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Comparison of phenyl-type columns in the development of a fast liquid chromatographic system for eighteen opiates commonly found in forensic toxicology

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Abstract

We report a precise and reliable method for the detection of 18 of the most commonly found opiates on the Belgian legal and illicit market, by ion-exchange, reversed-phase high-performance liquid chromatography, using a conventional phenyl-type analytical column (150×4.6 mm I.D., particle size 5 μm) and diode-array detection. We also describe a performance (efficiency and sensitivity) comparison of this column to a recently developed ''high-speed'' column (53×7.0 mm I.D., particle size 3 μm) packed with the same stationary phase, and used under slightly adjusted flow and gradient conditions. The final method, using the ''high-speed'' column, showed a significant reduction (55%) in analysis time without loss of resolution and sensitivity.

Keywords: Forensic analysis; Stationary phases, LC; Opiates

1. Introduction

Opiates and their derivatives are very potent analgesics. Commonly used as therapeutic agents some of these components are also frequently abused as illicit drugs. Although considerable progress has been made in elucidating their mode of action [1] and in the development of analytical techniques to measure morphine [2–4], its derivatives and metabolites [5–9], controversy still exists as to the most appropriate method of analysing these drugs.

Up to a few years ago, gas chromatography combined with mass spectrometry (GC–MS) was often used because of its sensitivity but the necessity of sample derivatisation and the cost of the technique itself restricted its applicability [10,11]. On the other hand, high-performance liquid chromatography (HPLC) appeared as a technique that could separate both the lipophilic and hydrophilic analytes without any chemical pretreatment. As such, it became the preferred technique in most applications, using a variety of detection methods such as ultraviolet [5,12], fluorescence [8,13], electrochemical [14] or a combination of them in series [9], and most recently, mass spectrometry [7,15].

Lately, the need for newer, faster HPLC methods has become an important issue. Analysts want to reduce the analysis time involved and hence increase sample throughput. In addition, such methods had to be amenable to existing instrumentation, to minimise investment costs. Finally, decreased solvent consumption also became increasingly important to minimise expenses and waste disposal problems

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[16]. This study evaluates a column configuration to meet these goals.

Theoretically, by using columns with particles smaller than the widely used 5 μm materials, these goals could be attained. On the one hand, they permit high column bed efficiency and better separation. On the other hand, higher mobile phase flow-rates are allowed without sacrificing the improved column efficiency [17]. In practice, however, disadvantages such as the tendency to ''plug'', instability of the column, high back pressure and extra-column effects (peak broadening, etc.) decrease these advantages [18]. The unique feature of the proposed column type, only recently commercially available, is a combination of desirable and practical characteristics such as reduced column length (53 mm), large internal diameter (7.0 mm), and smaller particle size (3 μm).

To the best of our knowledge, up to now, no fast assay exists which allows the detection of a wide range of opiates within one HPLC run, combined with diode-array detection. It is the purpose of the present paper to describe such a method for 18 of the most commonly found opiates on the Belgian legal and illicit market and compare the usefulness of the recently developed ''high-speed'' phenyl-type columns to the conventional and widely used analytical columns.

2. Experimental

2.1. Materials

The opiates studied were acetyldihydrocodeine, acetylcodeine, buprenorphine, butorphanol (internal standard), codeine, dextromethorphan, ethylmorphine, heroin (diacetylmorphine), hydrocodone, morphine, 6-monoacetylmorphine (6-MAM), methadone, naloxone, norcodeine, normethadone, normorphine, noscapine, papaverine and thebacone, all of which were supplied by Sigma (Bornem, Belgium).

Methanol (Romil, Merelbeke, Belgium), acetonitrile (Fisher, Leicestershire, UK) and water (Prosan, Merelbeke, Belgium) were all of HPLC grade. Ammonium acetate and formate buffer solutions (purity min. 98%), as well as triethylamine (purity min. 99%) were also supplied by Sigma, while formic acid (purity min. 98%) was purchased from Fluka (Bornem, Belgium).

2.2. Instrumentation

The HPLC–diode array detector system consists of a Hewlett-Packard 1090 liquid chromatograph and a 1040A Hewlett-Packard detection system. The LC unit is linked to a 9133 Workstation equipped with Hewlett-Packard 7099A Analytical Workstation software.

