time points. The decreases in the concentration in solution, at each of the concentrations, over time were significant. In all concentration series, the concentration reached a plateau after approximately 20 hr. No significant loss of fentanyl from solution was observed at any of the concentrations for the analogous aqueous phosphate buffer experiments.

The amounts of fentanyl detected in the 30-sec methanol washes are shown in fig. 8. All amounts were normalized as nanograms per milligram of hair, to facilitate comparison. No fentanyl was detectable in the washes of the hair exposed to 10 ng/ml initial concentrations. Fentanyl concentrations in the methanol washes in the 50 and 100 ng/ml series were not significantly different, but the appearance of fentanyl in the wash was significant after 4 hr. No fentanyl was detectable in the methanol wash of hair loaded in phosphate buffer.

No significant relationship between initial loading concentrations and levels of fentanyl extractable from exogenously loaded hair was detected (fig. 9). A significant time-dependent effect was evident,

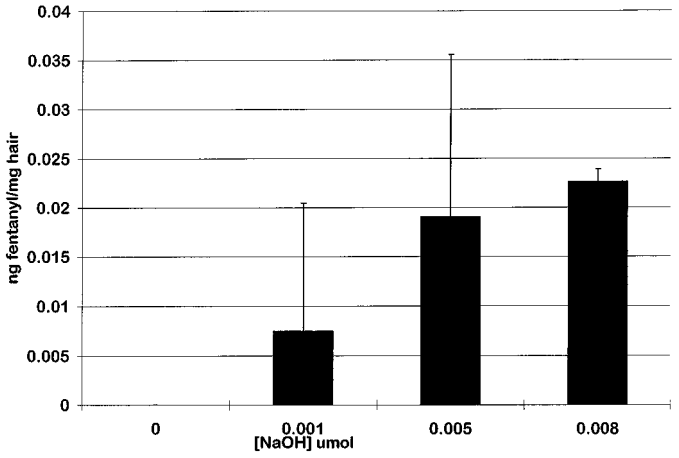

FIG. 6. *Aqueous extractable fentanyl from hair exposed to fentanyl in methanolic solutions containing various concentrations of NaOH (mean ± SE,* N = *3).*

The plot represents the amount of fentanyl detected in the phosphate buffer extractions of hair after loading in methanolic solutions with varying concentrations of NaOH. The pH effect was significant at the $\alpha = 0.01$ level.

with the 20-, 44-, and 68-hr time points differing significantly from the 0- and 4-hr points. As can be seen in fig. 9, the extractable fentanyl level also plateaued at approximately 20 hr. Additionally, amounts of fentanyl approximately 10 times the extractable fentanyl amounts in the *in vivo* experiments were extracted from the externally loaded hair. No additional fentanyl was detected in a subsequent 24-hr, 40°C methanol extraction. No significant fentanyl was extractable from the hair loaded in phosphate buffer, nor was any fentanyl detected in the methanol wash of the phosphate buffer-loaded samples.

The amounts of fentanyl lost from solution and fentanyl recovered from the hair were also analyzed as a mass balance. Table 1 shows a sample mass balance calculation for the 10 ng/ml initial loading concentration at 44 hr. All recovery rates for other times and concentrations were similar to or lower than the approximately 6% presented in table 1.

Modified Hair Experiments. *pH Titration Results.* Results of pH titrations of control, esterified, and acetylated hair are shown in fig. 3. TMS-derivitized hair was not titrated, because the TMS derivatives are labile in aqueous solutions (Nakamuro and Kuwajima, 1993). Results from titrations of two separate aliquots of esterified, acetylated, and control hair are shown in fig. 3, to demonstrate reproducibility.

Acetylated hair demonstrated a more acidic nature, as shown by the shift of the titration curve to the right. The loss of the buffering capacity evident with the control hair at approximately pH 7.4 was also observed for the acetylated hair. These results were consistent with the loss of amino groups within the hair and subsequent loss of the basic nature of the hair.

Esterified hair demonstrated a more basic nature, as evidenced by the shift of the titration curve to the left of the control sample curve. The esterified hair also demonstrated a loss of the buffering capacity observed in the control hair. These results are consistent with the loss of carboxylic acid groups and, thus, the loss of the acidic component of the hair.

FT-IR Results. FT-IR results for each of the three modification methods are presented in figs. 10– 12. All figures show two scans from two separate aliquots of control hair and modified hair. In all cases, the two control spectra agree closely. FT-IR spectra demonstrated no significant differences between control and TMS-derivitized hair samples, either in the full spectra (400–4000 cm^{-1}) or in the second derivative of the amide I region (fig. 10). These data are

