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Determination of opiates in urine by capillary electrophoresis

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Abstract

A method for the separation of a mixture of opiates comprising pholcodine, 6-monoacetylmorphine, morphine, heroin, codeine and dihydrocodeine by capillary electrophoresis using a running buffer of 100 mM disodium hydrogenphosphate at pH 6 is described. The characteristics of an analytical method based on this separation for the determination of these drugs following extraction from urine and using levallorphan as internal standard are reported. Detection limits in the region of 10 ng cm^{-3} are achieved when using electrokinetic injection. A comparison is made of the sensitivity and reproducibility of electrokinetic and hydrodynamic injection for these drugs. Data are presented to show the results obtained when the proposed method is applied to urine spiked with all the above opiates and also to urine from a subject following consumption of dihydrocodeine and pholcodine. The concentrations found are compared with those obtained by LC.

Keywords: Opiates; Pholcodine; 6-Monoacetylmorphine; Morphine; Heroin; Codeine; Dihydrocodeine

1. Introduction

In the screening of human urine samples to detect opiate abuse, an initial immunoassay method is usually adopted [1]. A positive result must subsequently be confirmed by a more specific method capable of distinguishing among the individual opiates since several commonly available and legal opiates produce a positive result in immunoassay [2,3]. To confirm the consumption of an illegal opiate the benchmark technique currently used is coupled GC–MS [4,5]. Using this technique it is possible to determine the concentrations of different opiates present in the sample. Presumption of heroin or morphine consumption is usually taken as positive on the basis of a high morphine to legal opiate concentration [6,7]. An alternative criterion for detecting consumption of heroin is the detection of the heroin metabolite 6-monoacetyl morphine [8]. In spite of the extensive use of coupled GC–MS there is a need for alternative techniques which are more generally available and can act as an additional screening method to eliminate samples showing evidence of the consumption of legal opiates only. The application of a normal-phase HPLC separation in this context has recently been demonstrated [9].

The technique of capillary electrophoresis (CE) is currently the focus of considerable attention as an alternative orthogonal separation technique to high-performance liquid chromatography for the separation and quantification of low molecular mass drug species in various matrices.

It has been shown that capillary electrophoresis operating in the capillary zone electrophoretic (CZE) mode using unmodified silica capillaries is capable

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of high resolution and peak capacity for basic drugs [10]. It is less well established that this technique is capable of the required concentration sensitivity to produce low enough quantification limits in biological fluids to provide an alternative to HPLC. This is a result of both the low concentration sensitivity of the short path length of detection and the small volume of sample which can be introduced into the capillary. The most commonly used method of sample injection in CE is hydrodynamic either by applying pressure at the inlet or vacuum at the detector end of the capillary. This allows a small volume, representative of the analysis matrix, to be introduced into the capillary. Alternatively, ionised analytes, particularly those which are cationic can be introduced into the capillary electrokinetically by temporarily applying a relatively low potential across the capillary with the injection end of the capillary immersed in a sample solution [11]. If such a potential is applied at low pH values, such that the electroosmotic flow is minimal, protonated basic species are selectively introduced into the capillary [12]. It has been demonstrated practically and explained theoretically that the mass of analyte introduced in this way can be increased by decreasing the ionic strength of the solution from which the sample is injected [13]. It has also been shown that, by using solid-phase extraction and electrokinetic injection from aqueous and methanolic solutions, it is possible to realise detection limits low enough to allow quantification of combinations of antimalarial drugs in urine [14].

It is the purpose of the present work to show that capillary zone electrophoresis is capable of resolving a mixture of commonly available legal opiates, heroin and its metabolites and of quantifying these in urine at levels comparable with those obtained using alternative techniques. In the process of this, some of the factors found to be significant in obtaining resolution and optimum sensitivity will be investigated and discussed. In this context, it can provide a useful preconfirmation test for opiate abuse. Previous publications on opiate separation and quantification [15,16] utilised micellar electrokinetic chromatography (MEKC) and have been restricted to much higher sample concentrations. Because of the lack of information in the literature concerning the quantitative characteristics of electrokinetic injection compared with that on hydrodynamic methods, a comparison of both the relative sensitivity and the precision of these injection methods will also be included for these analytes.