Separation was initially performed using a Hypersil BDS Phenyl analytical column (150×4.6 mm I.D., particle size 5 μm) preceded by a Hypersil BDS pre-column (7.5×4.6 mm I.D., particle size 5 μm), both supplied by Alltech (Lokeren, Belgium). The alternative fast separation was performed using a ''high-speed'' Hypersil BDS Phenyl Rocket column (53×7.0 mm I.D., particle size 3 μm), which was a gift from Alltech.

2.3. Analyte preparation

An individual standard solution of 1 g/l of each opiate was prepared in methanol or acetonitrile, according to the solubility of the solute, and stored in the dark at −20°C until use. Under these conditions all solutions proved stable for more than 6 months.

A working solution, containing 20 mg/l (1 μg/50 μl injected on column) of all opiates, was prepared by mixing an aliquot (100 μl) of each stock solution, evaporating the mix, at room temperature, under a constant flow of nitrogen, and redissolving the dry residue in 5 ml of solvent A (see Section 2.4) in an amber, volumetric flask. This solution was stored at −20°C.

2.4. Chromatography

Initial experiments started with a conventional, 4.6 mm I.D., phenyl column, under chromatographic conditions previously applied in our laboratory for the determination of cocaine and its metabolites [19]. Two solvent reservoirs, (A) water–methanol–acetonitrile (90:5:5, v/v) and (B) methanol–acetonitrile (50:50, v/v), both containing 50 m*M* ammonium acetate, were used to separate the opiates with a gradient elution programme starting with 100% A

and reaching an equilibrium of the two solvents, by linear increase, 20 min later. This was followed by a steep gradient to 100% B in 5 min and a washing period of an additional 5 min under these conditions. The programme took another 5 min to return to the initial conditions and a further 10 min to equilibrate before the next injection. Separation was performed at room temperature with a flow-rate of 1 ml/min and the opiates were detected by diode-array detection, monitoring at 280 nm for chromatogram construction.

Successively, different buffer solutions (ammonium acetate and ammonium- and triethylammonium formate), solvent compositions [75:25, 25:75, 20:80, 10:90 acetonitrile–methanol mixtures (all v/v) and 100% methanol] and buffer concentrations (5, 10, 15, 20, 25 and 50 m*M* triethylammonium formate) were tested.

Separation was initially performed on a conventional phenyl column (150×4.6 mm I.D., particle size 5 μm) by the following method: (A) water–methanol (90:10, v/v), (B) methanol, both containing 25 m*M* triethylamine and 30.3 m*M* formic acid [solvent A: apparent pH (pH_{app})=4.5]. The elution programme started with 100% A, changed linearly to 50% A and 50% B in 20 min and was programmed further to 100% B in 5 min. The latter solvent composition was held for 1 min and the return to the initial conditions was performed within 4 min (see Fig. 1). After 10 min of equilibration the next sample was analysed. This resulted in a method in which the last eluting component, i.e., methadone, was eluted after approximately 25 min and a total analysis time (elution, wash and equilibration period) of 40 min.

By adjusting the flow to 2.0 ml/min and accelerating the gradient elution programme (see Fig. 1), a similar separation, under exactly the same solvent and detection conditions, was achieved with the ''high speed'' column (53×7.0 mm I.D., particle size 3 μm).

