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Screening for drugs of abuse in hair with ion spray LC–MS–MS

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Abstract

Analyzing hair for many substances can be tedious and expensive, and a rapid screening method should prove helpful. Generally, screening has been performed using immunological tests, mainly in workplace drug testing, where the number of samples has been high.

The aim of this study was to develop an LC–MS–MS method for the simultaneous analysis of several drugs of abuse in human hair as an alternative to immunological screening tests.

In 75 randomly selected autopsy cases, hair was analyzed in addition to the usual specimens of blood and urine. The method included nicotine, cotinine, morphine, codeine, 6-acetylmorphine, ethylmorphine, amphetamine, methamphetamine, MDA, MDMA, benzoylecgonine, cocaine, 7-aminoflunitrazepam and diazepam.

The LC–MS–MS analysis was performed on a SCIEX API 2000 MS–MS instrument equipped with an electrospray interface. To 20–50 mg of hair, 0.5 ml of mobile phase A (acetonitril:methanol:20 mM formate buffer, pH 3.0 (10:10:80)) and 25 μl of internal standard were added and the sample was incubated in a water bath at 37 °C during 18 h. Using a threshold of 20 ng/sample, equivalent to 1 ng/mg if 20 mg hair is used, 26 positive results were found in 16 cases. Three of the 26 positive detections could not be confirmed by GC–MS. Two of the cases were not previously known as drug users. Of the 59 negative cases, only one case had a positive blood sample showing 0.01 and 0.07 μg/g femoral blood of 6-acetylmorphine and morphine, respectively. This might indicate drug abstinence resulting in decreased tolerance or even a ''first time'' use of heroin resulting in death. We conclude that the use of hair analysis in postmortem cases can reveal both unknown drug use, as well as confirm a period of drug abstinence prior to an acute fatal overdose. The proposed LC–MS–MS method showed high sensitivity, was very easy to perform and seemed appropriate for screening purposes.

Keywords: Liquid chromatography mass spectrometry; Hair; Drugs of abuse; Postmortem

1. Introduction

A common opinion in overdose cases is that the death is caused by lack of tolerance, after a period of abstinence. However, there is little evidence for this theory. An objective way to evaluate past drug use or abstinence is the analysis of hair from the deceased. Since the period of abstinence not necessarily has to be long, segmental analysis of hair is of paramount importance. Before such an extensive analysis, a rapid screening, particularly for cocaine, opiates, amphetamines and bensodiazepines is helpful. Even in other forensic cases, hair analysis may add valuable information to the investigation.

The analysis of drugs in hair usually involves several measures to ensure reliable and valid results. Besides washing, the extraction or digestion procedures come in such a variety that the comparison of results is very difficult. Several papers have evaluated different procedures for removing drugs and metabolites from the hair matrix [1–13]. The methods used involve digestion by enzymes, digestion by strong acid or base at elevated temperatures, direct solvent or buffer extraction of the hair, as well as subcritical fluid extraction. Basic conditions degraded substances such as heroin, cocaine and benzodiazepines,

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whereas the softer enzymatic digestions and the solvent extractions seemed to work well. In general though, the complete dissolution of the hair matrix produced the best recoveries, whereas direct extraction of hair with organic solvents seemed to give lower recoveries. Baumgartner and Hill [13] have proposed enzymatic digestion at neutral pH as a universal extraction procedure for all substances.

After the drugs have been liberated from the hair matrix, extraction and detection procedures are very similar to the ones used for the extraction of drugs from blood, plasma or urine. Routine hair testing, especially in workplace drug testing for drugs of abuse, is usually performed with an initial screening by immunoassay followed by confirmation of positive results by mass spectrometry [14–16]. Hair analysis has also been used in forensic casework in various contexts. Many papers have been published where hair–drug concentrations from living drug addicts or suspects as well as fatal overdoses were reported [17–29].

