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Analysis of Morphine by RIA and HPLC in Fingernail Clippings Obtained from Heroin Users*

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ABSTRACT: Heroin is abused around the world and is frequently reported as the cause of death in overdose cases. Analysis of morphine in hair has been used in the past in forensic toxicology to study the addiction history of heroin addicts. The purpose of the present study was to evaluate the usefulness of the nail as an analytical specimen in the identification and quantification of morphine in fingernail clippings of known heroin users. Fingernail clippings were obtained from 26 consenting patients of the Glasgow Drug Problem Service. At the time of sampling, the participants provided answers to a questionnaire regarding their drug use patterns. Samples were decontaminated by sonication in SDS, deionized water and methanol, and the methanolic washes were screened for analyte presence. The washed nail clippings were then hydrolyzed and extracted. RIA was used for the screening and HPLC for the confirmation of morphine. Positive RIA results were obtained with nail clippings from 25 of the 26 heroin users. The levels ranged from 0.06 to 4.69 ng/mg with a mean morphine concentration of 1.67 ng/mg. HPLC results were positive for 22 of the 26 nail samples. The mean morphine level by HPLC was 2.11 ng/mg with a range from 0.14 to 6.90 ng/mg. Based on these results, we suggest that nails have the potential of becoming a powerful alternative to hair for the detection of past heroin use in forensic cases.

KEYWORDS: forensic science, forensic toxicology, drug analysis, alternative specimens, nail, fingernail, heroin, morphine

In Scotland, excluding cannabis, heroin predominates in drug misuse, accounting for almost half of the new individual notified addicts (49%) and being involved in at least 30 deaths per annum according to recent official epidemiological data (1). Injecting is still the predominant route of use in those cases where heroin is involved: 47% of heroin users inject whereas only 4% take heroin orally and 32% smoke the drug (1).

Heroin or diacetylmorphine or diamorphine has been extensively studied and is known to be rapidly deacetylated in the body to 6-acetylmorphine. This metabolite undergoes further hydrolysis to morphine at a slower rate (2). Morphine analysis is frequently used in forensic toxicology to determine the use of heroin (3).

Nails have been used in the past for the determination of voluntary or involuntary exposure to substances. Specifically, fingernail analysis has been used for the determination of arsenic exposure (4) as well as for the qualitative and quantitative determination of amphetamines (5–7), cocaine and its metabolites (8–10) and cannabinoids (11). To determine the usefulness of the nail as an analytical specimen in a forensic environment where radioimmunoassay (RIA) and high-pressure liquid chromatography (HPLC) are used for routing opiate analysis, we examined nail clippings of known heroin users for the presence of morphine by the two aforementioned techniques.

Materials and Methods

Standards and Reagents

All organic solvents were high-pressure liquid chromatography grade, and all chemicals were reagent grade. Morphine hydrochloride was purchased from Carlo Erba (Milan, Italy). Sodium dodecyl phosphate (500 mM), sodium hydroxide (1 M) and sodium phosphate buffer (50 mM) were prepared from HPLC grade reagents in deionized water.

Samples

Nail clippings (3.0 to 96.0 mg) were collected from consenting adults ($n = 26$) attending the clinics of the Glasgow Drug Problem Service in Glasgow, Scotland. The procedures followed were in accordance with the ethical standards of the responsible regional committee on human experimentation. At the time of sampling, the participants were asked to provide answers to a short questionnaire regarding their drug use patterns. Nail clippings were generated using commercially available cosmetic nail clippers. The nail clippings of each participant were pooled and stored in a plastic bag at room temperature until the time of analysis. Demographic and epidemiological data for each participant are shown in Table 1. Our study population was comprised of 22 Caucasian males and 4 Caucasian females. Their average age was 26.5 years and ranged from 19 to 33 years.

