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Hair analysis for abused drugs by capillary zone electrophoresis with field-amplified sample stacking

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Abstract

The present paper describes the methodological optimisation and validation of a capillary zone electrophoresis method for the determination of morphine, cocaine and 3,4-methylenedioxymethamphetamine (MDMA) in hair, with injection based on field-amplified sample stacking. Diode array UV absorption detection was used to improve analytical selectivity and identification power. Analytical conditions: running buffer 100 mM potassium phosphate adjusted to pH 2.5 with phosphoric acid, applied potential 10 kV, temperature 20°C, injection by electromigration at 10 kV for 10 s, detection by UV absorption at the fixed wavelength of 200 nm or by recording the full spectrum between 190 and 400 nm. Injection conditions: the dried hair extracts were reconstituted with a low-conductivity solvent (0.1 mM formic acid), the injection end of the capillary was dipped in water for 5 s without applying pressure (external rinse step), then a plug of 0.1 mM phosphoric acid was loaded by applying 0.5 psi for 10 s and, finally, the sample was injected electrokinetically at 10 kV for 10 s. Under the described conditions, the limit of detection was 2 ng/ml for MDMA, 8 ng/ml for cocaine and 6 ng/ml for morphine (with a signal-to-noise ratio of 5). The lowest concentration suitable for recording interpretable spectra was about 10–20-times the limit of detection of each analyte. The intraday and day-to-day reproducibility of migration times ($n=6$), with internal standardisation, was characterised by R.S.D. values $\leq$0.6%; peak area R.S.D.s were better than 10% in intraday and than 15% in day-to-day experiments. Analytical linearity was good with R^2 better than 0.9990 for all the analytes.

Keywords: Capillary electrophoresis; Diode-array detection; Field-amplified sample stacking; Hair analysis; Morphine; Cocaine; MDMA

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1. Introduction

Hair is a complex tissue, annex of skin, originating from a germination centre located in the hair follicle, where matrix cells are in active proliferation. While the germination centre has high proliferation and metabolism rates, the hair stalk has no metabolic activity. Drug incorporation into hair occurs from the blood circulating in a capillary located close to the germination centre, but also from sebum and sweat secreted by glands associated with the hair root. Also, it has been demonstrated that external contamination by drugs in powder or liquid forms may lead to penetration of drugs into the hair matrix. After embedding in the hair matrix, drugs are protected from degradation and metabolism and, consequently, last unaltered for a long time in this tissue.

Hair growth rate varies from person to person and also in different body regions from the same person, but in the scalp it is generally in the range between 0.7 and 1.5 cm/month.

The fundamentals of hair composition and physiology have recently been described by Harkey [1].

On the basis of these peculiar features, after the pioneering research by Baumgartner et al. [2], hair analysis has been used in different contexts (violation of laws on narcotics, probation and parole monitoring, assessment of compliance to detoxication treatments, revocation of driving licence etc.) for investigating past, chronic exposure to abused or illicit drugs. For comprehensive information on the application of toxicological analysis of hair for forensic purposes, the readers are referred to a recent specific book edited by Kintz [3].

From the analytical toxicology point of view, hair poses specific problems, including sample collection, preparation and extraction, development of highly sensitive and selective methods for drug determination in minute specimens, and interpretation of results. In general hair analysis mainly relies on immunometric screenings and gas chromatographic (GC) or high-performance liquid chromatographic (HPLC) confirmation methods. Mass spectrometry (MS), because its high sensitivity and selectivity, is the favourite detection technique, recently in MS–MS configurations. Reviews of the current analytical methods have been published by Moeller [4] and Tagliaro et al. [5].

In the 1990's capillary electrophoresis (CE) has been successfully applied to the analysis of illicit or controlled drugs in clandestine preparations [6] as well as in biological samples [7].

CE shows potential advantages for hair analysis, including minimal need of sample, negligible consumption of reagents, high mass sensitivity and suitability for coupling with MS. Another important characteristic of CE is the possibility of applying different ''orthogonal'' separation methods by simply changing the separation buffer, without modifying the instrumental hardware. This offers an additional tool for confirmation of analytical data, by comparison of results obtained by multiple noncorrelated separation methods [8].