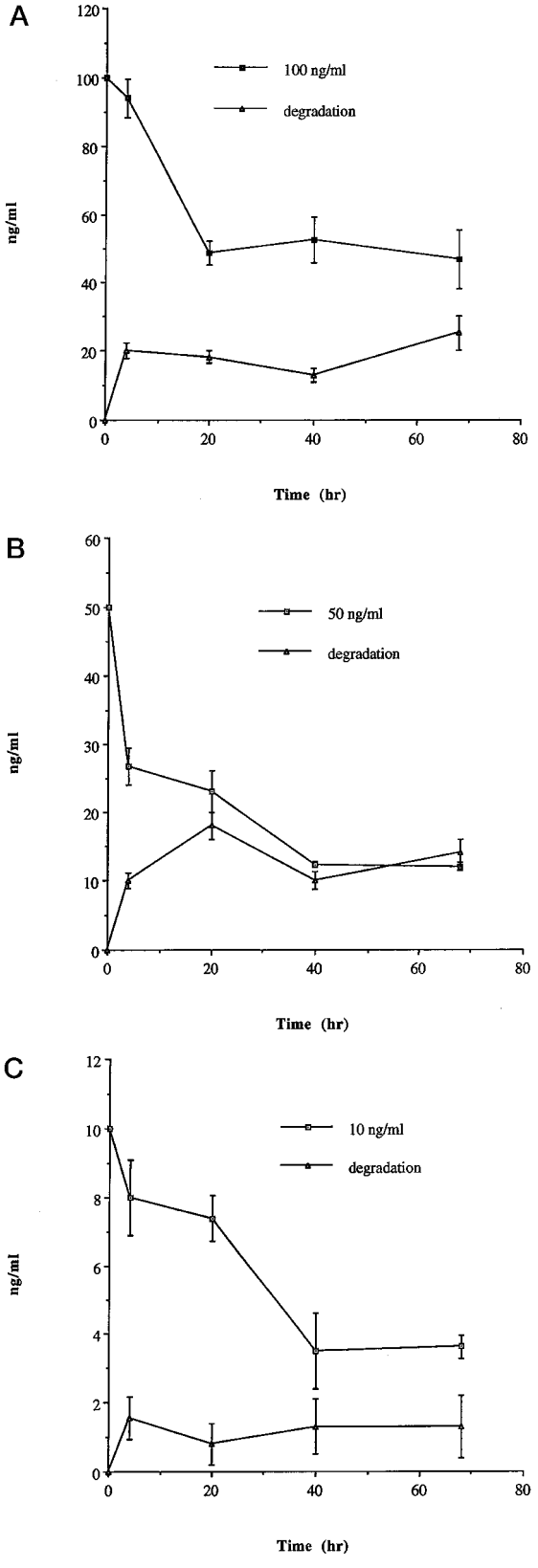

FIG. 7. *Concentrations of fentanyl remaining in methanolic solutions used to load hair* vs. *time (mean ± SE,* N = *3).*

A, Results for loading solutions with the initial fentanyl concentration of 100 ng/ml; *B,* results for loading solutions starting with 50 ng/ml; *C,* results for loading solutions starting with 10 ng/ml. *Upper lines,* concentrations of fentanyl in solution; *lower lines,* concentration of fentanyl lost at each time point in matched controls with no hair. Trends with time were significant at the $\alpha = 0.01$ level.

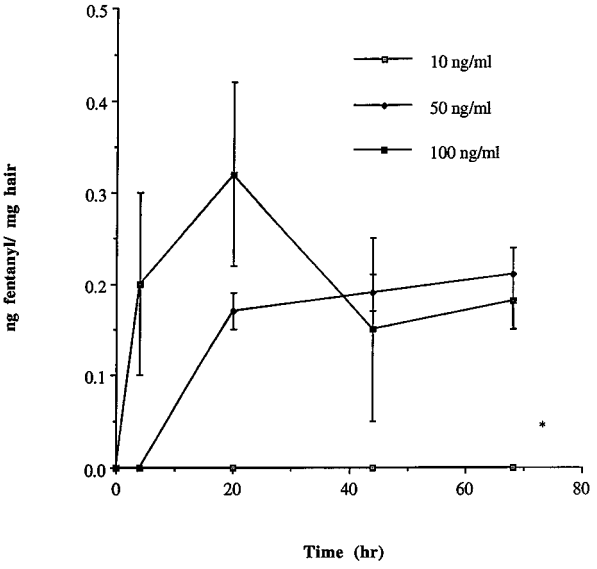

FIG. 8. *Fentanyl present in methanol washes of hair loaded in methanol* vs. *time (mean ± SE,* N = *3).*

The plot shows fentanyl present after various times in the methanol washes of hair loaded in solutions containing the indicated fentanyl concentrations and 0.001 μM NaOH. *, below the limit of quantification.

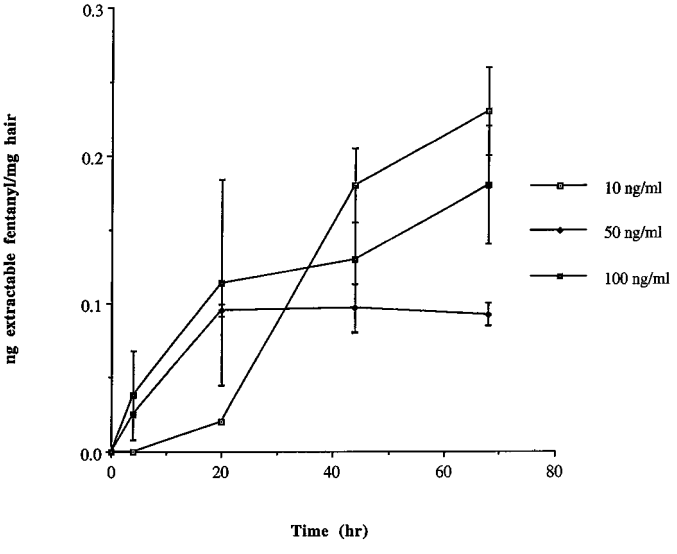

FIG. 9. *Aqueous extractable fentanyl from hair exposed to fentanyl in methanol* vs. *time (mean ± SE,* N = *3).*

The plot represents results of 24-hr 100 mM phosphate buffer extraction (45°C) of hair loaded in methanolic solutions with 0.001 μM NaOH.

consistent with the lability of the TMS derivatives in the methanolic fentanyl loading solution (Nakamuro and Kuwajima, 1993). Acetylation of hair resulted in a reduction in the intensity of absorbance at 1695 cm^{-1}, compared with controls (fig. 11). Esterified hair samples showed a reduction in the intensity of absorbance at 1594 cm^{-1} (fig. 12).