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TABLE 1—*Subject demographic and epidemiological data.*

Subject Number	Sex	Age*	Heroin Consumption Rate in GBP (£) or Weight Per Time Interval*	Other Drugs Taken*†
1	male	24	£20/day	M
2	male	29	£30-100/day	Cn, D, M, T
3	male	27	£60–80/day	Cn, Co, D, M
4	male	23	£60–80/day	Cn, Co, D, M, T
5	male	19	£10/day	M
6	male	33	£100–150/day	Ap, Cn, D, M, T
7	male	21	£10/day	Ap, Cn, M
8	male	28	£10/day	Cn, D, M, T
9	male	22	£10/day	D, M, T
10	male	26	£20–30/day	Ap, Cn, M, T
11	male	21	£80/day	Ap, Cn, Co, Dc, M, T
12	male	32	£10/day	Cn, M
13	female	23	£30/day	D, M
14	male	32	£40–60/day	Cn, Co, D, M, T
15	female	25	£30–40/day	Ap, Cn, D, M, T
16	male	31	£20/day	Cn, D, M
17	male	19	£10/day	M
18	male	24	£60/day	Ap, Co, M, T
19	male	28	£30/day	Ap, Cn, Co, D, M, T
20	male	27	£30/day	Cn, D, Dc, M, T
21	female	33	£10/day	Cn, D, Dc, M
22	male	24	1.5 g/day	Cn, Co, D, Dc, M, T
23	male	31	£40/day	Ap, Cn, D, M, P
24	male	31	£60/day	Ap, Cn, Co, D, M, T
25	female	29	£40/day	At, Cn, D, Dc, M, T
26	male	28	£20/day	Ap, Cn, D, M, T

* As reported on the questionnaires during sample collection at the clinics of Glasgow Drug Problem Service.

† At = Amitriptyline; Ap = Amphetamines; Cn = Cannabis; Co = Cocaine; D = Diazepam; Dc = Dihydrocodeine; M = Methadone; P = Paracetamol; T = Temazepam.

In addition, five sets of fingernail clippings from individuals who were unlikely to be heroin users (i.e., laboratory personnel, postgraduate research students and academics) were obtained and used as controls.

A conventional drug screening using radioimmunoassay followed by an HPLC confirmatory analysis was performed on the nail specimens at the Institute of Forensic Medicine, University of Verona, Verona, Italy.

Sample Decontamination

Using an ELMA-SINGEN HTW ultrasonication bath, the nail clippings were sonicated once in 10 mL of 500 mM sodium dodecyl sulfate (SDS) for 15 min and three times in 10 mL of deionized water for 15 min each time and all four resulting washes were discarded. Next, there were three sonications in methanol for 15 min each and these washes were collected, evaporated to dryness at 50°C under a stream of pressurized air, reconstituted in 200 μL of 50 mM sodium phosphate buffer (pH 5.0) and mixed by vortex. A 50 μL aliquot was then screened by RIA for morphine. In those cases in which the third methanolic wash tested positive for morphine, a further (fourth) sonication in methanol for 15 min was conducted and the resulting wash was collected and screened by RIA for methanol as before. Only when the final methanolic wash (third or fourth, as required) tested negative for morphine did the analysis of the nail clippings proceed.

Sample Extraction and Radioimmunoassay

The decontaminated nail clippings were allowed to air dry overnight and weighed. Alkaline hydrolysis was chosen as the extraction method for this study. This involved the incubation of the nail clippings for 60 to 120 min at 60°C in the presence of 1 mL of 1 M sodium hydroxide solution. The resulting nail hydrolysates were then evaporated to dryness at 50°C under a stream of pressurized air. Once dry, the residues were dissolved in 4 mL of deionized water, the pH was adjusted to 7.0, and the samples were sonicated for one hour and transferred to TOXI·TUBES A. The volume was brought up to the 5-mL mark using deionized water and the extraction tubes were agitated for 10 min. The extraction tubes were then centrifuged at 3500 rpm for 15 min and the resulting organic layer was stored. To each extraction tube containing the aqueous phase, 1 mL of dichloromethane:dichloroethane:heptane (19:18:63) was added and the extraction tubes were again agitated and centrifuged as before as part of a second extraction scheme. The organic layers from the two extractions were combined and evaporated to dryness under a stream of pressurized air. The dry residues were reconstituted in 200 μL of 50 mM sodium phosphate buffer (pH 5.0) and mixed by vortex. A 50 μL aliquot of the resulting solution was then analyzed for morphine using the Coat-A-Count® Morphine solid-phase ^{125}I radioimmunoassay by DPC and a Packard Instrument RIA counter (Downers Grove, IL). The RIA kits used contain antiserum which, according to their manufacturers, is highly specific to free (unconjugated) morphine with low cross-reactivity to 6-acetylmorphine (1%), codeine (0.06%), morphine-3-glucuronide (0.3%) and morphine-6-glucuronide (0.05%). The standard curve was prepared from morphine hydrochloride in 50 mM sodium phosphate buffer (pH 5.0) at the concentrations of 0.0, 7.8, 15.6, 31.2, 62.5, 125, and 500 ng/mL.