2. Experimental

2.1. Materials

The capillary electrophoresis equipment used was a Hewlett-Packard ^{3D}CE system fitted with a diode-array ultraviolet detector. The capillary was unmodified silica of internal diameter 50 μm. The overall length was 65 cm and effective length 60 cm. Although this equipment could be fitted with capillaries having an increased internal diameter at the detection region of the capillary, such capillaries were not used in the present work. Hydrodynamic and electrokinetic injection modes were available. Sample pretreatment was by Bondelut Certify (Analytichem, Harbor City, CA, USA) solid-phase extraction cartridges (300 mg) using a Vac-Elut ten-sample vacuum manifold (Jones Chromatography, Hengoed, UK).

Heroin (diacetylmorphine) (H) and levallorphan (I.S.), the internal standard, were obtained from D.M. Wood (Aberdeen, UK), 6-monoacetylmorphine (6-M) from MacFarlane Smith (Edinburgh, UK) and codeine (C), pholcodine (P), dihydrocodeine (D) and morphine (M) from Sigma (Poole, UK). Water was purified by a Millipore Milli-Q system. Methanol was obtained from Rathburn (Walkerburn, UK), dichloromethane and propan-2-ol from Fisons (Loughborough, UK) and all buffer salts and reagents were of AnalaR or equivalent grade.

2.2. Electrophoresis operating conditions

The capillary oven was thermostatted at 25°C. The capillary was preconditioned by flushing with running buffer for 2 min by applying 930 mbar pressure to the inlet end. Hydrodynamic injection was by applying 50 mbar for appropriate times.

2.3. Method development

It was found that the conditions found previously to give good resolution among several basic antimalarial drugs [12] gave inadequate resolution among the set of six opiates. The effects on resolution of buffer concentration (over a range of 20–160 m*M* in disodium hydrogen orthophosphate at a pH of 7.0) and pH (over a range of 3.9–8 at a buffer concentration of 100 m*M*) were therefore examined since these appear to be the major electrophoretic variables affecting separation. The peak shapes of the analytes on the resulting electropherograms and the reproducibility of the electromigration times were also noted during these measurements.

The pretreatment used for urine samples was based on that previously used for the extraction of opiates [9]. It was modified in that 0.5 cm^3 of urine and 0.5 cm^3 of a levallorphan solution (805 ng cm^{-3}) were applied to the conditioned cartridge. After washing, elution with a mixture consisting of dichloromethane–propan-2-ol–ammonia 8:2:0.2 (2 cm^3) and evaporated to dryness, the analytes and internal standard were redissolved in 100 μl methanol and this solution diluted to 1 cm^3 with water. Levallorphan was chosen empirically as an internal standard because of its appropriate electromigration time in the optimised buffer system coupled with its suitable retention and elution characteristics during the sample pretreatment.

It had previously been established from measurements on antimalarial drugs [12,14] that electrokinetic injection resulted in increased sensitivity over hydrodynamic injection. The variables of applied potential, duration of injection and injection solvent type were studied to ascertain optimum conditions for maximum sensitivity consistent with maintenance of resolution. Solvents comprising running buffer, water containing 2.5% methanol (to ensure complete solubility of the opiates), pure methanol and various proportions of methanol in water were examined. This latter was investigated because of the need, during the pretreatment of urine samples, to dissolve the extracted residue initially in methanol to ensure complete solution of the opiates. Since it was established during the optimisation of the separation that components of the matrix co-extracted during the pretreatment stage did not interfere with the determination of the analytes, the sensitivity of response was determined at several wavelengths ranging from 280 to 200 nm.

2.4. Validation

Using the conditions determined for optimum resolution among the analytes and for maximum peak areas consistent with this resolution the resultant assay was validated with respect to the following parameters. Linearity of response was determined by spiking 0.5-cm^3 aliquots of urine with four concentrations of each of the opiates P, C, D, H, 6-M and M over the range 150 to 600 ng cm^{-3}. I.S. was also included at a concentration of 805 ng cm^{-3}. From the electropherograms, run under the optimised conditions, the individual peak areas were recorded and regression equations determined for each opiate of peak-area ratio (opiate/I.S.) against opiate concentration. The accuracy of the method was established by plotting the concentrations calculated from the resultant regression lines against the spiked concentrations, showing that the slope of the resulting regression line was not significantly different from one at a given confidence level and showing that the intercept was not significantly different from zero [17]. The within-day precision of the method was determined by spiking a urine sample with approximately 300 ng cm^{-3} of each opiate and with the appropriate concentration of I.S. The peak areas and area ratios were determined for ten replicate samples after extraction and electrophoresis. The day-to-day precision was determined in the same way over a period of six days using four replicate samples. Detection limits were determined under the standard conditions used by extracting spiked urine containing opiates of successively lower concentrations until the peak height was three times the baseline noise. To validate the use of the method in vivo, urine samples from human subjects were subjected to the proposed method. These samples were obtained from four separate subjects following the consumption of the following doses of individual opiates: D (15 mg), P (10 mg), C (16 mg) and M (2 mg). These samples had previously been subjected to enzyme multiplied immunoassay technique (EMIT)