Loading Experiment Results. Fig. 13 illustrates the solution concentrations of fentanyl upon the loading of control hair and chemically modified hair. The results represent the loss of fentanyl from the loading solution and are presented as the differences of the test groups from matched solution controls containing no hair. Results for both methanolic and methylene chloride solutions are shown in fig. 13. Significantly less fentanyl was lost from the methanolic and methylene chloride solutions of the esterified hair than from all other groups. Also, significantly less fentanyl was lost from the methylene chloride solutions of all groups than from the methanolic solutions.

TABLE 1

Mass balance calculation using results from the 44-hr, 10 ng/ml loading concentration samples

Treatment	Mass
	ng
Original mass of fentanyl in system	100
Fentanyl removed in two analytical aliquots	20
Fentanyl lost to degradation (2 ng/ml × 8 ml)	16
Fentanyl removed by vortex-mixing with methanol	0
Fentanyl left in solution (8 ml with 4 ng/ml)	32
Fentanyl unaccounted for and lost to 10 mg of hair	32
Fentanyl recovered in phosphate buffer extraction of 10 mg of hair	1.8
Recovery (recovered fentanyl/fentanyl lost to hair)	6%

These data show that only a fraction of the fentanyl lost from solution to the hair can be recovered.

Fig. 14 shows the fentanyl recovered in the 30-sec methanol washes of loaded hair. No detectable fentanyl was recovered in any of the washes of the methylene chloride-loaded hair. Detectable fentanyl was recovered only in the washes of the methanol-loaded control (unmodified) and esterified hair.

The results of 24-hr extraction of all samples with phosphate buffer, pH 6, are shown in fig. 15. Significantly less fentanyl was recovered from both methanol- and methylene chloride-loaded esterified hair samples than from control hair. Additionally, significantly more fentanyl was recovered from the methylene chloride-loaded acetylated hair than from either the methanol-loaded acetylated hair or control hair.

Discussion

The results of *in vivo* fentanyl administration indicated that fentanyl was significantly detectable in the hair of animals subjected to chronic fentanyl dosing in a controlled animal model. The range of mean levels of fentanyl extractable from hair (0.025–0.05 ng/mg of hair) is consistent with the concentrations reported by Selavka *et al.* (1995) for an assumed chronic user of fentanyl and by Wang *et al.* (1993) for acutely treated surgical patients. This supports the appropriateness of this animal model to examine the deposition of fentanyl into hair. This may also indicate that the three different extraction methods, *i.e.* methanol (Wang *et al.*, 1993), dilute HCl (Selavka *et al.*, 1995), and phosphate buffer, are equally effective in extracting fentanyl from the hair matrix.

The data from *in vivo* mouse studies also indicated that fentanyl deposition plateaus as the dose increases; no linear dose-response relationship was observed. This is also consistent with the lack of a linear dose-response relationship observed after acute treatment of surgical patients by Wang *et al.* (1993). This suggests that the pool of fentanyl extractable from hair by ionization at low pH or with methanol is rapidly saturated at low doses and additional drug might be deposited in less accessible compartments within the hair. Additionally, this suggests that chronic fentanyl intake might not be distinguishable from acute intake by hair sampling.

The results of the experiments using external loading in methanol demonstrate that the solution of fentanyl reaches an equilibrium with the hair after approximately 20 hr, as demonstrated by the plateauing in the solution fentanyl concentrations after this time (fig. 4). The distribution of fentanyl into the hair matrix appeared to be greatly affected by the pH of the loading solution. As the pH was increased by increasing the concentration of NaOH, the amount of fentanyl that partitioned into the hair increased (fig. 6). The significant amounts of

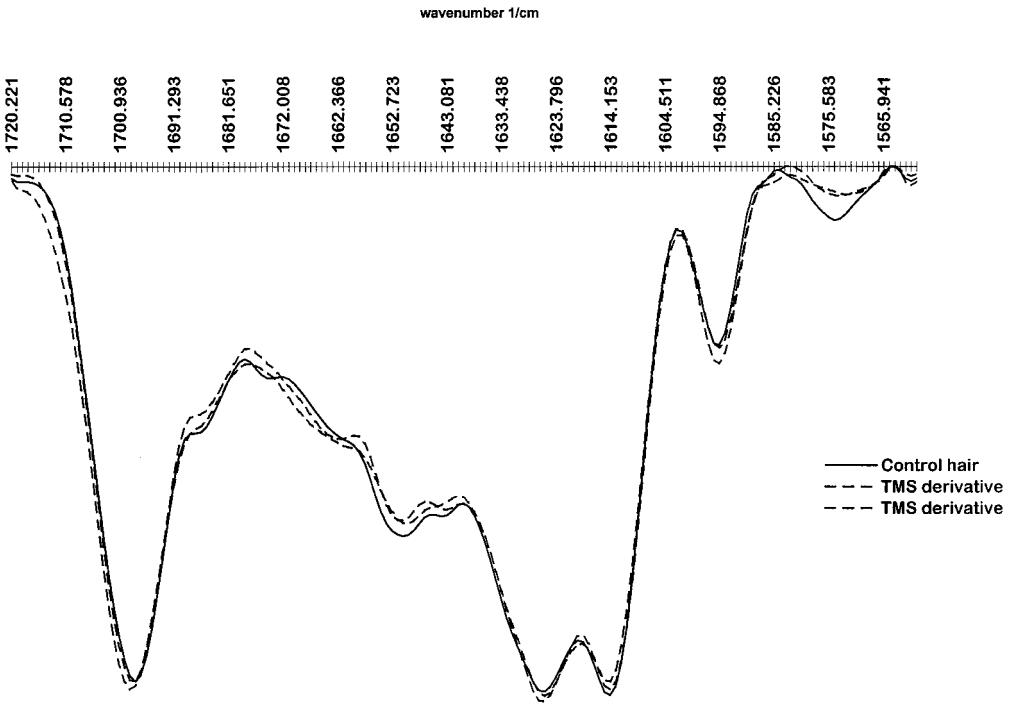

FIG. 10. *Second-derivative FT-IR spectra of the amide I region for TMS-derivitized hair, compared with control hair.*

