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High sensitivity simultaneous determination in hair of the major constituents of ecstasy (3,4-methylenedioxymethamphetamine, 3,4-methylenedioxyamphetamine and 3,4-methylenedioxyethylamphetamine) by high-performance liquid chromatography with direct fluorescence detection

Franco Tagliaro[a,b,*], Zeno De Battisti[a,b], Angelo Groppi[c], Yuji Nakahara[d], Daniele Scarcella[b], Roberta Valentini[b], Mario Marigo[b]

[a]*Institute of Forensic Medicine, Catholic University of the Sacred Heart, Rome, Italy*
[b]*Institute of Forensic Medicine, University of Verona, Verona, Italy*
[c]*Department of Legal Medicine and Public Health, University of Pavia, Pavia, Italy*
[d]*National Institute of Health Sciences, Tokyo, Japan*

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Abstract

A simple, but sensitive and specific high-performance liquid chromatographic assay for the simultaneous determination of the major constituents of ''ecstasy'' [i.e. 3,4-methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxyethylamphetamine (MDE)] with direct fluorimetric detection, particularly intended for the routine analysis of hair, is described. Hair samples (100 mg) were overnight incubated in 1 ml of 0.25 *M* HCl at 45°C and extracted with a commercial liquid–liquid method. The dried residue reconstituted with 500 μl of 0.05 *M* NaH_2PO_4 pH 5.2 was injected. Isocratic reversed-phase liquid chromatography was carried out on a column (250×4.6 mm I.D.) packed with spherical 5-μm poly(styrene–divinylbenzene) particles; the mobile phase was composed of 0.1 *M* potassium phosphate (pH 3)–acetonitrile (82:18). The excitation and the emission wavelengths were set to 285 and 320 nm, respectively. Under the described conditions, MDA, MDMA and MDE eluted in symmetric peaks with an analysis time of 30 min. The limit of detection was lower than 1 ng/ml, with a signal-to-noise ratio of 5, for each compound in solution, allowing a cut-off of 0.1 ng/mg in the hair matrix to be established. The intra-day precision ($n=6$) of the assay was characterised by RSDs between 1.0 and 3.0% and between 0.52 and 0.88% for concentrations of 10 and 100 ng/ml, respectively; in day-to-day precision tests ($n=6$), RSDs ranged between 5.12 and 11.12%, respectively, for the same concentrations. Interferences from as many as 92 therapeutic and/or abused drugs currently in use in the population were excluded, including *N*-methyl-1-(3,4-methylenedioxyphenyl)-2 butanamine (MBDB). © 1999 Elsevier Science B.V. All rights reserved.

Keywords: 3,4-Methylenedioxymethamphetamine; 3,4-Methylenedioxyamphetamine; 3,4-Methylenedioxyethylamphetamine; Ecstasy

*Corresponding author. Fax: +39-45-505-259; e-mail: ftmedl@borgoroma.univr.it

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1. Introduction

In the last decade, the use of methylenedioxy derivatives of amphetamines, including 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA) and 3,4-methylenedioxyethylamphetamine (MDE) has increased dramatically in Italy and in Southern Europe. These compounds are listed in the Schedule I of the Convention on Psychotropic Substances, 1971.

These ring-substituted amphetamines, besides well known stimulant effects, are believed to possess mild hallucinogenic and empathy-enhancing activity [1–3], which have prompted their popularity as recreational drugs, especially in the young population. Usually these drugs, known under the generic name of ''ecstasy'' (which traditionally applies to MDMA) are abused in discotheques or in the so-called ''raves'', where all-night dancing to high-tech music takes place without rest for many hours. Serious adverse reactions and fatal cases of intoxication from these hallucinogenic amphetamines have been reported [4–8], but even more major concern is due to the high number of street accidents occurring to people driving under the influence of ''ecstasy''.

As it is well known, urine testing is the standard method to investigate illicit drug abuse, but this type of biological sample has shown several pitfalls, among which is the short time window during which the drug is detectable after intake (2–3 days for most drugs), the embarrassing method of sample collection with the possibility of cheating, the impossibility of repeating the sample collection later to recheck a previous disputed sample, the poor correlation between the ''degree of addiction'' and the actual drug concentration.