High-Pressure Liquid Chromatography

Aliquots of the nail extracts were filtered using micro-filters (0.45 μm) attached to syringes and the filtrates (diluted 1:1 with 50 mM sodium phosphate buffer) were analyzed for morphine by HPLC following a published method (12). The HPLC instrument used for this analysis consisted of a Spectra-Physics AS 300 autosampler, a PRLP-S polymeric column (150 × 4.6 mm) operated at 63.8°C, a Jasco 880-PU pump, a BIO-RAD Electrochemical Detector and a Spectra Physics Chromjet integrator. During our sample analysis, morphine standards of known concentrations, blanks and spikes were also run and the morphine retention time was monitored.

Limit of Detection and Extraction Recoveries

To date, there is no published method available for spiking the intact nail matrix with morphine. Thus, the limit of detection and extraction recoveries of morphine were determined by spiking the solution obtained after hydrolysis of drug-free nail in sodium hydroxide. Ten different nail hydrolysates in sodium hydroxide (NaOH) (each equivalent to 10 mg of nail) were spiked with morphine and then analyzed as usual in order to determine the extraction recovery of the method. Furthermore, three nail hydrolysates in NaOH (each equivalent to 10 mg of nail) were spiked with decreasing amounts of morphine and then analyzed as usual in order to determine the limit of detection of the method.

Results

Our decontamination protocol produced contamination-free nail clippings. In almost two-thirds of our cases (65.4%), the SDS

TABLE 2—*Morphine levels in final methanolic wash (third or fourth, as required) measured by RIA and in the nail extracts screened by RIA and confirmed by HPLC.**

Subject Number	Sample Weight, mg	Wash 3, ng/mL	Wash 4, ng/mL	RIA Screening, ng/mg	HPLC Confirmation, ng/mg
S 1	22.8	7.81	NEG	1.10	1.30
S 2	47.0	3.87	NEG	4.10	6.60
S 3	61.0	NEG	N/R	0.80	0.90
S 4	16.0	NEG	N/R	1.80	1.80
S 5	35.1	3.36	NEG	1.40	2.00
S 6	16.9	2.98	NEG	1.90	1.40
S 7	11.0	NEG	N/R	1.50	1.90
S 8	7.0	7.96	NEG	3.20	3.90
S 9	10.9	NEG	N/R	1.60	1.30
S 10	13.3	NEG	N/R	0.69	0.51
S 11	15.3	NEG	N/R	1.24	1.54
S 12	35.4	NEG	N/R	0.32	0.00
S 13	96.0	NEG	N/R	2.70	6.90
S 14	36.9	NEG	N/R	2.05	2.90
S 15	17.5	9.82	NEG	3.38	2.68
S 16	8.8	3.76	NEG	1.53	1.22
S 17	30.0	NEG	N/R	0.39	0.45
S 18	9.2	NEG	N/R	1.16	0.50
S 19	23.4	NEG	N/R	0.62	0.57
S 20	29.2	NEG	N/R	0.06	0.00
S 21	15.4	5.79	NEG	4.69	4.51
S 22	6.3	NEG	N/R	1.39	0.00
S 23	3.0	NEG	N/R	0.00	0.00
S 24	38.5	NEG	N/R	0.32	0.14
S 25	48.8	NEG	N/R	0.91	0.72
S 26	31.6	1.09	NEG	2.80	2.59
Blank 1	23.6	NEG	NEG	0.00	0.00
Blank 2	17.0	NEG	NEG	0.00	0.00
Blank 3	9.5	NEG	NEG	0.00	0.00
Blank 4	45.8	NEG	NEG	0.00	0.00
Blank 5	4.3	NEG	NEG	0.00	0.00
Mean S 1–26	…	…	…	1.67	2.11
Median S 1–26	…	…	…	1.40	1.40
Range S 1–26	…	…	…	0.06–4.69	0.14–6.90

* NEG = Negative morphine screen by RIA; N/R = fourth methanolic wash not required.

wash, the three water washes and three methanol washes were sufficient in removing any superficial contamination and to produce a negative third methanol screen for morphine. However, in the remaining one-third of the cases (34.6%), a fourth methanol sonic wash was necessary before a negative methanol screen for morphine was obtained.