Hair was derivitized with bis(trimethylsilyl)trifluoroacetamide/1% trimethylchlorosilane. No significant differences were evident in the FT-IR analysis. TMS derivatives were possibly labile under the experimental conditions (Nakamuro and Kuwajima, 1993).

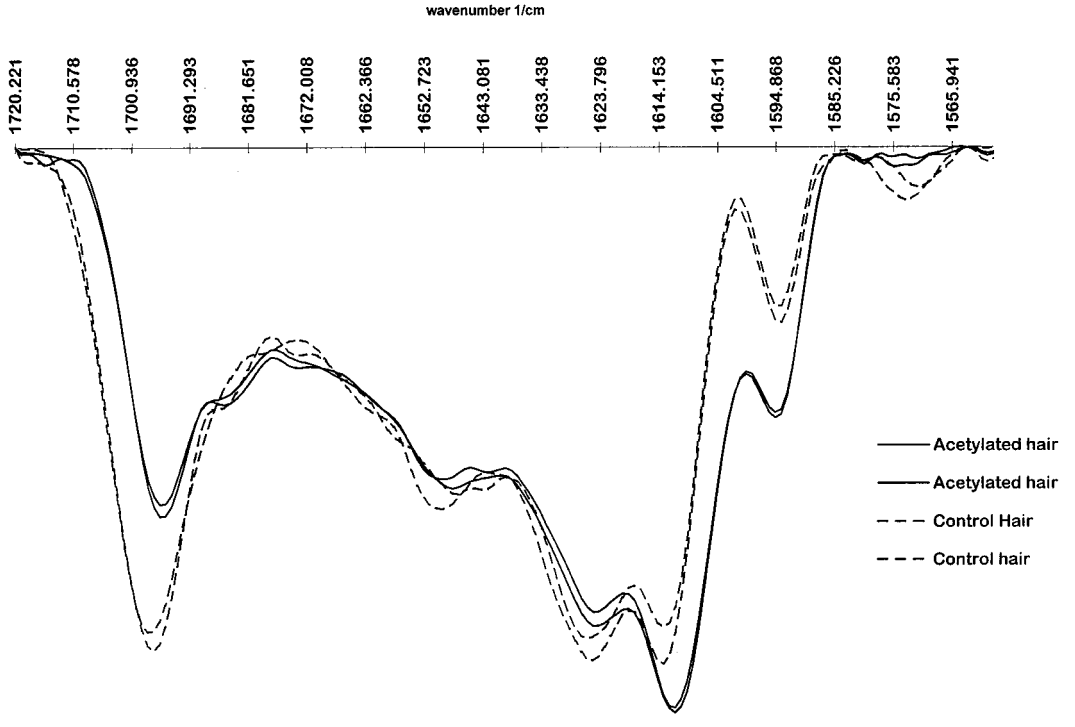

FIG. 11. *Second-derivative FT-IR spectra of the amide I region for acetylated hair, compared with control hair.*

Hair was acetylated by heating with acetic anhydride and pyridine, to acetylate amino groups to amides. The loss of absorption at 1695–1700 cm^{-1} indicates a loss of anti-parallel β-sheet secondary structure associated with this band (Arrondo *et al.*, 1993). This is consistent with a loss of hydrogen bonding interactions, which typically stabilize β-sheet structures, as would be expected with the formation of amides and acetyl esters.

fentanyl lost from solution when the hair was soaked in more basic methanolic solutions indicated that the nonionized form of fentanyl has greater access into the hair than does the ionized form of fentanyl in phosphate buffer. Additionally, because the amount of NaOH added to the loading solution far exceeded the stoichiometric amount necessary to form the free base of fentanyl, functional groups within the hair matrix must also have a pH dependence for fentanyl to partition into the hair. This is somewhat contrary to the model,