Nakahara and Kikura [30] described the use of hair and hair root analysis in acute poisonings of MDMA and Tagliaro et al. [25], Kronstrand et al. [26] and Darke et al. [27] evaluated heroin metabolite concentrations in hair from fatal overdoses. All came to the conclusion that the low concentrations found in the hair of the deceased suggested abstinence from heroin and hence possible reduced tolerance resulting in the overdose death.

The aim of this study was to develop and validate an LC–MS–MS screening method for the analysis of overdose-related drugs of abuse in human hair. The method also included nicotine and cotinine.

2. Materials and methods

2.1. Chemicals and reagents

The reference materials nicotine, cotinine, morphine, 6-acetylmorphine, codeine, cocaine, bensoylecgonine, amphetamine, methamphetamine, MDMA, MDA, diazepam and 7-aminoflunitrazepam were obtained from Cerilliant Corp. (Austin, Texas, USA). The deuterated internal standards d3-morphine, d3-cocaine, d5-amphetamine and d5-diazepam were also purchased from Cerilliant Corp. Ethylmorphine was purchased from Sigma (St. Louis, Missouri, USA). All other solvents and inorganic chemicals were of gradient or analytical grade.

2.2. Cases

From 75 consecutive autopsy cases, hair samples were obtained as a part of the on-going investigation. Three hair samples were cut from the back of the head. Care was taken to cut the hair as close to the scalp as possible. One of the samples from each case was cut in small pieces (1–5 mm) and weighed directly, and put into a 10 ml screw-capped glass tube. This portion was used for screening of drugs with LC–MS–MS. The remaining hair samples were stored at room temperature pending confirmation analysis.

2.3. Instrumentation

The LC–MS–MS analysis was performed on a Perkin Elmer Series 200 chromatography system consisting of a

Table 1
Transitions and MS–MS conditions for each analyte and internal standard

Analyte	Rt	Q1/Q3		Voltages			Internal standard
		M+	Daughter	DP	CE	CXP	
Nicotine	0.76	163.1	130.0	20	30	8	M
Cotinine	1.04	177.2	79.9	20	34	13	M
Morphine	0.88	286.2	286.2	50	5	9	M
6-acetylmorphine	1.76	328.2	328.2	60	5	6	M
Codeine	1.36	300.2	300.2	66	5	14	M
Ethylmorphine	2.02	314.2	314.2	60	5	8	M
Amphetamine	1.32	136.2	119.0	15	14	4	A
Methamphetamine	1.54	150.2	91.1	10	27	14	A
MDA	1.61	180.2	163.1	10	18	7	A
MDMA	1.86	194.2	163.1	10	18	7	A
Cocaine	3.15	304.2	182.1	30	25	8	C
Benzoylecgonine	2.33	290.2	168.0	35	25	8	C
Diazepam	6.84	285.0	257.0	50	30	9	D
7-aminoflunitrazepam	3.85	284.0	227.0	50	35	11	D
d3-Morphine	0.80	289.2	289.2	50	5	9	–
d5-Amphetamine	1.22	141.2	123.9	10	14	6	–
d3-Cocaine	3.05	307.2	185.2	30	25	8	–
d5-Diazepam	6.74	290.0	262.0	50	30	9	–

M = d3-morphine, A = d5-amphetamine, C = d3-cocaine, and D = d5-diazepam.

Series 200 pump, a "hot pocket" column oven, Series 200 autosampler and a SCIEX API 2000 MS–MS instrument (Applied Biosystems, Stockholm, Sweden) equipped with an electrospray interface (Turbo Ion Spray). Ion spray voltage was set to 5000 V. Nitrogen was used as nebulizer gas (25 psi), auxiliary gas (50 psi heated to 300 °C), curtain gas (30 psi) and as CAD gas (set on 5). We used a 50 × 2.1 mm Zorbax phenyl analytical column with 3-μm particle size (ChromTech, Congleton, UK). Mobile phase A was a 10:10:80 mixture of acetonitrile:methanol:20 mM formate buffer, pH 3.0, and mobile phase B was a 35:35:30 mixture. The system was run in a linear gradient from 100% A-phase to 35% A-phase from 0.5 to 7 min, followed by a 2-min equilibration with 100% A-phase. The total flow rate was 0.25 ml/min. The column oven was set at 30 °C. A 10-μl aliquot of the sample was injected. Chromatograms were evaluated with Analyst v1.1.