The results of our RIA screening and HPLC confirmation for the nail extracts and blanks are summarized in Table 2 together with data regarding their final methanolic wash (either the third or the fourth wash, as required). As illustrated in Table 2, the decontamination protocol employed in this study produced contamination-free nail clippings by the third (65.4% of the cases) or fourth (35.4% of the cases) methanolic wash and, had the nail samples not been decontaminated in this way, the final results would in most cases be heavily influenced. All blank samples and their methanolic washes tested negative for morphine by RIA and HPLC.

Hydrolysates of fingernail clippings were extracted using TOXI·TUBES A and liquid-liquid extraction and then assayed by RIA and HPLC for the presence of morphine. A typical RIA calibration curve showing the range of linearity of the technique is presented in Fig. 1 and chromatograms of analyses of a blank, a standard and a nail extract are shown in Fig. 2. Positive RIA results were obtained with nail clippings from 25 of the 26 heroin users. The levels ranged from 0.06 to 4.69 ng/mg with a mean morphine concentration of 1.67 ng/mg. Using high-pressure liquid chromatography, morphine was positive in 22 of the 26 nail samples. The mean morphine level by HPLC was 2.11 ng/mg with a range from 0.14 to 6.90 ng/mg.

Figure 3 presents the level of morphine seen in each of our samples by both RIA and HPLC plotted against the monetary level of heroin used (in GBP, £) as this was reported by each participant on his/her questionnaire at the time of sampling. The noticeable variations of morphine levels among individuals who declared to have consumed the same amounts of heroin might be due to several factors which are discussed below.

In regard to the limit of detection of our method, concentrations as low as 0.05 ng/mg morphine could be detected if the equivalent of at least 10 mg of nail was used for extraction with a signal-to-noise ratio of 5. This detection limit was determined by decreasing the concentration of the drug spiked in nail hydrolysate equivalent to 10 mg of nail. An extraction recovery was determined for morphine by spiking hydrolysate of drug-free nail clippings with

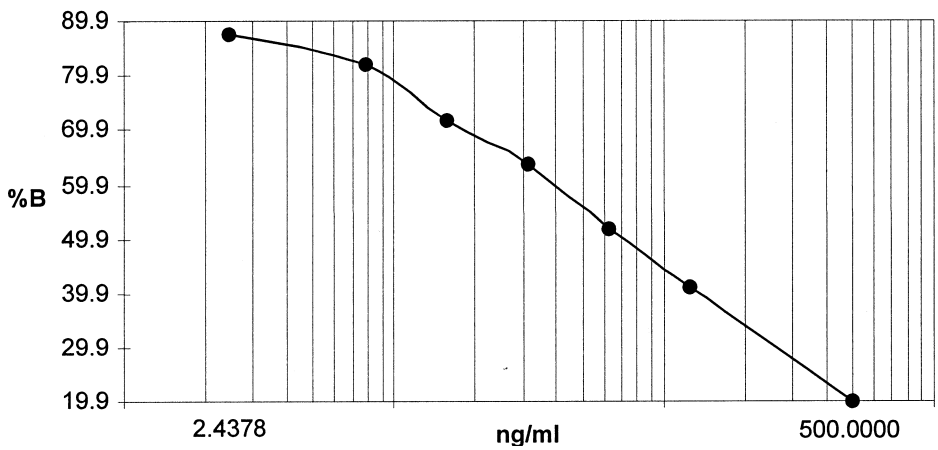

FIG. 1—*Typical RIA calibration curve generated using morphine calibrators showing the range of linearity for this assay and used for initial morphine screening of nail extracts.*