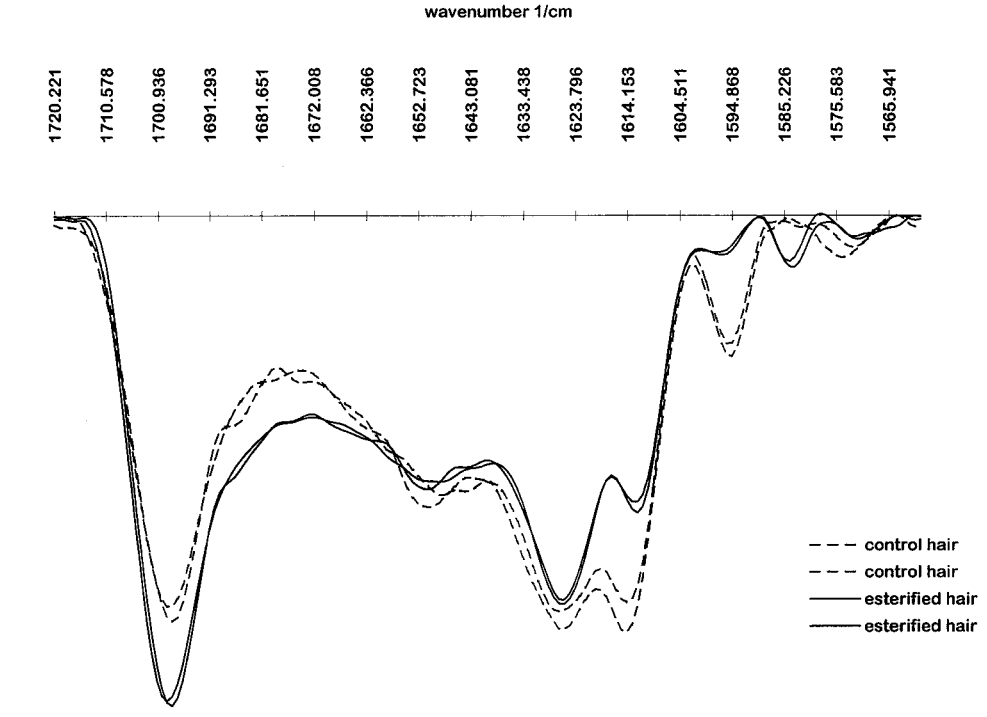

FIG. 12. *Second-derivative FT-IR spectra of the amide I region for esterified hair, compared with control hair.*

Hair was esterified by reaction with diazomethane in ether. The loss of absorption at 1595 cm^{-1} indicates a loss of the COO^- stretch. This is consistent with the esterification of carboxylic acid groups to methyl esters.

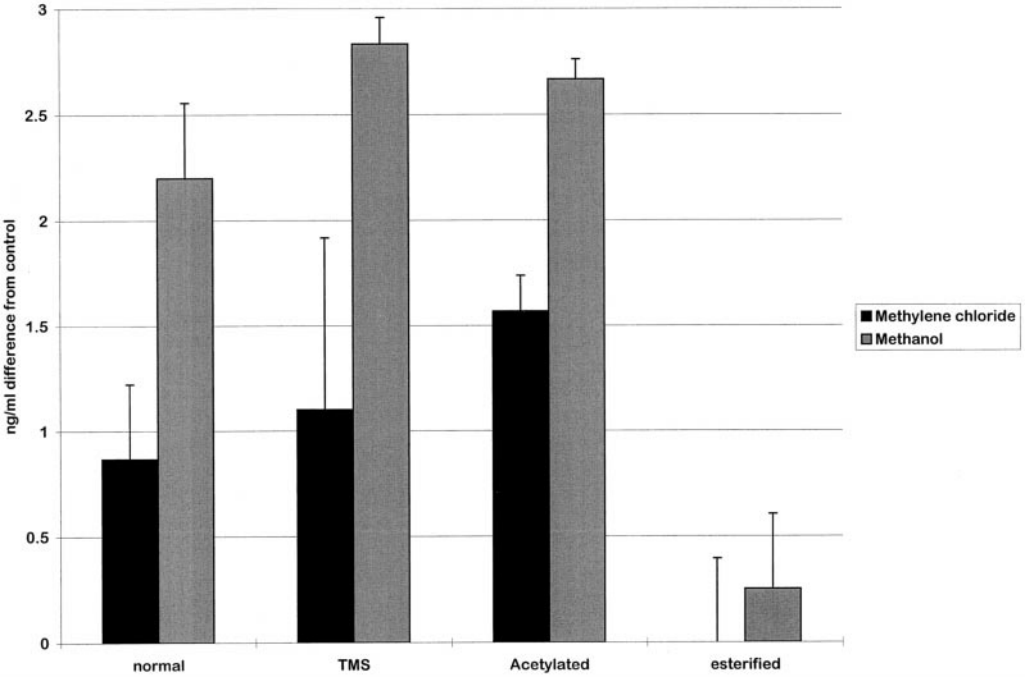

FIG. 13. *Loading experiment results.*

Results are presented as the differences between the test group and matched solution controls without hair. Significantly less fentanyl was lost from the loading solution of esterified hair, compared with all other groups. More fentanyl was lost from the methanol loading solutions than from the methylene chloride loading solutions. All results are the mean of three experiments; *error bars*, 1 SE.

proposed by Kidwell and Blank (1996) and Blank and Kidwell (1995), of hair as an ion exchange membrane that allows cations into the matrix more readily. However, our results may reflect the compound-specific nature of drug incorporation into hair, which may be more dependent on the structure of the drug and factors other than molecular charge (Uhl, 1997). This may reflect a greater affinity of nonionized fentanyl for more lipophilic compartments within the hair (see fig. 17).