2.4. Preparation of hair extracts

To 10–50 mg of hair were added 0.5 ml of mobile phase A and 25 μl of internal standard (2.0 μg/ml of d3-morphine, d3-cocaine, d5-amphetamine and d5-diazepam) and the sample was incubated in a water bath (with orbital shaking) at 37 °C during 18 h. A 150-μl aliquot was transferred to an autosampler vial and 10 μl were injected onto the chromatographic system (see Table 1 for retention times and measured transitions for each analyte and internal standard). Calibration was performed by addition of standard solutions to 20 mg of drug-free hair prior to incubation. Final concentrations were 0.5, 1.0, 1.5, 3.0, 5.0 and 7.5 ng/mg.

Table 2
LOD, LOQ and extraction recovery for the analytes in the final method

Analyte	LOD (pg/mg) ($N = 3$)	LOQ (pg/mg) ($N = 3$)	Extraction recovery at 18 h (%) ($N = 3$)
Nicotine	24	80	95
Cotinine	25	83	100
Morphine	9	30	84
6-acetylmorphine	15	50	81
Codeine	25	83	84
Ethylmorphine	4	13	n.a.
Amphetamine	33	110	100
Methamphetamine	6	20	91
MDA	12	40	n.a.
MDMA	4	13	100
Cocaine	3	10	88
Benzoylecgonine	5	16	77
Diazepam	70	232	97
7-aminoflunitrazepam	17	58	n.a.

Extraction recovery (normalized at 28 h incubation) was calculated from authentic samples, whereas LOD and LOQ were calculated from control samples. n.a. = not available.

Table 3
Between-day imprecision of the analytes calculated from control samples ($N = 10$)

	Added (ng/mg)[a]	Concentration found (mean) (ng/mg)	CV (%)	Accuracy (%)
Nicotine	1	0.65	30	65
	5	4.3	17	86
Cotinine	1	1.0	26	100
	5	5.9	11	118
Morphine	1	0.87	17	87
	5	5.2	11	104
6-acetylmorphine	1	0.92	6.6	92
	5	4.6	15	92
Codeine	1	1.0	9.1	100
	5	4.3	23	86
Ethylmorphine	1	0.88	11	88
	5	4.2	22	84
Amphetamine	1	0.92	20	92
	5	5.3	9.2	106
Methamphetamine	1	0.99	17	99
	5	5.6	15	112
MDA	1	0.94	26	94
	5	6.2	13	124
MDMA	1	0.97	16	97
	5	5.4	12	108
Cocaine	1	0.88	12	88
	5	4.8	10	96
Benzoylecgonine	1	0.77	16	77
	5	3.7	19	74
Diazepam	1	0.92	27	92
	5	5.3	7.7	106
7-aminoflunitrazepam	1	0.58	23	58
	5	5.0	13	100

[a] ng/mg is based on the addition of analytes to 20 mg of hair.

Table 4
Between-day imprecision of selected analytes calculated from authentic samples ($N = 10$)

	Concentration found (mean) (ng/mg)	CV (%)
Nicotine	38	13
Cotinine	1.83	27
Morphine	1.00	15
6-acetylmorphine	3.10	11
Codeine	0.36	5.9
Amphetamine	0.47	26
MDMA	5.58	16
Cocaine	3.67	13
Benzoylecgonine	4.58	19
Diazepam	0.60	24

2.5. Confirmation analysis

If a positive result was obtained at the screening, a new set of samples was prepared by cutting three to five segments of hair (usually 10 mm long), which were separately washed for 15 min with 2-ml 2-propanol, 3 × 30 min with 2-ml 0.01 M phosphate buffer (pH 6) and finally again with 2-ml 2-propanol. After drying the hair at room temperature, aliquots were weighed in 10-ml screw-capped tubes for target analysis with GC–MS for opiates and amphetamines [26] or LC–MS–MS for benzodiazepines [31] according to Kronstrand et al.