Our first paper on application of CE to hair analysis [9] reported morphine and cocaine determination using capillary zone electrophoresis (CZE) separation with UV detection. The electropherograms showed excellent efficiency and resolution of the

analytes, with minimal interferences from the matrix. However, due to the limitation in sample loading inherent to the technique, the analytical sensitivity was moderate. Preliminary experiences using a sample stacking method resulted in increased sensitivity (about five-times) [10], but not yet sufficient for application to hair samples from irregular/occasional users of illicit drugs.

The present paper describes methodological improvements to increase CZE sensitivity in hair analysis by using a high efficiency head-column field-amplified sample stacking technique reported by Zhang and Thormann for amiodarone analysis in serum [11]. Also, for the first time in hair analysis, diode array UV detection was used to improve the analytical selectivity and identification power of CZE.

2. Materials and methods

2.1. Reagents and standards

Salts used for buffer preparation were of analytical grade and solvents were of HPLC grade. All chemicals were furnished by Carlo Erba (Milan, Italy).

Standards of analysed drugs [morphine, cocaine, 3,4-methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxyethylamphetamine (MDE), nalorphine and tetracaine] were provided by Salars (Como, Italy). Stock solutions of each standard were prepared in methanol at the concentration of 1 mg/ml and stored at −20°C. Every day, working standards were prepared by dilution in water at suitable concentrations.

Toxi-Tubes A, used for liquid–liquid extraction of biological samples, were obtained from Marion (Irvine, CA, USA).

2.2. Instrumentation

A capillary electropherograph P/ACE 2200 (Beckman, Fullerton, CA, USA) fitted with a filter UV absorbance detector was used for quantitative work. Control of the instrumentation, data acquisition and analysis was performed with the software System Gold® version 8.1 (Beckman). Method development and on-line spectral analysis of peaks was performed with a HP ^{3D}CE instrument model 1600A (Hewlett Packard, Palo Alto, CA, USA) fitted with a diode array UV–vis detector.

Uncoated fused-silica capillaries from Composite Metal Services (The Chase, Hallow, Worcestershire, UK) were used throughout the experiments (47–57 cm total length, 40–50 cm effective length, 75 μm I.D.).

2.3. Analytical method

The electrophoretic separations were carried out using a running buffer composed of 100 mM potassium phosphate adjusted to pH 2.5 with phosphoric acid.

Analytical conditions were as follows: applied potential was 10 kV (unless otherwise

stated); temperature was 20°C; detection was by UV absorption at the fixed wavelength of 200 nm or by recording the full spectrum between 190 and 400 nm. Pressurised (0.5 psi for 5–10 s) or field-amplified sample injection were carried out using conditions which will be described in the specific section.

New capillaries were flushed in sequence with 1 M and 0.1 M sodium hydroxide for 10 min, water for 10 min and running buffer for 20 min. Between runs, washes with running buffer for 5 min were carried out.

As reference methods for hair analysis, we used HPLC with electrochemical detection for morphine [14] and MDMA [15] and HPLC with fluorescence detection for cocaine [16].

2.4. Sample preparation

Hair samples (100–200 mg) of about 4 cm in length were collected from the scalp of the vertex posterior of the head, by cutting with scissors as close to the skin as possible. ''Blank'' hair was obtained from well known subjects (personnel of the department) abstinent from drugs during the previous 6 months. When needed, blanks were spiked with suitable amounts of drugs to mimic concentrations usually found in real users (0.5–10 ng/mg). ''Positive'' hair samples from real drug users were preliminarily analysed by our routine methods based on radioimmunoassay screening and HPLC confirmation [12].

Hair was treated according to already described procedures [12], which can be summarised as follows. About 100 mg of hair was first washed with an aqueous solution of 0.3% Tween 20 (20 ml×2 times), in order to remove the lipids present on the surface. Then hair was cut into small fragments and incubated overnight in 2 ml of 0.25 M HCl at 45°C. Finally the incubation mixture was neutralised with equimolar amounts of NaOH and twice extracted into organic phase with ready-to-use Toxi-Tubes A. The organic layer (a mixture of heptane 63%–dichloromethane 19%–dichloroethane 18%) was evaporated under a stream of air and the dried residue redissolved in a suitable amount of solvent as described in the specific section.