and the concentration of opiate present in each sample determined by a previously published LC method [9]. To assist in the identification [15] of individual opiates, the diode-array detection facility of the equipment was used to store the ultraviolet spectrum of each peak on the electropherogram over the wavelength range 190 to 350 nm.

2.5. Comparison of injection methods

Using the set of opiates at a concentration of 800 ng cm^{-3}, five replicates of the area of each opiate peak on the resultant electropherograms using the conditions described for the proposed assay were determined. The measurements were repeated using a 10-s hydrodynamic injection while maintaining all other conditions constant. From these data the relative standard deviations of the electromigration time, the areas and the area ratios were determined. The relative responses, in terms of peak areas, resulting from electrokinetic and hydrodynamic injection were also calculated for each opiate.

3. Results and discussion

Fig. 1(a) shows the variation of migration time with buffer concentration and it is apparent that, while resolution increases as buffer concentration increases, there is little increase in resolution above 100 m*M*. In contrast, Fig. 1(b) shows that pH is a much more important variable in obtaining selectivity [10]. At low values of pH, migration times are large and this is attributed to the reduction in electroosmotic flow since the compounds will be maximally protonated at low values of pH. In addition, there is incomplete resolution between codeine and dihydrocodeine below pH 5 although all the opiates have pK_a values above 7.6. As pH increases there is a decrease in migration time for all compounds although this is reversed for pholcodine above pH 6.5. All six opiates are resolved between 5.7 and 6.5 and separations were found to be most reproducible in this pH range. Above 6.5 there are marked changes in selectivity due to the increase of migration time of pholcodine. On this basis a pH of 6.0 was chosen as optimum. It is assumed that resolution is being achieved on the basis of degree of ionisation in contrast to previously published work on the antimalarials where all analytes were completely protonated at the pH employed [12,14]. Fig. 2 shows a representative electropherogram of the six opiates and the internal standard levallorphan. Separation is complete in 12 min and the conditions used produced typical peak efficiencies in the region of $2 \cdot 10^5$ plates.

The expected increase in peak height using field-amplified sample injection (FASI), in which low ionic strength solutions rather than running buffer are used for electrokinetic injection [11], was realised for this set of opiates. The technique of separately introducing a water plug [18] before electrokinetic injection from the water solution of the opiate mixture was not found to offer any advantages in increased sensitivity of detection. The use of methanol which has previously been shown to increase sample stacking for a set of antimalarials [12,14] was not found to be effective in the present system. When opiates were injected from methanol, peak areas were considerably reduced from those obtained in running buffer. Since this also occurred during hydrodynamic injection from methanol it is assumed that precipitation, due to the higher concentration of buffer required to achieve resolution, occurs. Since a low residual concentration of methanol was present in all standard opiate solutions to ensure complete solution, the effect of the concentration of methanol in the injection solvent on the sensitivity of detection was examined. It was found that concentrations of methanol at or below 10% did not reduce the response. For subsequent work on samples extracted from urine a solvent containing 10% methanol was used to allow easy and complete reconstitution of extracts after evaporation to dryness.

As reported by previous workers, increasing both the time interval and the electrical potential of injection increased peak-area response [19]. At the higher values of the product of these variables loss of peak symmetry and resolution occurred and conditions of 10-s injection time at 5 kV applied potential were used in all subsequent experiments. This resulted in running currents of 90–100 μA.