Chemical modification of hair by reaction with diazomethane or acetic anhydride and pyridine to esterify or acetylate hair produced reproducible results, as verified by FT-IR. The band at 1695 cm^{-1} has

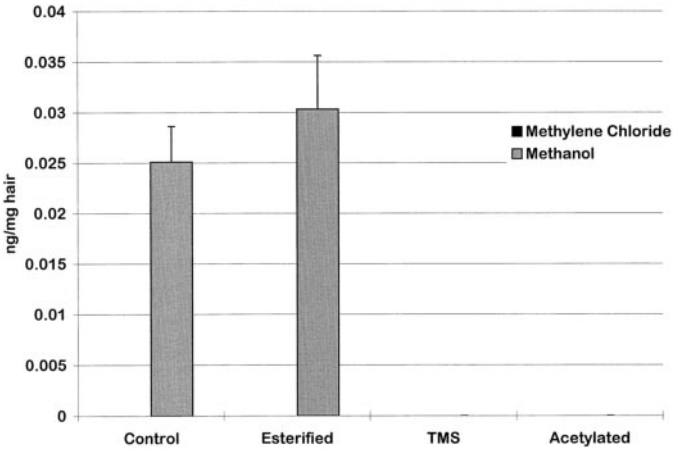

FIG. 14. *Fentanyl recovered from externally loaded hair in 30-sec methanol washes (mean ± SE, N = 3).*

Fentanyl was detectable in the washes of only the esterified and control hairs loaded in methanolic solutions. No fentanyl was detected in any of the methylene chloride-loaded hair. Results indicate that loading solvent has a significant effect and that methylene chloride may have access to more of the hair matrix than does methanol.

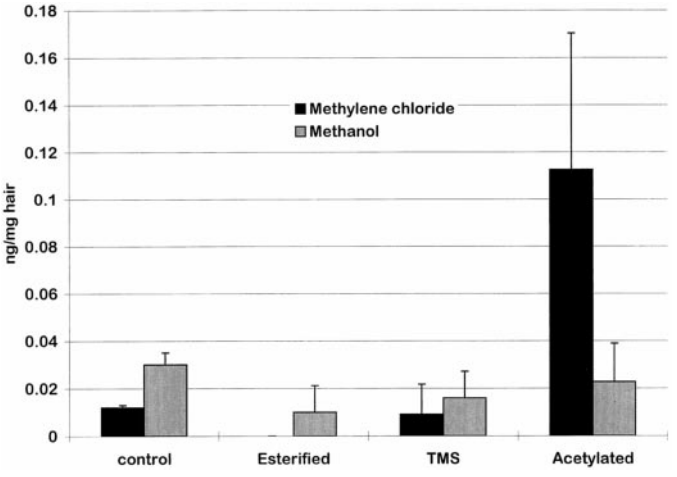

FIG. 15. *Fentanyl recovered in a 100 mM phosphate buffer, pH 6, extraction of externally loaded hair (mean ± SE, N = 3).*

Significantly less fentanyl was recovered from esterified hair than from control hair. Significantly more fentanyl was recovered from acetylated hair loaded in methylene chloride, compared with all other groups.

been associated with anti-parallel β-sheets (Arrondo *et al.*, 1993); therefore, these results suggest a loss of anti-parallel β-sheet structure of the proteins in acetylated hair. This is consistent with a loss of hydrogen bonding interactions, which typically stabilize β-sheet structures in proteins, as would be expected with the formation of amides and acetyl esters.

The absorbance band at 1594 cm^{-1} has been associated with the COO^- stretch (Venyeminov and Kalnin, 1990). The reduction in intensity of this band observed in esterified hair is consistent with the formation of methyl ester groups from carboxylic acid groups and the loss of that stretch component. The increase in absorption at 1585 cm^{-1} may be attributable to a slower stretch resulting from larger methyl groups substituted onto carboxylic acid groups. The reduced intensity of the band at 1594 cm^{-1} may be the result of reduction in some β-sheet characteristics secondary to the loss of hydrogen bonding by other groups in the protein.

By pH titration, acetylated hair demonstrated a loss of both buffering capacity and basic nature. Additionally, esterified hair lost both its buffering capacity and its acidic nature. Both of these results were consistent with the chemical modifications reducing the amino and carboxylic acid groups within the hair.

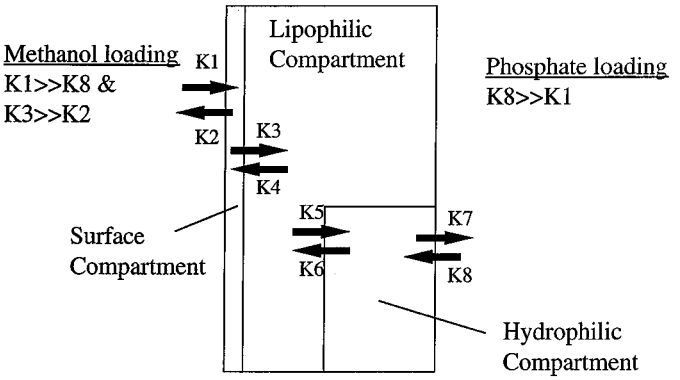

FIG. 16. *Proposed compartmental model for hair to describe the movement of fentanyl into and within the hair matrix for both ionized and nonionized drug application.*

K_1 and K_2 represent the kinetic coefficients describing movement into and out of a surface compartment. K_3 and K_4 describe the movement of fentanyl into a more interior lipophilic compartment. K_5 and K_6 describe movement between lipophilic and hydrophilic compartments. K_7 and K_8 describe transfer of fentanyl from the loading solution directly into a hydrophilic compartment. Thus, if fentanyl is present in the loading solutions as its free-base, lipophilic form, it partitions into the lipophilic compartment *via* the surface compartment ($K_1 \gg K_8$). Alternatively, phosphate buffer loading and extraction accesses the hydrophilic compartment ($K_8 \gg K_1$).

Loading experiments demonstrated that significant changes in both loading into hair and recovery from hair of fentanyl were associated with the modification of these functional groups. Esterification of hair resulted in significantly less uptake of drug and significantly lower recovery of drug, relative to controls, suggesting that carboxylic acid groups are necessary for the uptake of drug.