2.5. Head-column field-amplified sample stacking

In order to increase the sample loading without excessive band broadening, the dried hair extracts were reconstituted with 0.2 to 1 ml (depending on the needed sensitivity) of a low-conductivity solvent and injected by electromigration, according to a method reported by Zhang and Thormann [11]. In method development, 0.1 mM phosphoric acid, 0.1 mM formic acid and 0.1 mM glycine were tested. Injection conditions were typically as follows: first, the injection end of the capillary was dipped in water for 5 s without applying pressure (external rinse step), then a plug of low conductivity solvent (water, 0.1 mM phosphoric acid or 0.1 mM formic acid) was loaded for 10 s at 0.5 psi and, finally, the sample was injected electrokinetically for 10 s at 10 kV.

2.6. Calculation of separation parameters

Resolution (R) was calculated according to the equation: $R=[2(t_2-t_1)]/(W_1+W_2)$, where t_1 and t_2 are the migration times of two adjacent peaks, and W_1 and W_2 are the peak width at the baseline.

Efficiency, expressed as number of theoretical plates, was calculated as: $N=16[t/W]^2$, where t is the migration time, and W is the peak width at peak base.

3. Results and discussion

3.1. Optimisation of CZE conditions

After our first experiences on hair analysis for morphine and cocaine [9] using CZE at pH 9.2, which gave good separation but moderate sensitivity, we switched to an acidic running buffer (pH 2.5) in order to improve sensitivity by applying field-amplified sample injection, which requires the suppression of the electroosmotic flow.

The CZE separation of as many as 17 basic drugs, including compounds of forensic interest, has already been described by Chee and Wan [13] who used 50 mM NaH_2PO_4 adjusted at pH 2.35 with H_3PO_4.

In order to reduce any possible interaction between the basic (cationic) drugs and the residual ionised silanols (anionic) on the capillary wall, the buffer concentration was increased to 100 mM, compatibly with the resulting background current (about 100 μA).

Under the chosen analytical conditions, the analytes of interest migrated in the order: tetracaine, MDA, MDMA, MDE, cocaine, morphine and nalorphine, nicely separated in symmetrical peaks, with excellent resolution (R between 2.8 and 7.4) and efficiency ($N=150\,000$ plates). The overall analysis time was about 20 min. Using hydrodynamic injection (0.5 psi for 10 s), the limit of detection (LOD) at 200 nm wavelength was approximately 250 ng/ml. This sensitivity, although comparable to that reported by the majority of authors, was not completely satisfactory for hair analysis.

In reality, the low amount of hair (50–100 mg) which can be sampled and the moderate concentrations of analytes present in the matrix (from less than 1 up to 10 ng/mg) pose challenging problems of sensitivity in terms of concentration. In order to avoid the need of redissolving the hair extracts in extremely low volumes (5–20 μl), which are impractical in real application, a head-column field-amplified sample injection method was adopted. This procedure allowed extracts to be diluted with 1 ml of solvent, which was perfectly adequate for reproducible and complete dissolution of samples.

The comparison between pressure injection and electrokinetic injection under stacking conditions showed a remarkable increase in sensitivity using the latter technique. The comparison between 0.1 mM glycine, 0.1 mM phosphoric acid and 0.1 mM formic acid as sample solvents showed slight differences in terms of efficiency and signal-to-noise ratio. Table 1 summarises the results obtained by injecting MDMA at 1 μg/ml either by pressure or electrokinetically (similar data were obtained with morphine, cocaine, MDA,

Table 1
Variation of signal-to-noise ratio and efficiency of MDMA analysis (1 μg/ml) under different injection conditions

Injection method	Sample solvent	Signal-to-noise ratio	Efficiency (*N*)
Pressure: 0.5 psi, 10 s	0.1 mM Glycine	27	110 000
Electrok.: 10 kV, 10 s	0.1 mM Glycine	750	48 000
Pressure: 0.5 psi, 10 s	0.1 mM Formic acid	27	110 000
Electrok.: 10 kV, 10 s	0.1 mM Formic acid	750	49 000
Pressure: 0.5 psi, 10 s	0.1 mM Phosphoric acid	15	70 000
Electrok.: 10 kV, 10 s	0.1 mM Phosphoric acid	690	50 000

MDE, tetracaine and nalorphine). On this basis, 0.1 mM formic acid was chosen for sample dissolution.