Fig. 3 shows a representative electropherogram of urine, spiked with the six opiates and including the internal standard after solid-phase extraction and

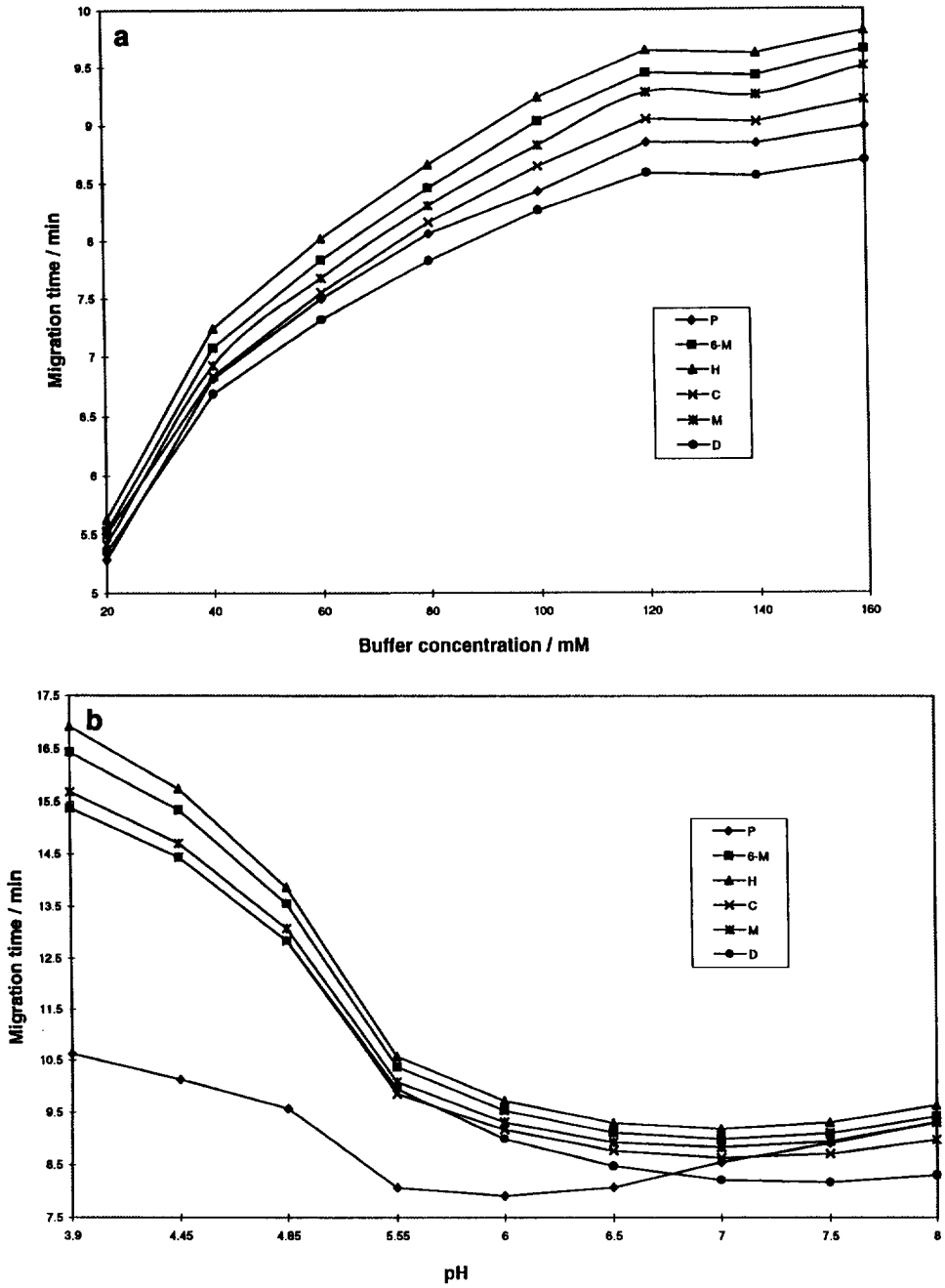

Fig. 1. Variation of migration time with (a) buffer concentration (pH 7) and (b) pH at 100 m*M* buffer for each of the opiates, pholcodine (P), 6-monoacetyl morphine (6-M), heroin (H), codeine (C), morphine (M) and dihydrocodeine (D). A running voltage of 20 kV and a 10-s electrokinetic injection at 5 kV was used.