2.6. Method validation

Hair from 75 autopsy cases was analyzed to test the method. All positive results were reanalyzed with another verification method. Recovery of the analytes from authentic

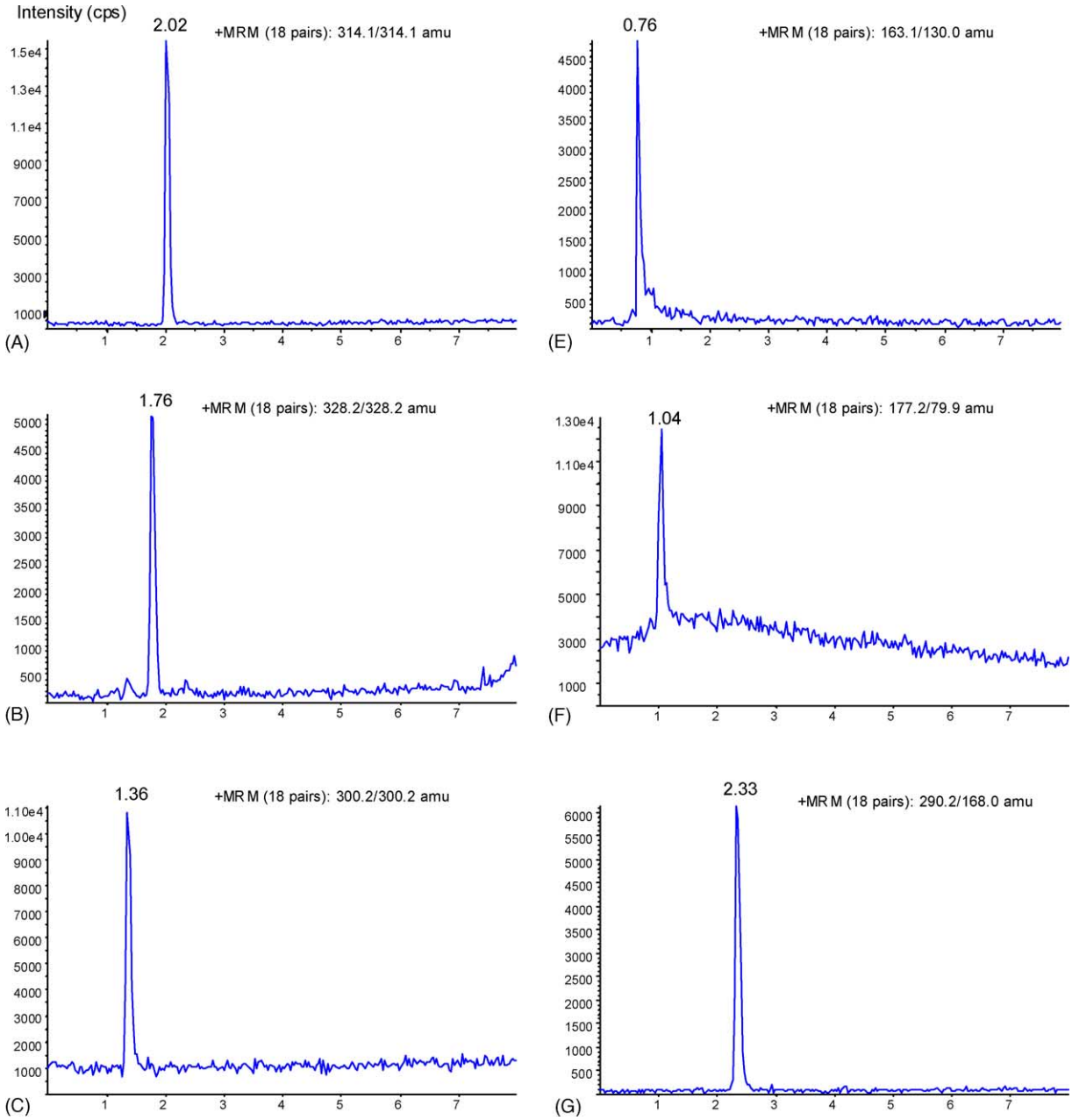

Fig. 1. Ion chromatograms from a control sample at 1 ng/mg hair. (A) Ethylmorphine, (B) 6-acetylmorphine, (C) codeine, (D) morphine, (E) nicotine, (F) cotinine, (G) benzoylecgonine and (H) cocaine.