The preliminary rinse of the capillary and the introduction of a plug of water or diluted acid before electrokinetic injection prevented contamination of the sample with the highly concentrated separation buffer, with consequent increase in sample conductivity and worsening of the stacking effect. On the other hand, an excessively long water plug (>10–15 s) worsened the efficiency of separation, because of local overheating of the injection end of the capillary. With water plugs >20 s the electric resistance in the capillary was dramatically increased, thus decreasing the current and the amount of ionic species injected. On the basis of these data, the optimal plug length chosen was 10 s. The best solvent for the preinjection plug to optimise sensitivity and efficiency was 0.1 mM phosphoric acid, as can be seen in Table 2.

Time and voltage were also optimised and the best compromise between sample load and efficiency was found at 10 kV for 10 s.

3.2. Validation

Under optimised conditions (i.e. sample dissolved in 0.1 mM formic acid, preinjection of 0.1 mM phosphoric acid at 0.5 psi for 10 s, electrokinetic injection at 10 kV for 10 s), the LOD values achieved were: 10 ng/ml for tetracaine, 3 ng/ml for MDA, 2 ng/ml for MDMA and MDE, 8 ng/ml for cocaine, 6 ng/ml for morphine and 8 ng/ml for nalorphine (with a signal-to-noise ratio of 5). In terms of drug concentration in hair, the theoretical sensitivity ranged from 0.02 to 0.01 ng/mg for the different analytes. Fig. 1 shows a typical electropherogram from a mixture of drug standards (10 ng/ml) analysed according to the conditions described above.

The lowest concentration suitable for recording interpretable spectra was about

Table 2
Variation of signal-to-noise ratio and efficiency of MDMA analysis (10 ng/ml) with different solvents forming the preinjection plug (10 s injection at 0.5 psi)

Injection method	Preinjection plug solvent	Signal-to-noise ratio	Efficiency (*N*)
Electrok.: 10 kV, 10 s	Water	15	110 000
Electrok.: 10 kV, 10 s	0.1 mM Formic acid	20	110 000
Electrok.: 10 kV, 10 s	0.1 mM Phosphoric acid	25	115 000