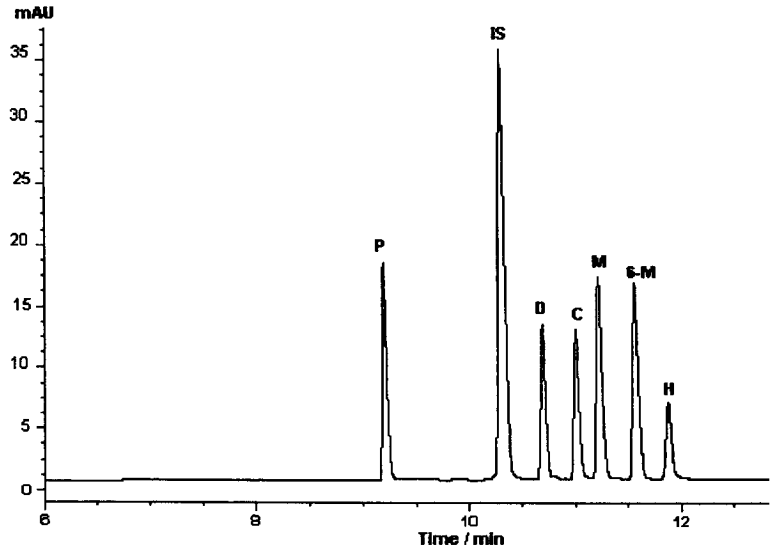

Fig. 2. Representative electropherogram of the six opiates, pholcodine (P), 6-monoacetyl morphine (6-M), heroin (H), codeine (C), morphine (M), dihydrocodeine (D) and levallorphan as internal standard (I.S.). The running buffer was 100 m*M* disodium hydrogen orthophosphate at pH 6, applied voltage 20 kV, electrokinetic injection for 10 s at 5 kV and detection at 200 nm.

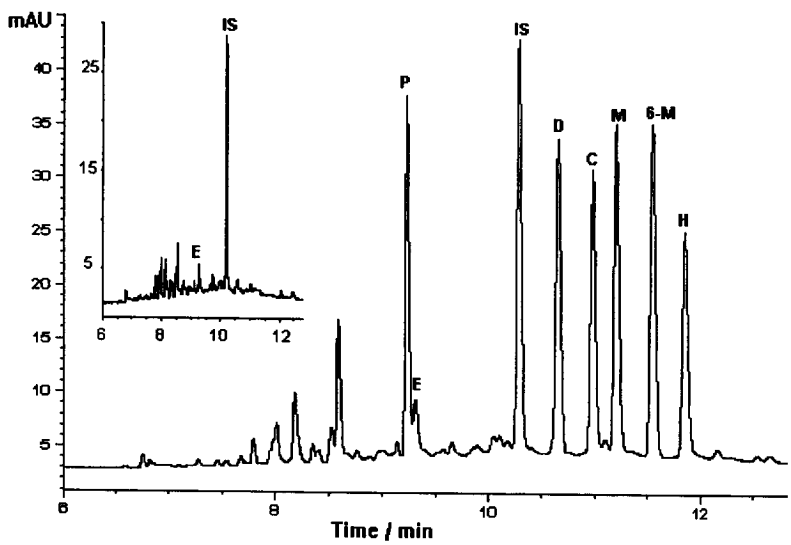

Fig. 3. Representative electropherogram of urine spiked with the six opiates pholcodine (P), 6-monoacetyl morphine (6-M), heroin (H), codeine (C), morphine (M), dihydrocodeine (D) and levallorphan (I.S.) as internal standard after solid-phase extraction and electrophoresis with detection at 200 nm. An endogenous compound (E) with a similar retention to pholcodine is identified in the spiked sample and the blank urine (inset). The running buffer was 100 m*M* disodium hydrogen orthophosphate at pH 6, applied voltage 20 kV, electrokinetic injection for 10 s at 5 kV and detection at 200 nm.

electrophoresis with detection at 200 nm. Shown as an inset on this is the electropherogram obtained by subjecting blank urine to the same treatment. It is evident that very few endogenous compounds in the urine are being extracted and applied to the capillary under the electrokinetic conditions used. Only one of these (E) is close to any of the opiate peaks. The remaining endogenous species detected have migration times shorter than the opiates. In practice, during the determination of pholcodine in spiked and subject urine samples, pholcodine can be reliably distinguished from E since the resolution between the two compounds is greater than 1.1 and the spectra over the range 190 to 350 nm are markedly different. The use of 200 nm as a wavelength of detection to some extent offsets the short path length of the detector. It allows an approximately 14 times increase in detection sensitivity over measurement at 280 nm which was the optimum wavelength required in a previous opiate determination by HPLC [9] due to the solvent front produced by endogenous materials co-extracted from the urine.