Acetylation of hair resulted in increased removal of fentanyl from methylene chloride loading solutions and increased recovery from these same samples. The same result was not observed in methanol loading solutions. This suggests that the loading solvent has a significant effect and that methylene chloride may have greater access to more lipophilic regions of the hair. Because the amides and acetyl esters formed by acetylation are less polar than the original amino and hydroxyl groups, acetylation potentially resulted in an increase in the lipophilicity of the hair matrix. Thus, the solvent differences observed might have resulted from differences in the polarity of the solvents used in loading.

These results demonstrate that solution-accessible ionizable functionalities of hair play a significant role in the accumulation and retention of fentanyl from external solution. Additionally, these results suggest that functional groups on proteins can play a significant role in the deposition and retention of drugs, specifically nonionized fentanyl, in hair without the presence of melanin.

A compartmental model is suggested in fig. 16, to describe the movement of fentanyl into and within the hair matrix for both ionized and nonionized drug application. K_1 through K_8 represent kinetic coefficients describing the partitioning of fentanyl between compartments. K_1 and K_2 represent the coefficients describing movement into and out of a surface compartment from the loading solution. K_3 and K_4 describe the movement of fentanyl into a more interior lipophilic compartment. K_5 and K_6 describe movement between lipophilic and hydrophilic compartments. K_7 and K_8 describe transfer of fentanyl from the loading solution directly into a hydrophilic compartment.

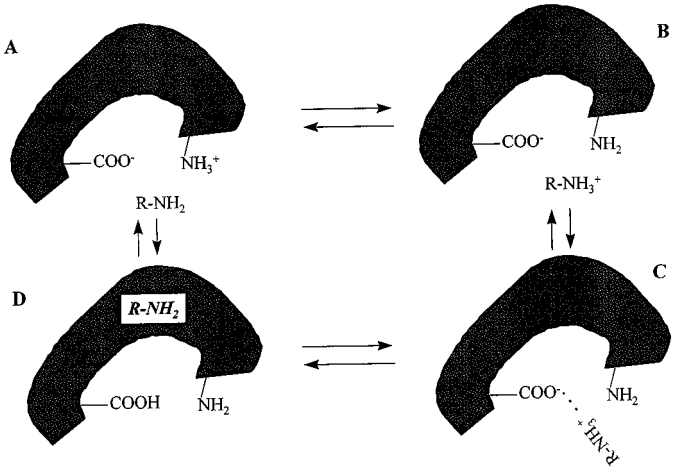

FIG. 17. *Proposed model for the mechanism of ionizable functional group participation in the deposition of nonionized drugs into hair.*

A, Generalized nonionized drug in proximity to an ionized protein in the hair matrix; *B*, proton exchange from the protein to the drug, thus adding a charge to the drug; *C*, facilitation, by this charge, of the association of the drug with the COO^- group, subsequent exchange of the proton back to the COOH group, and movement of the nonionized drug into a lipophilic compartment within the protein matrix. This model is based on the model of the H2 receptor proposed by Giraldo *et al.* (1992).

Thus, if fentanyl is present in its free-base lipophilic form, it would partition into the lipophilic compartment *via* the surface compartment. Aqueous loading of ionized fentanyl would partition directly into a hydrophilic compartment, as described by K_7 and K_8.

The amount of fentanyl extractable from the externally loaded hair also demonstrated no significant relationship with the concentration of the initial loading solution, also indicating possible saturation of the extractable compartment. This process again appeared to reach an equilibrium, because the significant time effect plateaued at about 20 hr. The lack of any relationship between loading concentration and extractable fentanyl is in contrast to the results of Selavka *et al.* (1995) and Wang *et al.* (1993), who found linear relations between their spiking concentrations and the amounts of fentanyl extractable from the hair. This may reflect the fact that, over the time that hair was exposed to the loading solutions, equilibria were reached between the solution and the hair and between compartments within the hair, thus saturating the extractable compartment (fig. 16).

The inefficiency of our aqueous extraction suggests that a large pool of tightly bound or inaccessible fentanyl within the hair was created by external loading with nonionized fentanyl. Kidwell and Blank (1996) and Blank and Kidwell (1995) found that significant amounts of phencyclidine may remain in hair exposed to phencyclidine vapor and then subjected to decontaminating washes. Cone *et al.* (1991) found that significant amounts of externally applied cocaine could be retained after numerous sequential washes of loaded hair. Therefore, our results are consistent with others in indicating that some drugs may be tightly bound or significantly occluded within the hair matrix even when applied exogenously.

A functionally larger, hydrophobic compartment, into which fentanyl is loaded, and a smaller, hydrophilic compartment, from which fentanyl is extracted, could explain the sequestration of a large amount of fentanyl from aqueous extraction. The surface compartment depicted in fig. 16, through which externally applied drug passes, could provide an easily extracted compartment from which the 30-sec methanol washes removed some drug.

Fig. 17 depicts a potential model for the roles of carboxylic acid and amino groups in the deposition of a free-base, nonionized, amine drug into hair. Proton transfers from protein to ligand are commonly invoked in receptor functioning (Giraldo *et al.*, 1992). A generalized amine drug is represented in fig. 17*A* by $R\text{-}NH_2$, with ionized amine and carboxylic acid groups of the protein matrix. A proton is exchanged to the drug from the charged amine (fig. 17*B*), thus facilitating the interaction of the drug with the COO^- group (fig. 17*C*). This interaction allows a nonionized drug to associate with a more polar, hydrophilic aspect of the hair matrix. Exchange of the proton back to the COO^- group (fig. 17*D*) removes the charge from the drug molecule and allows its association with a more lipophilic compartment of the hair matrix. Therefore, modification of COOH groups to nonionizable methyl esters would result in reduced storage of fentanyl.