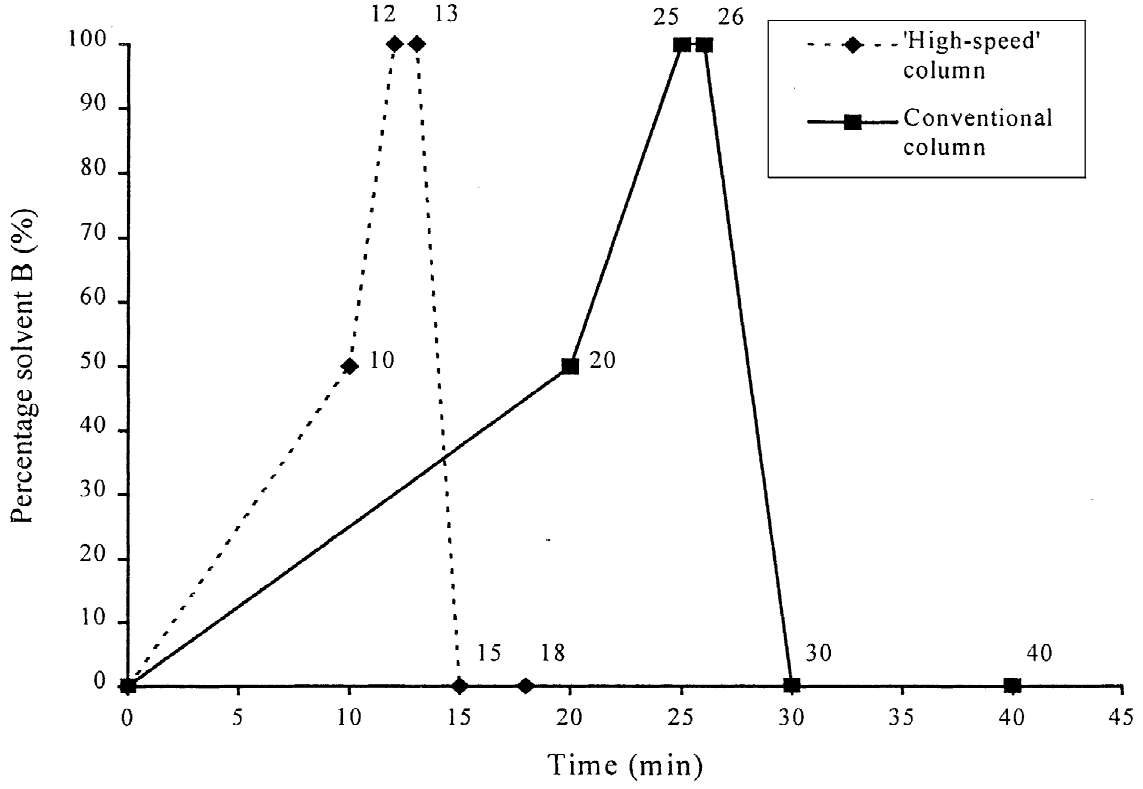

Fig. 1. Gradient elution programme for the conventional (——) and the ''high-speed'' (- - -) column.

2.5. Data analysis

The selection of the detection wavelength was a compromise made based on the spectral characteristics of all compounds of interest. In an aqueous acidic medium most opiates have an absorption maximum at approximately 280 nm. Although, in comparison with lower wavelengths, the sensitivity decreases, we decided to monitor at 280 nm because of the increased selectivity.

To establish the limit of quantitation (LOQ) the method described by the European Pharmacopoeia was used [20].

Relative retention times (RRTs), relative peak areas (RPAs) and their corresponding relative standard deviation (RSDs) were calculated for each component to increase our confidence that the method was indeed stable and reliable.

Finally, the performance of both columns was evaluated. For each column, the minimal statistically significant resolution for each pair of consecutive chromatographic peaks was calculated using the Student *t*-test.

3. Results and discussion

The objective of this study was to develop a fast HPLC method utilising a new ''high-speed'' column to analyse an 18-component opiate mixture and to compare its performance (efficiency and sensitivity) to that of a conventional analytical HPLC column.

3.1. Choice of the stationary phase

Although preliminary experiments were performed on the widely used octadecyl silica bonded phase, we soon switched to a phenyl-type column because of the higher retention and enhanced resolution, as predicted by theory [21]. Silica with aromatic ligates at the stationary phase surface can give significant differences in retention and separation as compared to the commonly used alkyl (C_{18} and C_8) bonded phases. Because of their smaller shape, their planar structure, their polarisable character and consequently better retention mechanisms, phenyl bonded phases are known to be more retentive for aromatic substances such as opiates.

3.2. Choice of the internal standard

Besides deuterated internal standards [22], opioid structures such as nalorphine [7,23], oxycodon [5], naltrexone [24], and nalbuphine [4] have been used previously.

Because we wanted the number of detectable opiates to be as large as possible and as most of the components mentioned above showed a poor resolution with particular representatives of the test mixture, we decided to use the narcotic analgesic and antitussive, butorphanol, which, as far as we know, has not been previously used for this purpose. This mixed opioid agonist–antagonist gave an excellent separation from the opiate mixture (see Fig. 2) and, moreover, is not available on the Belgian market. It is, however, widely used in the USA in human [25] and veterinary medicine [26].