Table 1 shows the main validation aspects for the optimised system as applied to spiked urine samples. The precision of the electromigration times is seen to be 1.1% R.S.D. or less. Resolution between adjacent peaks is greater than two for all pairs and the plate numbers are above $2 \cdot 10^5$. The peak-area ratio–concentration calibration lines show good linearity with correlation coefficients greater than 0.9917. The calibration range chosen was that thought to be appropriate for application of this method as a preconfirmation test to determine the nature of an opiate detected by immunoassay. The legal cut-off for such detection is 300 ng cm^{-3} with respect to total opiate present so that any sample to which the proposed method would be applied is likely to have an opiate concentration greater than this. The within-day and day-to-day precision of the proposed method are seen to be comparable in the range of 1 to 4% when peak-area ratios are used. These are lower than have been reported in the literature for electrokinetic injection [14]. The variances of replicate areas used directly are very much greater than these values suggest. It appears from the literature that the very considerable advantages in sensitivity which can be realised by using electrokinetic injection are not being exploited as a result of reported lack of adequate reproducibility. Table 3 below shows data comparing the characteristics of electrokinetic and hydrodynamic injection to allow evaluation of the alternative injection methods. The detection limits shown in Table 1 are in the region of 4–9 ng cm^{-3} using the conditions specified for the optimised assay method described. These are more than adequate for confirmation of particular opiate consumption given that enzyme immunoassay cut off values are usually taken as 300 ng cm^{-3}. In the more general analytical context it is apparent that these detection limits can be decreased in a variety of ways. As was discussed above the resolution is capable of accommodating injection for longer time periods and at higher applied voltages without losing specificity. Alternatively the sample volume used for the analysis can be increased by a factor of 10 and 5-cm^3 aliquots of urine used as is the case in GC–MS methods. The method was shown to be accurate at the 95% confidence level as described above.

Table 2 shows the results of the in vivo measurements of opiate concentrations determined in four urine samples from human subjects who had consumed various legal opiates. All of these samples tested positive for opiate by enzyme immunoassay. For the subject samples tested, the identification of the peak on the electropherogram was on the basis of electromigration time and confirmed by comparison with standard stored spectra of individual opiates. Also included in this table are the results of opiate determination previously obtained by a recently published HPLC method [9]. It can be seen that the results are in good agreement. In practice it was found that the CE method produced better resolution among the opiates, that matrix interference was virtually absent and that the separation and quantitation were considerably faster. This last was due to a combination of the intrinsically faster separation and also the higher degree of automation both of sample application and data treatment possible with the CE equipment used. Fig. 4(a) and Fig. 4(b) show representative electropherograms obtained by analysis of urine from a subject after consumption of dihydrocodeine and pholcodine, respectively. It is seen that at the concentrations found few matrix peaks are evident. It was found that the N-de-

Table 1
Validation data for the optimised system as applied to urine samples spiked with the six opiates pholcodine (P), 6-monoacetyl morphine (6-M), heroin (H), codeine (C), morphine (M), dihydrocodeine (D) and levallorphan (I.S.) as internal standard

	P		I.S.		D		C		M		6-M		H
Migration time (min) (n=5) (R.S.D., %)	9.18 (1.1)		10.18 (0.80)		10.68 (0.71)		10.99 (0.77)		11.21 (0.79)		11.55 (0.79)		11.87 (0.74)
Resolution		13.4		4.7		3.7		2.3		3.9		3.4	
Efficiency of peak ($\times 10^{-5}$)	2.5		2.7		2.8		2.6		2.2		2.2		2.8
Linearity													
Slope of calibration line ($\times 10^3$) (R.S.D., %)	1.3 (5.1)		–		1.6 (5.2)		1.5 (4.2)		1.4 (3.4)		1.5 (3.2)		1.2 (2.6)
Intercept ($\times 10^3$)	15		–		33		2.0		4.0		12		1.3
Correlation coefficient	0.9917		–		0.9924		0.9944		0.9966		0.9967		0.9980
Precision													
Within-day at 300 ng cm^{-3} (n=5) R.S.D. (%)	3.8		–		1.8		1.4		2.1		0.6		3.4
Day-to-day at 300 ng cm^{-3} (n=4) R.S.D. (%)	2.9		–		2.2		2.1		2.5		1.4		2.8
Detection limit (ng cm^{-3})	4		–		8		7		8		9		8