Although this model differs, with respect to the function of carboxylic acid groups, from that proposed by Kidwell and Blank (1996), it reconciles the increased storage of nonionic fentanyl, relative to ionic fentanyl, and the observed participation of ionizable functionalities in drug uptake and retention. This model is consistent with the possibility of binding sites for drugs within the hair, as proposed by Joseph *et al.* (1997). This type of binding interaction is not dependent on the presence of melanin and could account for drugs that appear to bind independently of hair color, such as methamphetamine (Ishyama *et al.*, 1983).

In the preparation of externally loaded samples as control or calibration samples, it is unclear whether externally applied drug enters and leaves the hair matrix in a manner analogous to that of drugs deposited from the circulation. This is supported by the observation that the externally loaded hair samples, after 44 hr of exposure to either 10, 50, or 100 ng/ml fentanyl, showed extractable fentanyl levels 10 times those of the *in vivo* samples after administration of 0.02, 0.05, or 0.1 mg/kg for 21 days. This makes the use of externally loaded hair samples as calibration standards problematic. These calibration standards are used in a legitimate effort to eliminate matrix effects from the analysis of unknown samples but, if the extractable drug concentration within the hair is dependent on the time of exposure to the loading solution and the pH of the loading solution, resulting in differential partitioning of the drug within the hair, then these calibrators may not be representative of the concentrations of the drug in unknown samples. The large amount of fentanyl that remains in the hair after extraction also suggests, as do the results reported by Kidwell and Blank (1996) and Blank and Kidwell (1995), that endogenously deposited drug and exogenously deposited drug may be difficult to distinguish from one another.

Acknowledgments. The authors thank Dr. Brent Kendrick and Dr. John Carpenter for help with the FT-IR experiments.

References

Arrondo JL, Muga A, Castresana J and Goni FM (1993) Quantitative studies of the structure of proteins in solution by Fourier-transform infrared spectroscopy. *Prog Biophys Mol Biol* **59:**23–56.

Bandekar J (1992) Amide modes and protein conformation. *Biochim Biophys Acta* **1120:**123–143.

Baumgartner WA and Hill VA (1992) Hair analysis for drugs of abuse: decontamination issues, in *Recent Developments in Therapeutic Drug Monitoring and Clinical Toxicology* (Sunshine I ed) pp 577–597, Marcel Dekker, New York.

Blank DL and Kidwell DA (1995) Decontamination procedures for drugs of abuse in hair: are they sufficient? *Forensic Sci Int* **70:**13–38.

Cone EJ, Yousefnejad D, Darwin WD and Maguire T (1991) Testing of human hair for drugs of abuse. II. Identification of unique cocaine metabolites in hair of drug abusers and evaluation of decontamination procedures. *J Anal Toxicol* **15:**250–255.

Giraldo J, Martin M, Campello M and Pardo L (1992) Theoretical studies on the histamine H_2 receptor: molecular mechanism of action of antagonists. *Mol Pharmacol* **42:**373–381.

Gygi SP, Colon F, Raftogianis RB, Galinsky RE, Wilkins DG and Rollins DE (1995) Dose-related distribution of codeine and its metabolites into rat hair. *Drug Metab Dispos* **24:**282–287.

Hamilton E, Howard A and Potten CS (1974) Cell kinetics in the sebaceous glands of the mouse. *Cell Tissue Kinet* **7:**399–405.

Ishyama I, Nagai T and Toshida S (1983) Detection of basic drugs (methamphetamine, antidepressants, and nicotine) from human hair. *J Forensic Sci* **28:**380–385.

Joseph RE, Tsai W, Tsao L, Su T and Cone EJ (1997) *In vitro* characterization of cocaine binding sites in human hair. *J Pharmacol Exp Ther* **282:**1228–1241.

Kidwell DA and Blank DL (1996) Environmental exposure: the stumbling block of hair testing, in *Drug Testing in Hair* (Kintz P ed) pp 17–68, CRC Press, New York.

Nakamuro E and Kuwajima I (1993) Copper catalyzed conjugate addition of a zinc homoenolate, ethyl-3-[3-(trimethylsiloxy)cyclohex-2-enyl]propionate, in *Organic Synthesis Collective* (Freeman JP ed) pp 277–281, Wiley & Sons, New York.

Poklis A (1995) Fentanyl: a review for clinical and analytical toxicologists. *Clin Toxicol* **33:**429–447.

Schwartz JG, Garriott JC, Igler EJ and Orr MD (1994) Measurements of fentanyl and sufentanil in blood and urine after surgical application. *Am J Forensic Med Pathol* **15:**236–241.

Selavka CM, Mason AP, Riker CD and Crookham S (1995) Determination of fentanyl in hair: the case of the crooked criminalist. *J Forensic Sci* **40:**681–685.

Shingu K, Eger EI, Johnson BH, Lurz FW and Hickey RF (1983) MAC values of thiopental and fentanyl in rats. *Anesth Analg* **62:**151–154.

Uhl M (1997) Determination of drugs in hair using GC/MS/MS. *Forensic Sci Int* **84:**281–294.

Venyeminov SY and Kalnin NN (1990) Quantitative IR spectrophotometry of peptide compounds in water (H_2O) solutions. I. Spectral parameters of amino acid residue absorption bands. *Biopolymers* **30:**1243–1257.

Wang WL, Cone EJ and Zacny J (1993) Immunoassay evidence for fentanyl in hair of surgery patients. *Forensic Sci Int* **61:**65–72.