Since the first report by Baumgartner et al. [9] hair analysis has been widely recognised as a useful method for investigating chronic exposure to drugs [10–13], offering, in comparison to urinalyses, advantages in terms of ease of sample collection, sample stability and, above all, a much wider diagnostic window (from weeks to months).

The time window of detectability is particularly crucial for ecstasy, which is used irregularly (during weekends or parties) and, due to a rapid elimination ($t_{\frac{1}{2}}$; 8 h), is present for few hours in urine. This causes a very low diagnostic sensitivity for urine testing and makes hair analysis particularly suitable for this purpose.

So far, the determination of MDMA, MDA and MDE in biological samples, including hair, has mainly been carried out by using gas chromatography–mass spectrometry (GC–MS) [14–17]. GC–MS provides excellent sensitivity and selectivity, but needs derivatisation and, due to high instrumental and service costs and moderate productivity, is generally believed to be unsuitable for heavy routine work.

High-performance liquid chromatography (HPLC) is instrumentally more robust and much cheaper than GC–MS, and, not requiring derivatisation, is often preferred for routine drug analysis. The adoption of ''selective'' electrochemical, fluorescence or diode-array UV detectors partially overcomes the limited selectivity of the single-wavelength UV detection, and this technique has recently been introduced successfully in the forensic toxicology environment.

Tedeschi et al. [18] described a HPLC method with UV absorbance detection at 480 nm for the simultaneous identification of amphetamine, methamphetamine, MDA and MDMA in urine after rapid extraction on celite cartridges and, derivatisation with sodium 1,2-naphtoquinone-4 sulphonate. The reported limit of detection (LOD) was 40–60 ng/ml for all derivatives. Hemlin et al. [19] used cation-exchange solid-phase extraction and HPLC with photodiode array detection achieving limits of quantitation (LOQ) of 7 and 5 ng/ml for MDMA and MDA, respectively, in plasma and urine.

Garret et al. [20] using HPLC with UV absorption detection at the wavelength of 280 nm reported analytical sensitivities as low as 2.7 ng/ml for MDMA and 1.6 ng/ml for MDA in plasma.

Michel et al. [21] with electrochemical detection achieved the sensitivity of 1 ng/ml for MDMA, MDA and MDE in microsamples of whole blood.

The coupling of HPLC with mass spectrometry (MS) for the analysis of ring-substituted amphetamines has been proposed by Verweij and Lipman [22] and later by Bogusz et al. [23] who compared HPLC with atmospheric pressure chemical ionisation (APCI) MS and HPLC–UV absorption spectrometry with diode array detection (DAD). The LOD in

serum or urine ranged from 1 to 5 ng/ml using HPLC–APCI-MS and from 10 to 30 ng/ml with HPLC–UV.

A high sensitivity HPLC method with chemiluminescence/fluorescence detection (HPLC–FL) proposed by Hayakawa et al. [24] needed analyte post column derivatisation.

Due to a native fluorescence of methylenedioxylated amphetamines, their HPLC determination with a direct fluorimetric detection looks attractive and was recently reported by Sadeghipour and Veuthey [25] for the analysis of illicit ''ecstasy'' tablets and of serum samples.

The aim of the present work was to develop, a simple, but very sensitive and specific HPLC–FL method for the simultaneous determination of MDA, MDMA and MDE in hair samples, suitable for application in the routine toxicological analysis to investigate ''ecstasy'' abuses.

2. Experimental

2.1. Reagents and standards

Ready-to-use Toxi-tubes A (Marion Labs., Irvine, CA, USA) were adopted for liquid–liquid extraction of hair incubation mixtures.

Stock solutions of MDMA, MDA and MDE (Salars, Como, Italy) were prepared in methanol at a concentration of 1 mg/ml and stored at −18°C; working solutions of standards at suitable concentrations were prepared every day in water or drug-free extracts of hair, from the stock solutions.

Standards of 91 therapeutic or abusive drugs (10 μg each), supplied dried onto glass microfiber discs impregnated with silicic acid, were from the Toxi Disc® Library (Toxi-Lab, Irvine, CA, USA). *N*-methyl-1-(3,4-methylenedioxyphenyl)-2 butanamine (MBDB) extracted from high purity illicit preparations and checked by gas chromatography–mass spectrometry was obtained as a kind gift from Dr. Aldo Polettini, Department of Legal Medicine and Public Health, University of Pavia, Pavia, Italy.