3.3. Stability of heroin and noscapine

A problem we encountered very early in our experiments, was the deterioration of heroin and noscapine during analysis. Under the initial eluent conditions, 50 m*M* ammonium acetate (pH_{app} 6.2) was used as a buffer and added to both solvents A and B to maintain the secondary equilibrium [27] on the column during the gradient run. The pH-dependent hydrolysis of heroin (to morphine via 6-monoacetylmorphine) and of noscapine (to noscapinic acid) has previously been reported. Poochikian and Cradock confirmed that the optimal pH value for minimal degradation of diacetylmorphine in aqueous solutions was 4.0–4.5 [28]. They also proved that hydrolysis was reduced with increasing proportions of ethanol [28] and that an acetate buffer showed a catalytic effect on the disappearance rate of the parent compound [29]. Johansson et al. studied the pH-dependent equilibrium between noscapine and noscapinic acid and concluded that up to a pH of 5, no hydrolysis of noscapine took place [30].

Since we aimed to measure the intact components, we consequently decided to work at a pH_{app} of 4.5.

3.4. Effect of different buffers

3.4.1. Effect of different anions

By adsorption of the anionic part of the buffer to

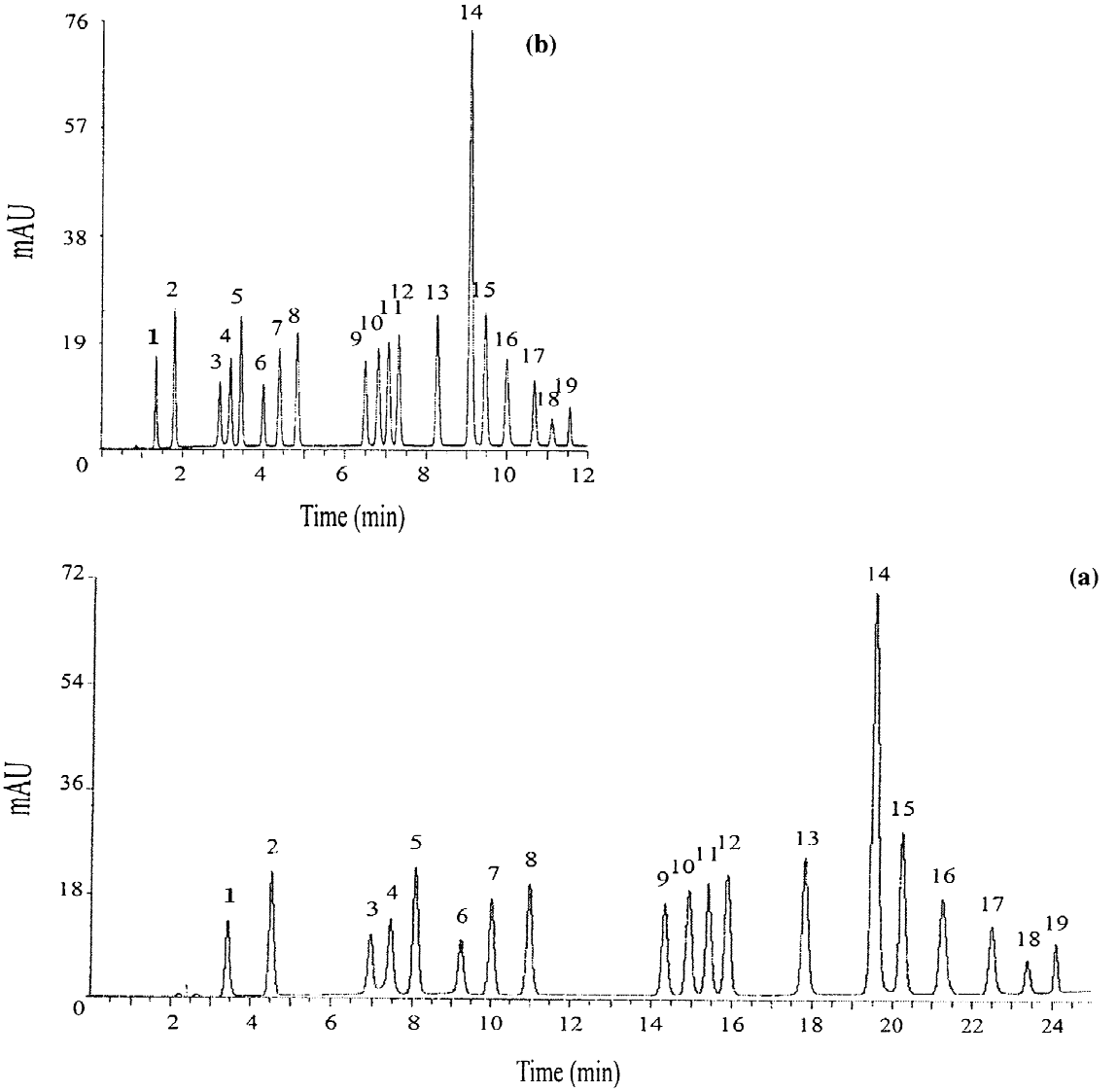

Fig. 2. HPLC chromatogram (λ=280 nm) of the opiate mixture on the ''high-speed'' (b) and the conventional (a) phenyl column. Chromatographic conditions are given in the text. (1) Normorphine, (2) morphine, (3) norcodeine, (4) naloxone, (5) codeine, (6) hydrocodone, (7) 6-monoacetylmorphine, (8) ethylmorphine, (9) acetyldihydrocodeine, (10) thebacone, (11) acetylcodeine, (12) heroin, (13) I.S., (14) papaverine, (15) dextromethorphan, (16) noscapine, (17) buprenorphine, (18) normethadone, and (19) methadone.

the phenyl structure of the stationary phase the charged surface acts as an ion exchanger. Counter ions from the aqueous buffer solution are concentrated near the surface and during analysis the compounds, containing a protonated tertiary amine function, are assumed to exchange with the counter ions. A similar mechanism for a C_{18} bonded-phase has been previously described by Deelder et al. [31].

We investigated the influence of 50 m*M* ammonium acetate and formate buffers on the final separation of the opiate mixture.

We noticed no significant differences in separation