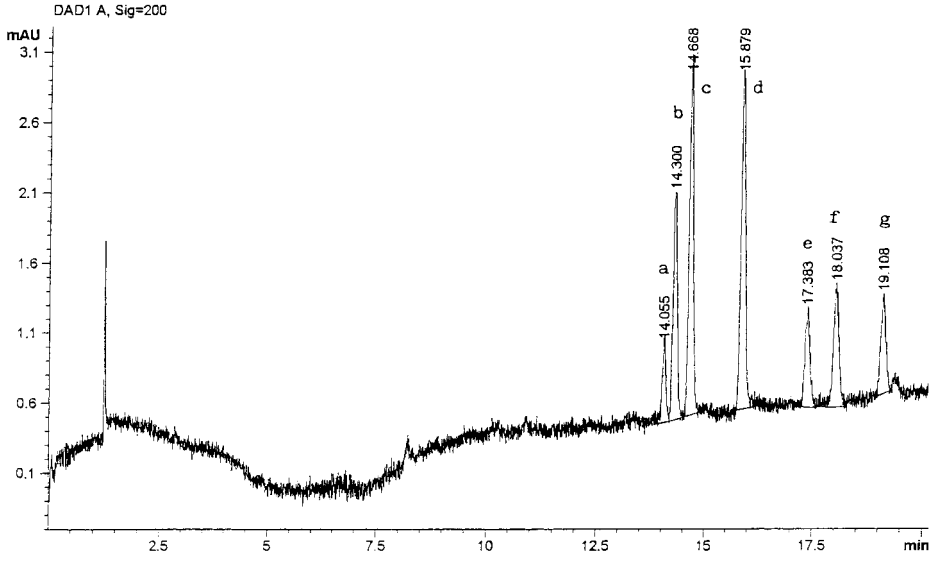

Fig. 1. Electropherogram of a mixture of drug standards (10 ng/ml of each in 0.1 mM formic acid) analysed under the following conditions: injection, 0.1 mM phosphoric acid at 0.5 psi for 10 s, sample at 10 kV for 10 s; separation, 100 mM phosphate pH 2.5, 10 kV; detection, UV absorbance at 200 nm. Peak identification: (a) tetracaine; (b) MDA; (c) MDMA; (d) MDE; (e) cocaine; (f) morphine; (g) nalorphine.

ten-times higher than the LOD of each analyte (see Fig. 2), still useful for confirmation of results from moderate consumers.

The quantitative validation was carried out using hair samples from real users of morphine, MDMA and cocaine spiked with fixed amounts (1 μg/ml) of nalorphine [as internal standard (I.S.) for morphine] and tetracaine (as I.S. for cocaine and/or MDMA). The used hair samples contained 1.5, 2.3 and 3.2 ng/mg of morphine, cocaine and MDMA respectively. In the present study, MDA and MDE were excluded from quantitative validation, as drugs of minor relevance in real samples.

The reproducibility of absolute migration times ($n=6$) (Table 3) was characterised by R.S.D. values of 1–2% and 1.8–4.5% in intraday and day-to-day experiments, respectively, but with the use of an I.S., R.S.D. values were ≤0.6%.

As expected, absolute peak area reproducibility with electrokinetic injection was unacceptable, but with internal standardisation R.S.D. values were better than 10% intraday and than 15% in day-to-day experiments ($n=6$) (Table 3), being suitable for analysis of biological samples.

Analytical linearity was studied on seriate 1:2 dilutions of extracts from real positive hair samples, in which the drug concentration had been determined by the reference methods. The linear regression equation was calculated by using the least square method between hair content (ng/mg) and peak area ratios drug/I.S.

The resulting equations were:

MDMA $y = 1.4106x + 0.004$; $R^2 = 0.9990$; range: 0.5 − 0.025 ng/mg

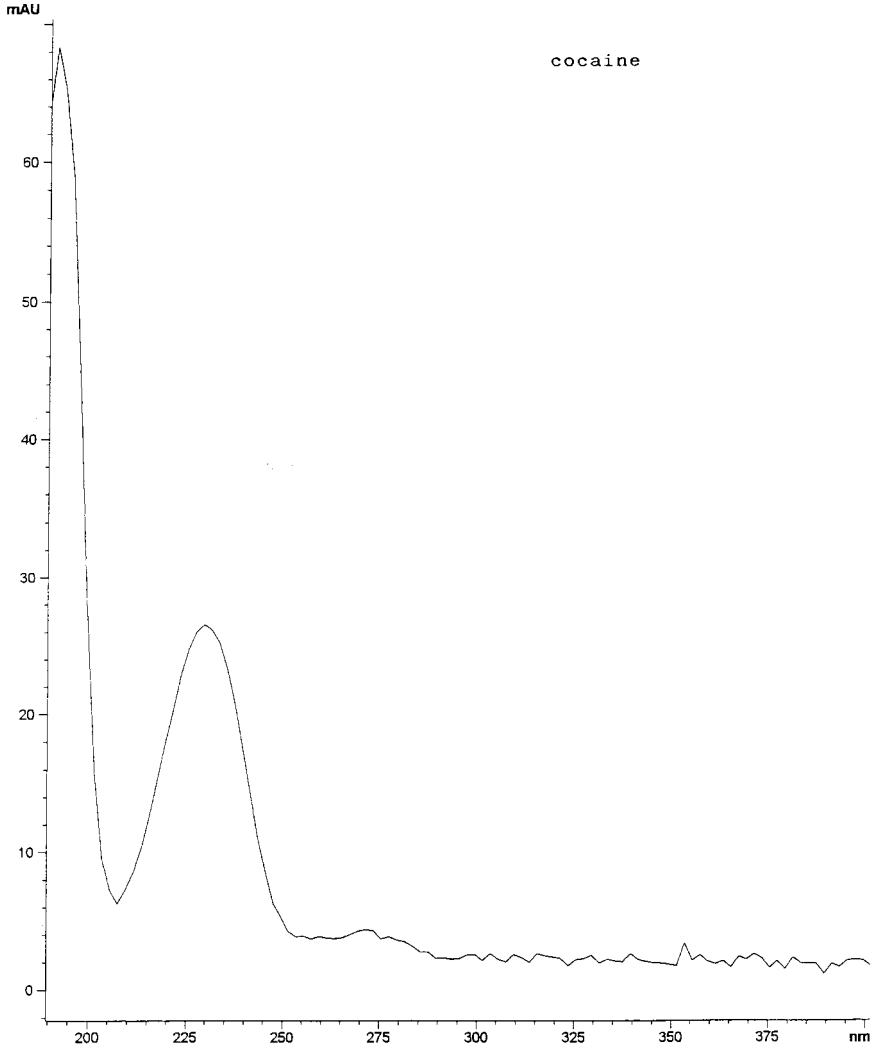

Fig. 2. On-line UV spectrum (190–400 nm) of the cocaine peak recorded during CZE separation of the drug mixture described in Fig. 1 at the concentration of 1 μg/ml (conditions as in Fig. 1).

cocaine $y = 0.5003x + 0.003$; $R^2 = 0.9998$; range: 1.0 − 0.050 ng/mg

morphine $y = 2.2361x - 0.042$; $R^2 = 0.9997$; range: 1.0 − 0.050 ng/mg

showing a linear range including the lowest concentrations of illicit drugs in hair which can be of forensic interest.

Typical examples of electropherograms from hair samples of real users positive for

Table 3
Analytical reproducibility (R.S.D.%) of CZE analysis of MDMA, cocaine and morphine in hair

Compounds	Migration times		Peak areas	
($n=6$)	Intraday	Day-to-day	Intraday	Day-to-day
MDMA	2.0	4.5	32.9	37.5
MDMA/I.S.	0.3	0.6	6.0	8.0
Cocaine	1.0	1.8	39.0	12.0
Cocaine/I.S.	0.1	0.2	9.0	14.0
Morphine	1.7	3.8	20.0	26.3
Morphine/I.S.	0.1	0.5	5.0	7.0

MDMA at the concentration of 4.0 ng/mg and cocaine at 3.4 ng/mg are shown in Figs. 3 and 4, respectively.

4. Conclusions

In recent years, CZE has gained popularity as an alternative analytical tool in forensic sciences and has been applied successfully to toxicological analysis of hair [9].

However, as with any other capillary analytical technique, CE has excellent sensitivity in terms of mass, even with simple UV detection (fractions of pg), but, due to the