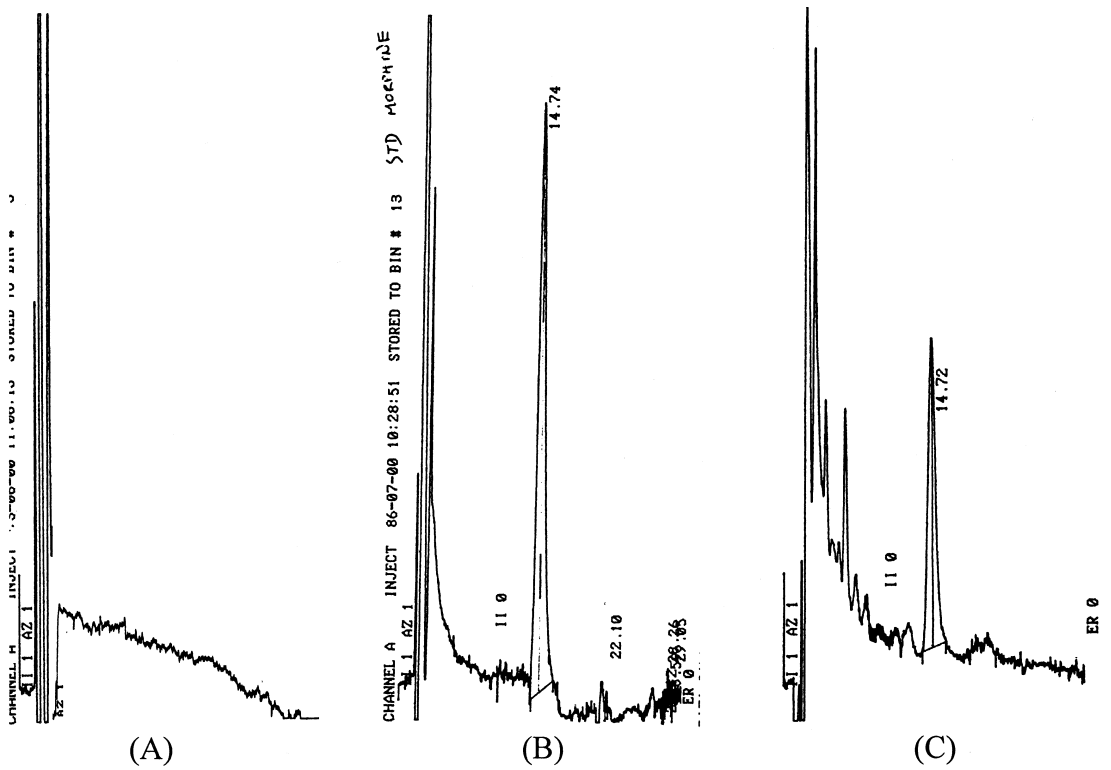

FIG. 2—*High-pressure liquid chromatograms of* (A) *a drug-free nail extract,* (B) *a standard morphine solution, and* (C) *a nail extract from a participant (Sample 9: 1.30 ng/mg morphine).*

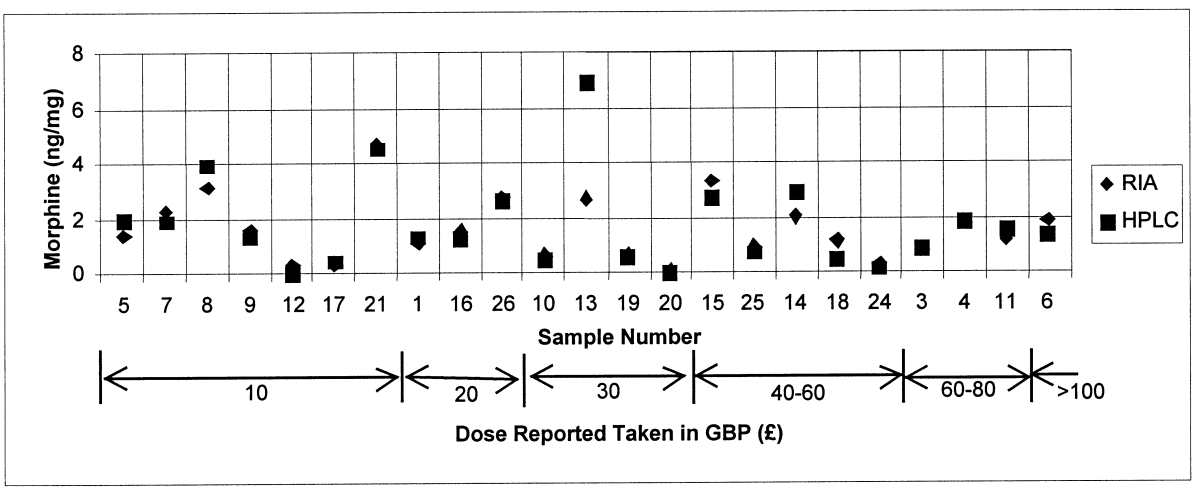

FIG. 3—*Morphine levels in ng/mg measured by RIA and HPLC in each of our samples plotted against the monetary value (in GBP, £) of heroin used by each participant as was declared on his/her questionnaire at the time of sampling.*

known quantities of morphine, subjecting them to our extraction method and then analyzing for them using the instrumental methods described earlier. The extraction recovery thus calculated was 80.5% for morphine by RIA and 86.3% for morphine by HPLC.