Table 2
Results of the in vivo measurements of opiate concentrations determined in four urine samples from human subjects who had consumed various legal opiates and had tested positive by EMIT

Time since first dose (h)	Preparation ingested	Drug dose	HPLC		CZE	
			Drug found	Concentration (ng cm^{-3})	Drug found	Concentration (ng cm^{-3})
1	Paramol	Dihdrocodeine tartrate BP. (15 mg)	Dihydrocodeine	10 900	Dihydrocodeine	10 000
1	Pholcodine	Pholcodine Linctus BP. (10 mg)	Pholcodine	1963.4	Pholcodine	1337.6
			Morphine	60.0	Morphine	58.7
−96	Pholcodine	Pholcodine Linctus BP. (10 mg)	Codeine	3190.0	Codeine	3163.4
			Pholcodine	199.5	Pholcodine	157.0
1	Panadeine Co.	Codeine phosphate BP. (16 mg)	Morphine	60.4	Morphine	64.8
−120	Pholcodine	Pholcodine Linctus BP. (10 mg)	Pholcodine	51.2	Pholcodine	32.8
3	J. Collis Browne Mixture	Morphine (2 mg)	Morphine	248.1	Morphine	215.0

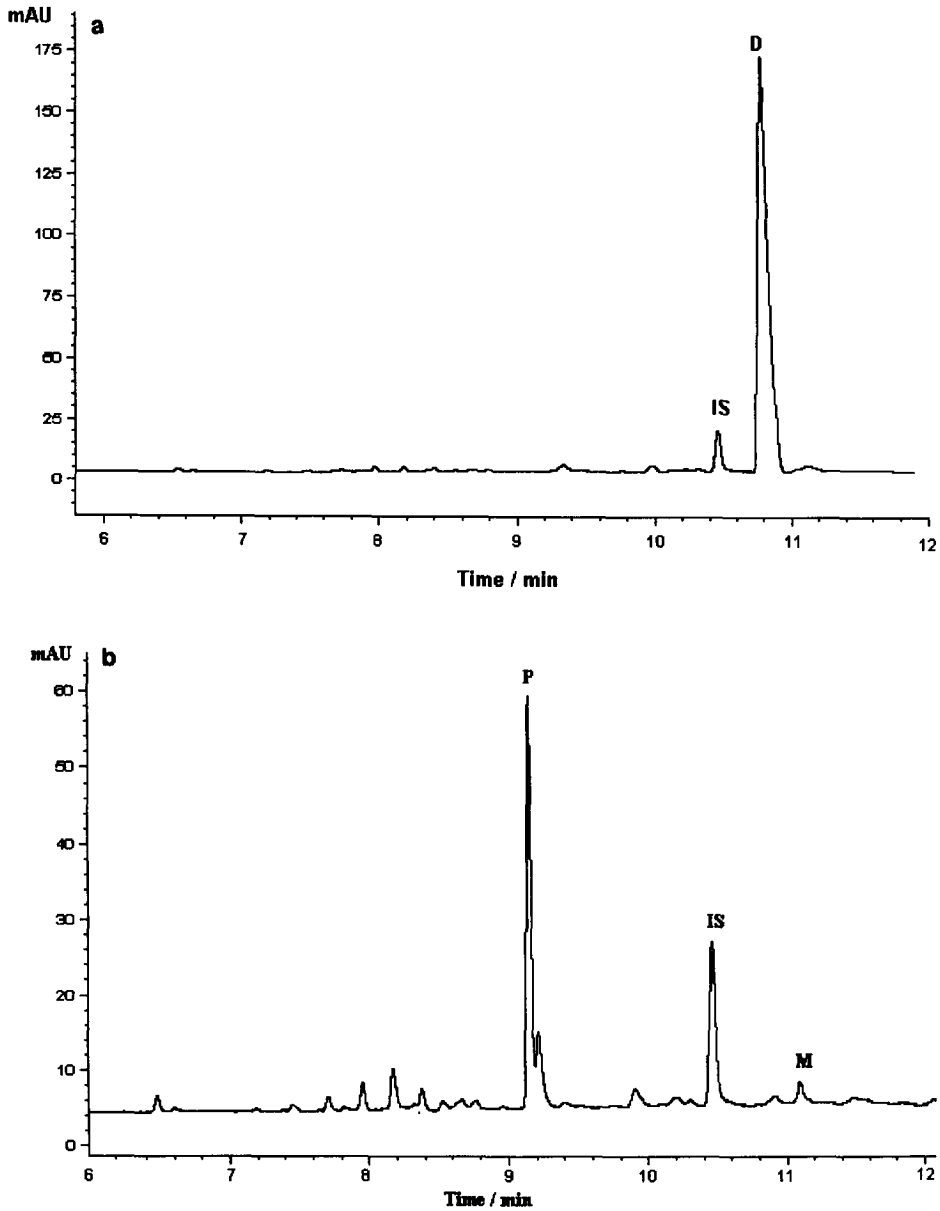

Fig. 4. Representative electropherograms obtained by analysis of human subject urine collected one hour after consumption of (a) 15 mg dihydrocodeine, (b) 10 mg pholcodine. Electrophoretic conditions as in Fig. 3.

methylated metabolites norcodeine and normorphine migrated at times comparable with the parent opiate.

Table 3 shows the results of controlled experiments to compare the precision and relative sensitivity of the alternative methods of electrokinetic and hydrodynamic injection at the sub μg cm^{-3} level. An opiate concentration of approximately 0.8 μg cm^{-3} was used. This concentration was chosen as the lowest concentration which could be detected following hydrodynamic injection. The R.S.D. (%) of the electromigration time for electrokinetic is approximately twice that for hydrodynamic injection

Table 3
Precision and relative sensitivity from five replicates each of electrokinetic and hydrodynamic injections

	P	I.S.	D	C	M	6-M	H
Electrokinetic (0.8 μg cm^{-1})							
Migration time (min)	9.18	10.18	10.68	10.99	11.21	11.55	11.87
R.S.D. (%)	1.1	0.80	0.71	0.77	0.79	0.79	0.74
Peak-area	351.4	600.7	176.9	183.1	248.0	225.6	160.79
R.S.D. (%)	16.0	14.7	15.8	15.8	16.1	16.1	16.2
Peak-area ratio	0.584	–	0.294	0.304	0.412	0.375	0.267
R.S.D. (%)	2.96	–	1.54	1.72	2.18	3.39	3.60