Water and other solvents were of HPLC grade and salts of analytical grade and were purchased from Carlo Erba (Milan, Italy).

2.2. HPLC instrumentation and analytical conditions

The isocratic HPLC system used consisted of a Model 302 single piston high pressure pump (Gilson, Villiers-le-Bel, France), a Model 802 C pulse damper (Gilson), a Model 7125 manual injector (Rheodyne, Cotati, CA, USA) with a 200-μl loop and a Model 821 FP double monochromator fluorimeter (Jasco, Tokyo, Japan). The excitation and the emission wavelengths were set at 285 and 320 nm, respectively. The width of both the excitation and emission slits was 18 nm. The detector signal was recorded with an Oracle 3 integrator (Indtech Instruments, Bombay, India).

The analytical column (150×4.6 mm I.D.) was packed with 5-μm spherical poly(styrene–divinylbenzene) (PLRP-S, 100 Å, Polymer Labs., Chruchstretton, Shropshire, UK). The mobile phase, pumped at 0.5 ml/min flow-rate, was composed of 0.1 *M* potassium phosphate (pH 3)–acetonitrile (82:18). Usually, 100 μl of sample was injected with partial loop filling.

2.3. Sample preparation

Hair samples (20–200 mg, usually 100 mg), cut close to the scalp, were washed with 20 ml×2 of 0.3% Tween 20 (Sigma, St. Louis, MO, USA) solution in water (overall washing time 10 min) and then thoroughly rinsed with distilled water. After drying at 37°C, the hair samples, manually cut in small fragments, were subjected to alkaline hydrolysis in 1 *M* NaOH at 45°C overnight. As an alternative, the hair was also extracted in 2 ml of 0.25 *M* HCl at 45°C overnight, as is carried out in our laboratory for the routine hair analysis for morphine and cocaine [26]. The resulting mixtures were neutralised with equimolar amounts of 1 *M* HCl or NaOH and twice extracted into an organic phase with ready-to-use Toxi-Tubes A. The pooled organic layers were evaporated to dryness and the residue usually reconstituted with 1 ml of 0.05 *M* NaH_2PO_4 (pH 5.2).

The recovery studies were carried out by adding known amounts of analytes to blank hair acid extracts, which were then processed according to the

specific extraction procedures. The extracts were then injected and the peaks compared with the corresponding standards directly injected. Quantification was carried out using external standardisation.

3. Results and discussion

3.1. HPLC determination

Under the described conditions, MDA, MDMA and MDE eluted in symmetrical peaks with capacity factors (k') of about 1.9, 2.8 and 4.4, respectively. The efficiency of separation was about 62 000 plates/metre.

The ring-substituted amphetamines exhibited a good native fluorescence, with excitation and emission maxima at 285 and 320 nm, respectively. No spectral differences were found between the different compounds.

This allowed their sensitive and selective detection by direct fluorescence. The limit of detection (LOD) of the present method in pure solutions of MDA, MDMA and MDE was about 0.4, 0.5 and 0.9 ng/ml, respectively, with a signal-to-noise ratio of 5.

The linearity of the method for MDA, MDMA and MDE was fairly good in the range of concentrations from 0.9 to 250 ng/ml, being described by the following equations:

MDA: $y = 1.011x - 0.205, r^2 = 0.9998$

MDMA: $y = 0.728x + 0.153, r^2 = 0.9997$

MDE: $y = 0.485x - 0.238, r^2 = 0.9992$

(where x = analyte concentration and y = fluorescence response).

The intra-day precision is described in Table 1. Peak areas were characterised by RSDs of 1–3% and 0.52–0.88% for concentrations of 10 and 100 ng/ml, respectively ($n = 6$). In day-to-day repeatability tests ($n = 6$) RSDs were 5–11% and 2–3% for concentrations of 10 and 100 ng/ml, respectively ($n = 6$).

A great advantage of the proposed HPLC fluorimetric method in comparison to those using UV detection, is the possibility of analysing biological matrices, such as hair, without complex and time-consuming sample pretreatments, because of the high selectivity provided by direct fluorimetric detection.