Discussion

The use of nail material for the determination of voluntary or involuntary exposure to substances is not a recent scientific development. In particular, exposure to arsenic by its detection in the nail of human subjects was among the first developments in this area. In one report, arsenic was determined in fingernails of five subjects who had been given therapeutic doses of arsenious oxide (4) in an effort to develop an alternative way for the determination of time of exposure to arsenic. Fingernail clippings from the five subjects were collected prior to the start of the experiment and thereafter at regular intervals over the following six months. When the measured amount of arsenic was plotted against the days on which the clippings were collected, two arsenic peaks were observed. Scrap-

ing the underside of the nail reportedly removed the second (smaller) arsenic peak. It was suggested that one peak (the larger) was the result of arsenic deposition into the nail root from the bloodstream while the second, smaller peak was the result of exogenous contamination of the nail, probably from sweat.

Drugs remain in nails for extended periods of time thereby providing information on the history of drug exposure of an individual. Amphetamines were among the first drugs of abuse to be identified in nail clippings (5). In that study, the clippings were subjected to alkaline hydrolysis after washing. N-pentane was used to extract amphetamines from the nails and the extracts were derivatized and analyzed by gas chromatography/mass spectrometry (GC/MS). The mean concentrations of methamphetamine and amphetamine were 4.75 ± 2.34 (range 0.00 to 17.7) and 0.14 ± 0.06 (range 0.00 to 0.40) ng/mg, respectively. These were similar to the measured drug concentrations in hair from the same individuals. Average toenail methamphetamine and amphetamine concentrations were always higher than the corresponding fingernail concentrations of the same subjects. The authors suggested that the slower growth of toenails (1.1 mm/month) as compared to fingernails (3 to 5 mm/month) provided a possible explanation for the consistent difference in drug levels between the two specimens. Furthermore, Cirimele et al. (6) reported that several amphetamines were present in hair and, using a similar extraction method as Suzuki et al. (5), were able to identify amphetamine (AMP), 3,4-methylenedioxyamphetamine (MDA) and 3,4-methylene dioxymethamphetamine (MDMA) in one fingernail. The concentrations determined in this one sample by GC/MS were 12.0 ng/mg for AMP, 9.7 ng/mg for MDA and 60.2 ng/mg for MDMA. These levels appear to be in agreement with the concentrations of Suzuki et al. where the drug analysis was carried out by mass fragmentometry (7).

Cocaine has also been measured in the nail. Nail and hair concentrations of cocaine in a man who died of an overdose were measured by enzyme immunoassay and GC/MS (8). The nail specimens were washed prior to analysis and the wash solutions were analyzed and found positive for cocaine (range 4.1 to 5.8 μg/mg). The nail concentrations of cocaine also measured excessively high at 2.2 to 2.3 μg/mg. Toenail specimens were also positive for cocaine (range 6 to 16 ng/mg). The authors suggested that the deceased had recently handled cocaine because they found high concentrations of cocaine in the fingernail wash solutions. The washing protocol, however, would have removed the significant superficial contamination that was measured had it been more extensive and efficient.

Garside et al. have also been successful in determining several cocaine metabolites in nail (9). Nails were cut into small sections and washed with methanol. Methanolic reflux was then used to extract the drugs from the nail matrix and the solid phase extracts were analyzed by GC/MS. Cocaine and benzoylecgonine (0.0 to more than 10 ng/mg in both fingernail and toenail for both drugs) were the predominant analytes in all cocaine positive nail specimens. Other cocaine by-products (anhydroecgonine methyl ester, ecgonine methyl ester, ecgonine ethyl ester, cocaethylene, norcocaine, and benzoylecgonine) were also detected in the nails. The pattern of drug abuse could be established based on these results: detection of cocaethylene suggested concomitant use of ethanol and cocaine and detection of anhydroecgonine methyl ester indicated use of crack cocaine. Moreover, the detection of cocaine metabolites in the nail indicated drug ingestion followed by metabolism and ensuing incorporation into the nail.