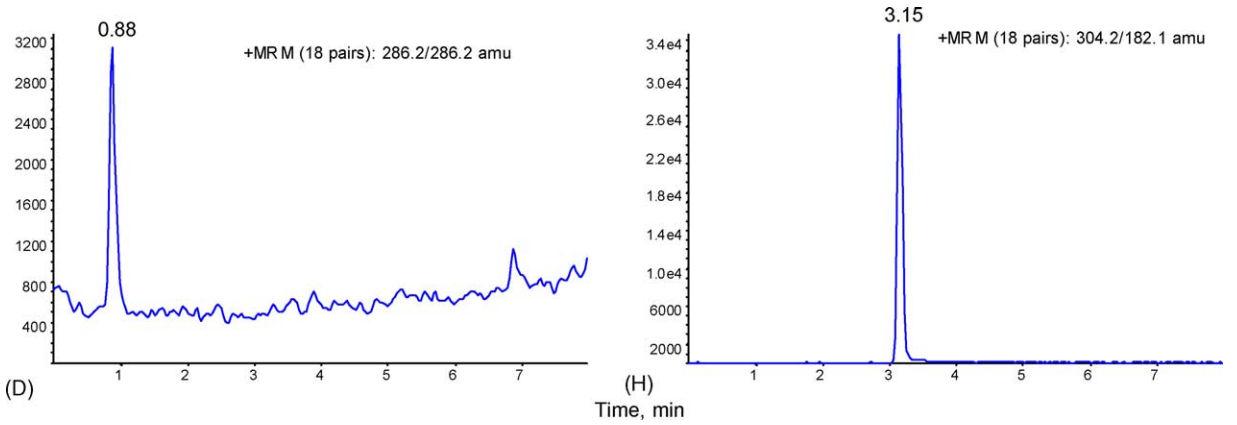

Fig. 1. (*Continued*).

samples was determined by incubating aliquots (10–20 mg) of authentic hair during 1, 2, 4, 6, 8, 10, 12, 24 and 28 h ($N = 3$). The data for each analyte were normalized and then fitted to a regression curve. From the curve, the estimated recovery at 18 h was calculated.

Recovery for the final method (18 h incubation time) was also compared with results from GC–MS analysis ($N = 5$).

The stability of the analytes during the incubation was tested at 5 ng/mg by addition of standard solutions before and after incubation and comparing the results ($N = 5$).

The between-day imprecision was estimated by analysis of control samples at low (1 ng/mg) and high (5 ng/mg) concentrations on 10 different days. The between-day imprecision was also estimated by analysis authentic samples on 10 different days. LOD ($S/N = 3$) and LOQ ($S/N = 10$) were determined by analyzing low concentrations of analytes ($N = 3$) and calculating the S/N using the Applied Biosystems script S/N 3.1.dll.