Table 1
Precision of retention times and peak areas (RSD %)

	Intra-day ($n = 6$)		Day-to-day ($n = 6$)	
	Time	Area	Time	Area
MDA 10 ng/ml	0.73	1.30	1.45	5.12
MDA 100 ng/ml	0.73	0.52	1.45	3.41
MDMA 10 ng/ml	0.33	1.00	0.95	9.16
MDMA 100 ng/ml	0.32	0.75	0.95	2.95
MDE 10 ng/ml	0.78	3.07	1.38	11.12
MDE 100 ng/ml	0.77	0.88	1.38	3.87

In fact, a simple and rough liquid–liquid extraction with ready-to-use Toxi-tubes A was sufficient for obtaining extracts suitable for analysis, even at the highest sensitivity. Fig. 1 shows typical chromatograms of blank hair and hair from a user of ''ecstasy'', in which the peak of MDMA corresponds to a content in hair of 3.08 ng/mg. Also, trace amounts of MDA, a metabolite of MDMA, and MDE, sometimes present in ''ecstasy'' preparations, can be identified in the chromatogram. The sensitivity in real hair matrix was calculated as ten times higher than in pure solutions, thus being about 0.1 ng/mg.

The comparison between hair incubation in acid, which is routinely carried out in our laboratory for morphine and cocaine analysis, and the basic hydrolysis, reported for amphetamine extraction by other authors [27–31], shows that the latter method gives substantially higher recoveries from the hair matrix (Table 2).

Using Toxi-tubes, the average ($n = 6$) recoveries (relative standard deviations) from 100 mg samples of blank hair added with 100 ng of MDA, MDMA and MDE were 77.2% (RSD 3.9%), 87.5% (RSD 4.1%) and 86.6% (RSD 4.5%) respectively. A recovery test from blank hair samples (100 mg) spiked with 10 ng/mg of the same compounds gave similar figures, i.e. 77.4% (RSD 4.9%) for MDA, 86.8% (RSD 4.0%) for MDMA and 85.8% (RSD 3.8%) for MDE.