In a study examining eight postmortem specimens to determine cocaine and its metabolites in the nail, GC/MS in the selected ion monitoring (SIM) mode was employed (10). All eight cases were positive for the parent drug as well as benzoylecgonine and ecgonine methyl ester. Cocaethylene and norcocaine were also found in some of the specimens. The same study reported the results of opiate analysis for a further eight cases. Morphine was found present in seven of the eight cases whereas 6-monoacetylmorphine and codeine were found positive in five cases. Finally, in two cases hydromorphine was also detected.

Cannabinoids have recently been identified in the nail (11). Fingernail clippings of known cannabis users were collected and decontaminated following a sonication protocol in sodium dodecyl sulfate, deionized water and methanol. The methanolic washes were screened for analytes and only when they were negative, the authors proceeded with the drug extraction from the washed nail clippings. The decontaminated samples were subjected to alkali hydrolysis, extraction and analysis for cannabinoids by radioimmunoassay and GC/MS. The average Δ^9-tetrahydrocannabinol concentration in fingernails was reported to be 1.4 ng/mg with a range from 0.1 to 6.9 ng/mg. The average concentration of Δ^9-tetrahydrocannabinol-9-carboxylic acid was 19.8 ng/mg with a range from 9.8 to 29.6 ng/mg.

The relatively small size of the population examined in the present study ($n = 26$) does not allow for the construction of proper dose-response relationships. The heterogeneity of the street heroin available in Scotland may also be an important factor. Furthermore, having to rely on the self-reporting figures of what the participants think they have consumed as opposed to conducting experiments under controlled dosage parameters adds at least two potential sources of errors (what the participants think they have consumed and what the participants declare to have consumed based on what they believe their answers should be). Variations in the heroin consumption patterns of our volunteers and the varying length of heroin use within this group may also be factors of variation. Finally, the mechanism(s) of substance incorporation into the nail matrix is (are) not yet understood and there may be substantial inter-individual variation due to physiological, biochemical and pharmacokinetic factors.

Although there is a significant portion of the scientific literature which reports on the detection of drugs in nails, the mechanisms of drug incorporation into this matrix have not yet been fully characterized. Literature reports exist which suggest that drugs may gain quick access to the distal nail plate during nail production by incorporation into the cornified cells of the nail bed. Johnson et al. have reported that there exists minimal incorporation of drug by diffusion from the nail bed to the ventral portion of the nail plate (13,14). Norton and Zaias have separately shown that drug incorporation occurs in the lunular germinal matrix as the nail grows from the base of the nail along the distal axis (15,16). There exist other potential sources of drug entry into the nail such as environmental contamination, and contamination from sweat, saliva, sebum, urine, etc. Finally, the chemical properties of the drugs in consideration could also play a protagonistic role in their incorporation into the nail. However, further studies are needed to determine the actual mechanism(s) of drug incorporation into the nail matrix and the roles played by physiological, biochemical and pharmacokinetic factors.

The addiction history of heroin users in forensic toxicology cases is often determined by morphine analysis in hair. In this study nail clippings were successfully evaluated as analytical specimens. The nail decontamination protocol proposed herein consistently produced negative morphine screenings for the final methanol wash.

Morphine was determined in decontaminated nail clippings by RIA (mean: 1.67 ng/mg) and confirmed by HPLC (mean: 2.11 ng/mg).

The nail matrix offers several advantages to the forensic toxicologist. Drugs remain trapped in the nail matrix for extensive periods of time, thus allowing the determination of exposure to drugs for periods from months to years. Furthermore, the collection of nail clippings is a noninvasive procedure as compared to the collection of blood or urine and only a small sample size is required as demonstrated in this study (mean sample size: 26.4 mg). Nails are easily stored in plastic bags at room temperature (11), allow for increased stability of drugs, and are less likely to suffer any melanin race bias (9). It is because of these advantages that we suggest that more work be done in drug analysis in the nails. Based on our work and understanding of these specimens, we believe that nails have the potential of becoming a powerful alternative to hair for the detection of past heroin use in forensic cases.

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