3. Results and discussion

Linear calibration curves were established from 0.5–7.5 ng/mg for all analytes. The relative incubation recoveries as well as the LOD and LOQ of the analytes are shown in Table 2. The recovery from authentic samples showed a steep increase in the beginning of the incubation period and then the curve came to a plateau or slightly increasing slope. The incubation time of 18 h was chosen because it enabled the incubation to be carried out overnight with good recoveries for most analytes. All analytes were stable during the 18-h incubation. The between-day imprecision and accuracy of the analytes are presented in Tables 3 and 4. Figs. 1 and 2 show ion chromatograms from a control sample with analytes added at 1 ng/mg hair. Nicotine is the most polar of the analytes and elutes very early and just after the column dead volume. Since the samples are extracted into mobile phase A, there is almost no ''solvent peak'', but a small dip in the baseline can be seen in Fig. 2C. A comparison of the quantitative results from authentic samples analyzed both with the LC–MS method and by GC–MS confirmation methods is shown in Fig. 3. For opiates and cocaine, the extraction methods for LC–MS and GC–MS were similar, the latter using methanol extraction of finely cut hair; still the results from measurement of cocaine and codeine differed significantly ($P < 0.01$) between the methods. Amphetamines, on the other hand, are confirmed using sodium hydroxide to disintegrate the hair matrix, thus the recovery might be better than from incubation in aqueous buffer. The mean concentrations of those analytes showed good agreement between the methods and support the findings of Kintz and Cirimele [11], who found that concentrations of amphetamine, MDMA and MDA were statistically indistinguishable regardless of extraction conditions.

The purpose of screening methods differs depending on the context. Today, screening tests are used primarily to rapidly and with low cost dismiss lots of negative samples in a vast amount of samples. This is achieved with immunological tests such as RIA [13,14] and ELIZA [16]. The main goal is to save time and money. Another purpose of screening tests originates from the valuable forensic concept of using two independent methods to confirm a positive result. The main goal then is to add quality to the results. The use of an LC–MS method for screening does not fulfill the former criteria, but instead it is an excellent choice for the latter. Another advantage with chromatography is that one can design the screening method to measure exactly what the confirmation method does. In contrast to immunological tests, that suffer from cross-reactivity making both qualitative and quantitative data difficult to extrapolate to specific confirmation results; LC–MS provides the analyst with comparable results.

Of the 75 cases, 59 were negative and the other 16 cases showed 26 individual positive results with the LC–MS method. Three of those 26 could not be confirmed with GC–MS. These three results were low codeine (<0.7 ng/mg) positives. An explanation is that the hair samples were not washed before the screening as they were prior to the confirmation methods. Thus, a portion of the codeine may