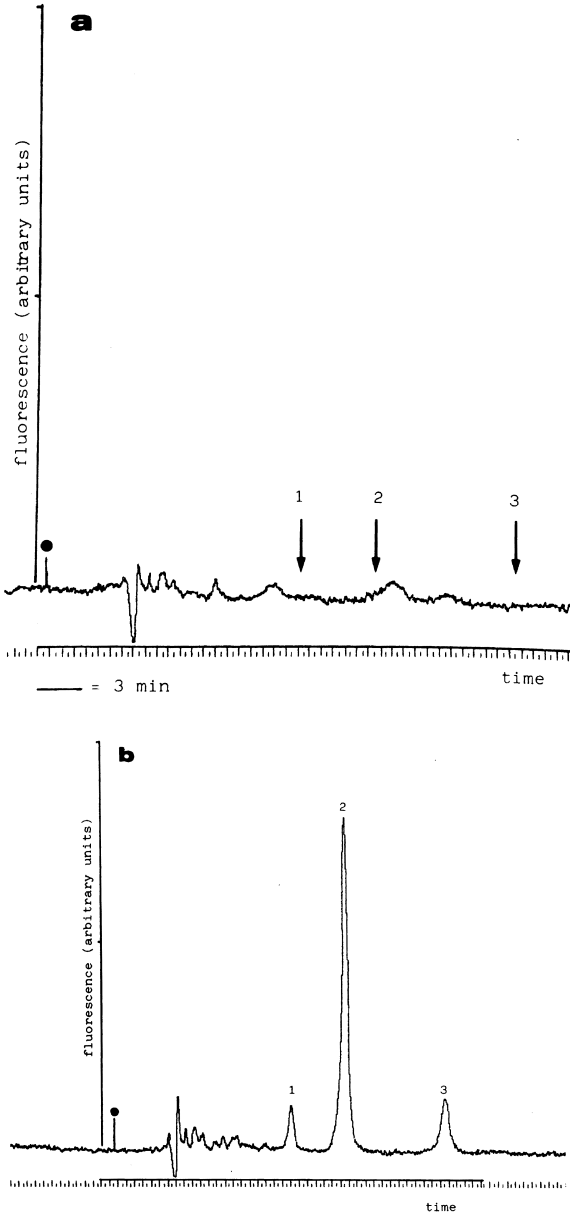

Fig. 1. (a) chromatogram of a ''blank'' hair sample; (b) chromatogram of a hair sample from an ''ecstasy'' user, containing 0.180 ng/mg of MDA (1), 3.08 ng/mg of MDMA (2) and 0.40 ng/mg of MDE (3). The black dots indicate injection. Conditions: column PLRP-S, 5 μm (150×4.6 mm I.D.), mobile phase: 0.1 *M* potassium phosphate (pH 3)–acetonitrile (82:18), flow-rate: 0.5 ml/min, injection: 100 μl; detection: $\lambda_{ex.}$ 285 m, $\lambda_{em.}$ 320, chart speed: 0.3 cm/min.

Table 2
Comparison between basic and acid extraction from hair

Samples	Analyte	1 *M* NaOH (ng/mg)	0.25 *M* HCl (ng/mg)
# 1	MDA	0.60	0.22
	MDMA	13.60	6.30
	MDE	0.56	0.23
# 2	MDA	0.10	<0.10
	MDMA	0.42	0.15
	MDE	0	0
# 3	MDA	<0.10	<0.10
	MDMA	0.50	0.10
	MDE	0	0
# 4	MDA	0	0
	MDMA	<0.10	0
	MDE	0	0
# 5	MDA	<0.10	<0.10
	MDMA	0.10	<0.10
	MDE	0.55	0.23

Lacking a suitable certified hair with known content of analytes of interest, analytical accuracy was evaluated by comparing the results from the present method with those obtained with an independent reference procedure based on GC–MS, carried out at the National Institute of Health Sciences, Tokyo, Japan. The sample extraction and analysis methods were reported in detail by Kikura et al. [16].

The results from five samples of hair from ecstasy users analysed in parallel by HPLC–FL and GC–MS are shown in Table 3. A good concordance between the two methods (even if carried out in different laboratories and with different sample pretreatments) over a wide range of concentrations (from 0.1 to 13.6 ng/mg) is evident, which demonstrates a good analytical accuracy for the proposed liquid chromatographic method.

In order to exclude interferences from therapeutic and/or abused drugs currently in use in the population, as many as 91 standards from the Toxi Disc® Library (Table 4) were injected at a concentration of 1 μg/ml under the described analytical conditions. Also, *N*-methyl-1-(3,4-methylenedioxyphenyl)-2 butanamine (MBDB), a compound closely related to MDE and known to have similar gas chromatographic retention and to give similar fragmentation in mass spectrometry, was tested for interference. No interfering peaks with MDA, MDMA or MDE were

Table 3
Accuracy: comparison between HPLC–FL and GC–MS

Samples	Analyte	HPLC–FL (ng/mg)	GC–MS (ng/mg)
# 1	MDA	0.60	0.79
	MDMA	13.60	12.51
	MDE	0.56	0.74
# 2	MDA	0.10	0.18
	MDMA	0.42	0.48
	MDE	0	0
# 3	MDA	<0.10	<0.10
	MDMA	0.50	1.05
	MDE	0	0
# 4	MDA	0	0
	MDMA	<0.10	<0.10
	MDE	0	0
# 5	MDA	<0.10	<0.10
	MDMA	0.10	0.15
	MDE	0.55	0.70

identified for any of the screened compounds. MBDB, in particular, eluted after MDE in a symmetrical and completely resolved peak. Due to the scarce distribution of MBDB in Italy, we could not find biological samples from real users.

4. Conclusion

The peculiar pattern of abuse of the so-called hallucinogenic amphetamines is generally characterised by irregular/occasional intake and not by daily use, such as happens for heroin and other substances causing high physical dependence. This hampers the efficacy of urinalysis, which has too narrow a diagnostic window to detect a reasonable percentage of occasional abusers. Hair analysis offers a much wider time window in which drug use can be identified by toxicological analysis and, conceivably, has a higher diagnostic value.