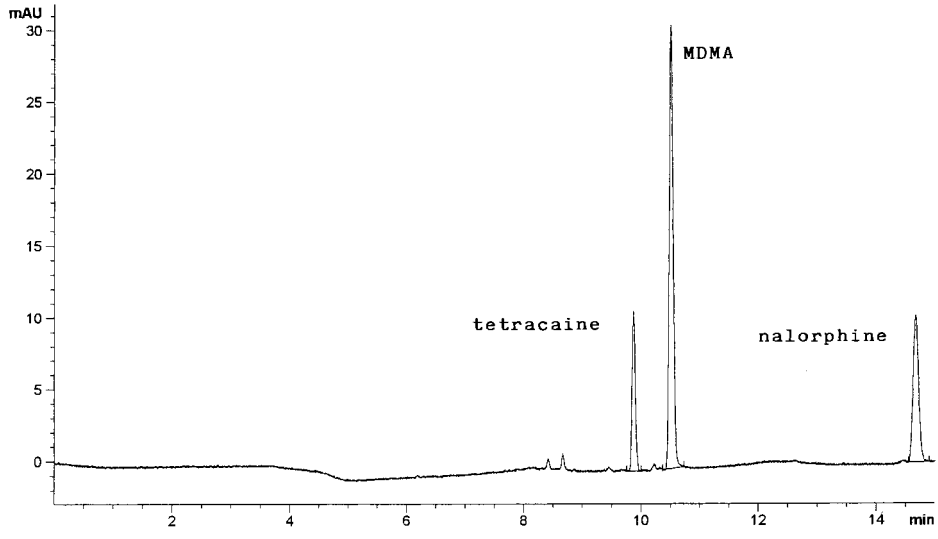

Fig. 3. Typical electropherogram of a hair sample from an ''ecstasy'' user positive for MDMA at the concentration of 4.0 ng/mg (analytical conditions as in Fig. 1).

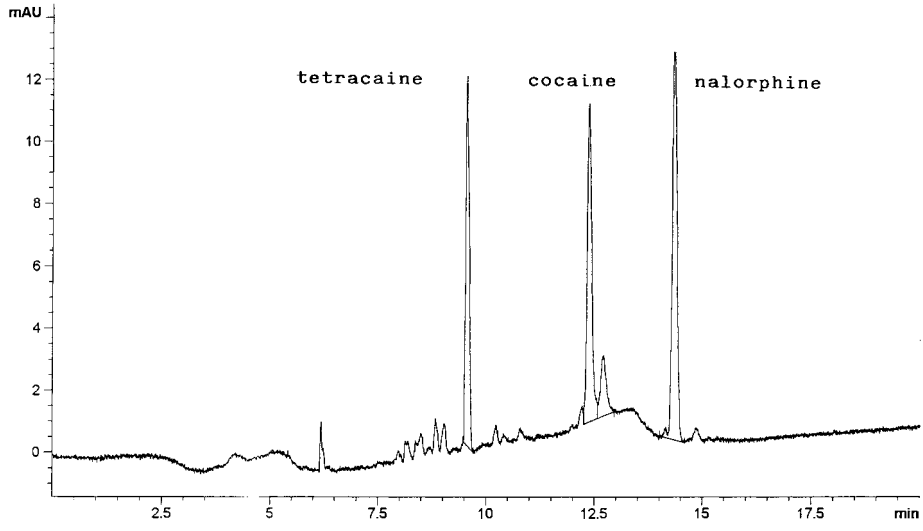

Fig. 4. Typical electropherogram of a hair sample from a cocaine user positive for cocaine at the concentration of 3.4 ng/mg (analytical conditions as in Fig. 1).

minimal volumes which can be injected (nl), only moderate sensitivity in terms of concentration (fractions of μg/ml).

This has limited the application of the technique to biological fluid analysis, in which the analytes to be determined are often present at ng/ml concentrations. Hair poses additional problems due to the limitations in the amounts which can be sampled because of aesthetic reasons.

A peculiar injection method of CE, such as field-amplified sample stacking proved to permit remarkable increases in injectable amounts of samples, without compromising separation efficiency, and was recently applied also to the analysis of biological fluids, reportedly providing over 1000-fold sensitivity enhancement [11].

In the present paper, head-column field-amplified sample stacking was tested and optimised for hair analysis, proving to offer adequate sensitivity and resolution power for determining some major drugs of abuse or drug metabolites, including cocaine, MDMA and morphine. The adoption of a suitable I.S. (i.e. nalorphine for morphine and tetracaine for cocaine and MDMA) provided excellent reproducibility in terms of migration times and acceptable quantitative precision and linearity.

The increase of the sample load provided by sample stacking also gave the opportunity to perform spectral analysis of the separated peaks by diode array detection, thus offering an additional possibility of peak identification and peak purity testing at drug concentrations which cannot be dealt with by standard CZE diode array detection approaches.

In conclusion, CZE coupled to head-column field-amplified sample stacking proved to offer a sensitive, precise, easy, low cost and rugged method for toxicological analysis of hair. Being based on separation mechanisms not correlated with the drug screening methods used in analytical toxicology (e.g. immunoassays) nor with gas and liquid

chromatography, CZE looks an interesting complementary technique for confirmation of results obtained with traditional techniques.

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