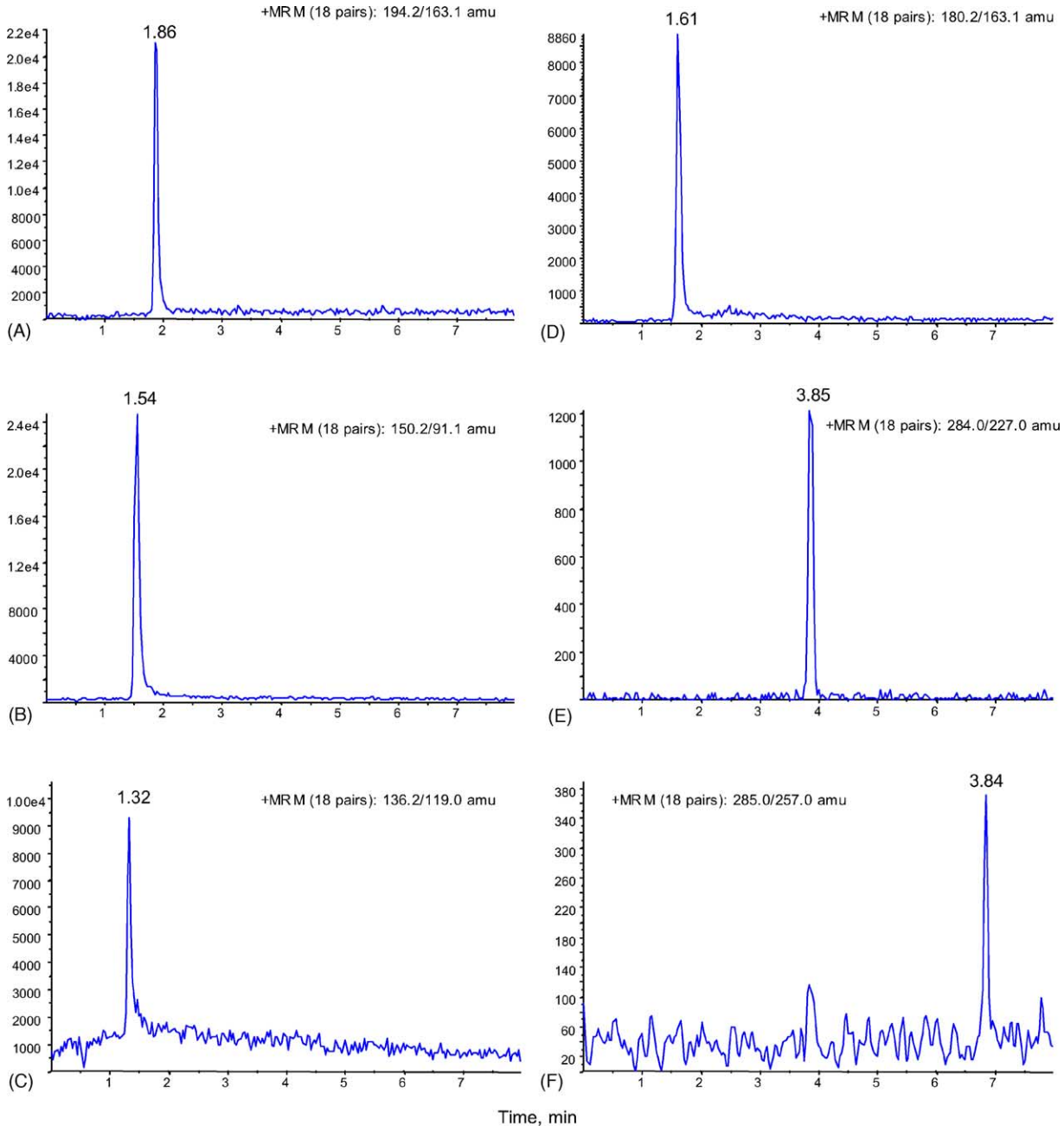

Fig. 2. Ion chromatograms from a control sample at 1 ng/mg hair. (A) MDMA, (B) methamphetamine, (C) amphetamine, (D) MDA, (E) 7-aminoflunitrazepam and (F) diazepam.

have been washed out. Codeine was also one of the analytes that showed significantly higher concentrations using LC–MS. The segmental analysis performed after a positive screening test did not reveal any remarkable concentration differences between the segments.

Two of the positive cases were previously unknown drug users and the use of hair analysis revealed this. Also, one of the 59 negative cases had an opiate positive blood sample. In postmortem blood was found 0.07 μg/g of morphine and 0.01 μg/g of 6-acetylmorphine. This was an 18-year-old girl with no previous history of drug abuse, and these circumstances point towards an accidental overdose death, or even a ''death at first use''.

We conclude that the use of hair analysis in postmortem cases can reveal both unknown drug use as well as confirm a period of drug abstinence prior to an acute fatal overdose.

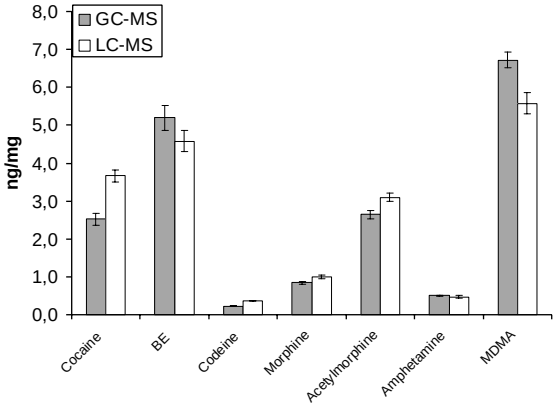

Fig. 3. Comparison between the LC–MS screening method ($N = 10$) and GC–MS confirmation methods ($N = 5$) for selected analytes. Error bars represent standard error of the mean. BE = benzoylecgonine.

The proposed extraction method, followed by LC–MS, seems appropriate for screening purposes because it has very good sensitivity and is very simple to perform.

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