However, the moderate doses of ring-substituted amphetamines which are usually taken by abusers are paralleled by correspondingly moderate concentrations in hair. Rothe et al. in a recent epidemiologi-

Table 4
Drugs investigated in order to exclude interferences in MDA, MDMA or MDE determinations[a,b]

Opiates and antagonists	Codeine	Dextromethorphan	Dihydrocodeine	Diphenoxilate
	Ethylmorphine	Hydrocodone	Hydromorphone	Meperidine
	Methadone	Morphine	Naloxone	Oxicodone
	Papaverine	Propoxyphene	Terpin hydrate	
Central nervous system active drugs	Amphetamine	Amitriptyline	Benztropine	Carbamazepine
	Caffeine	Chlorprothixene	Chlorpromazine	Diazepam
	Diphenylhydantoin	Doxepin	Ethinamate	Flurazepam
	Imipramine	Loxapine	Meprobamate	Methamphetam.
	Methaqualone	Methylphenidate	Nordiazepam	Nortriptyline
	Pentobarbital	Phenmetrazine	Phentermine	Phencyclidine
	Phenobarbital	Phenytoin	Phetidine	Prazepam
	Protriptyline	Secobarbital	Strychnine	Thioridazine
	Thiothixene	Trifluperazine	Trflupromazine	Amobarbital
	Aprobarbital	Butabarbital	Barbital	Cocaine
Miscellaneous	Acetaminophen	Atropine	Benzoylecgonine	Carisoprodol
	Chlorpheniramine	Cimetidine	Diphenhydramine	Disopyramide
	Doxylamine	Emetine	Erythromycin	Glutethimide
	Hydrocortisone	Hydroxyzine	Lidocaine	Methapyrilene
	Methocarbamol	Nicotine	Orphenadrine	Pentazocine
	Phenacetin	Pyrilamine	Phenolphthalein	Phenylpropanola.
	Propranolol	Procaine	Procainamide	Pseudoephedrine
	Quinine	Salicylamide	Spironolactone	Triamterene
	Triexyphenidyl	Trimeprazine	Trimetobenzamide	Trimethoprim

[a] Substances injected at the individual concentration of 1 μg/ml.
[b] Abbreviations: methamphetam = methamphetamine; phenylpropanola = phenylpropanolamine.

cal study have found concentrations of these compounds ranging from a few nanograms to fractions of nanogram per milligram of hair in 20 ''ecstasy'' users [32].

On these grounds, the development of a sensitive and specific, but rapid and simple assay for MDMA and congeners in hair is of high interest for forensic toxicologists.

Due to the extremely poor specificity of the immunoassays available for the analysis of amphetamines, the proposed HPLC method with fluorimetric detection looks particularly interesting for heavy routine application.

The results of the HPLC–FL method reported here are substantially in agreement with the paper recently published by Sadeghipour and Veuthey [25], which was focused on the analysis of illicit tablets of ''ecstasy'', but was also tested on spiked serum samples. Sadeghipour and Veuthey's method, based on C_{18} columns, gave faster analyses than the present method using polymeric columns, with the same elution order of MDA, MDMA, MDE and MBDB. However, it was not tested on hair samples, which, because of cosmetic treatments, frequently contain highly fluorescent compounds, which may interfere at the detection stage. In order to improve the separation selectivity, particularly towards possible interferences from the hair matrix, we chose a very hydrophobic poly(styrene–divinylbenzene) column and a slower elution of the compounds of interest. The increased analysis time does not negatively affect the usefulness of the method, while it appears to improve the selectivity and consequently the reliability of the results with critical samples such as human hair.

The method's high selectivity is proved by the absence of any interference on the chromatogram from as many as 91 drugs tested at concentrations 1000 time higher than the method's LOD for the analytes of interest. Also remarkable is the baseline separation of MDE from MBDB, which has been occasionally reported as present in the illicit market.

The sensitivity of the HPLC–FL method is also very good, being comparable to that reported with GC–MS [16]; accuracy and precision are comparable to other HPLC methods and the sample pretreatment is easy.

Due to its simplicity and intrinsic ruggedness, the proposed method is susceptible to automation, and easily transferable to other laboratories.

In conclusion, hair analysis for MDMA and congeners in hair by the proposed HPLC–FL method is an important new tool for the study of ''ecstasy'' abuses in the population and for forensic and/or administrative applications.

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