Hydrodynamic (0.8 μg cm^{-3})							
Migration time (min)	9.11	10.24	10.67	10.98	11.18	11.49	12.03
R.S.D. (%)	0.66	0.27	0.21	0.31	0.32	0.36	0.33
Peak area	2.21	6.31	1.50	1.83	1.92	1.63	1.74
R.S.D. (%)	5.97	5.39	12.43	6.04	5.93	16.29	16.73
Peak-area ratio	0.350	–	0.237	0.290	0.304	0.259	0.275
R.S.D. (%)	4.10	–	11.1	4.40	3.85	17.3	12.7
Relative sensitivity	159.2	–	118.3	100.0	129.3	138.5	92.2

Pholcodine (P), 6-monoacetyl morphine (6-M), heroin (H), codeine (C), morphine (M), dihydrocodeine (D) and levallorphan (I.S.).

but both are below 1% which is comparable with accepted HPLC values for retention times. The variation of the areas of the respective peaks are comparable for both electrokinetic and for hydrodynamic methods. When the areas are normalised using the internal standard the electrokinetic method appears more precise than hydrodynamic injection. This is not consistent with data in the literature and is probably a result of operating near the limit of quantification for the hydrodynamic method. The relative sensitivity of the two methods is also shown in this for the individual opiates and it is seen that this varies from 90 to 160 times in favour of the electrokinetic method.

4. Conclusions

The proposed method is capable of detecting and quantifying opiates present in urine following consumption of legal and illegal opiates. It is useful, therefore, as a technique for the determination of the individual opiate consumed in situations where an immunoassay-based screen has shown the presence of opiate in a urine sample. The criteria normally applied to establish this, namely the detection of the 6-monoacetyl morphine metabolite or the ratio of morphine to legal opiate detected can be based on data obtained using this general technique. The use of spectral matching of individual peaks on the electropherogram with standards reinforces the identification of particular opiates.

The development of the method and its validation have shown the applicability of electrokinetic injection methods for opiates using FASI and has provided self-consistent data on the reproducibility of this injection method compared with the more generally used hydrodynamic techniques.

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