between the two buffer systems. Since we already knew that acetate buffers had a catalytic effect on the hydrolysis of heroin (see Section 3.3) [29] we decided to continue the experiments with formic acid as the buffer anion and to adjust the pH_{app} of solvent A to 4.5.

3.4.2. Effect of different cations

At a pH_{app} 4.5 the components (containing amino groups) are mainly present in the ionized form. They compete with a counter cation, present in the mobile phase, for the available ion-exchange sites in the stationary phase. Retention can be affected by changing the nature and concentration (see Section 3.6) of the counter ion [32]. Also, the cationic part of the ion-exchange buffer serves as a masking agent for free silanol groups resulting in improved peak shape and lower k' values (silanophilic interaction).

We examined the influence of two different counter ions, i.e., the ammonium ion and protonated triethylamine, having both a volatile conjugate base. The pH_{app} of solvent A was maintained at 4.5 by addition of formic acid.

Although there was no noticeable improvement of the peak shape, the use of triethylamine as a counter ion resulted in a drastic improvement of the separation.

3.5. Effect of solvent composition (organic modifier)

In a next step, we investigated the influence of the methanol and acetonitrile content in the mobile phase. We originally started with 50% of both solvents in solvent B. Successively we tested the following acetonitrile–methanol mixtures: 75:25, 25:75, 20:80, 10:90 (all v/v) and methanol 100%. By adjusting the composition of solvent B we automatically changed solvent A.

It was clear from the resulting separations that a reduction of the percentage of acetonitrile in the mobile phase improved the resolution. This was already reported by Nehmer who studied the effect of acetonitrile as an organic modifier on the separation of components containing ternary and quaternary amine functions [33]. Since acetonitrile has a greater elution strength than methanol an increase in the amount of acetonitrile resulted in a faster elution but concomitant decreasing resolution. Also, the baseline increased with an increasing percentage acetonitrile because of its higher UV absorbance at 280 nm. We therefore decided to work with a 100% methanolic solvent B.

3.6. Effect of buffer concentration

Finally, we investigated the influence of different buffer concentrations in the mobile phase. The following triethylammonium formate concentrations were tested: 50, 25, 20, 15, 10 and 5 m*M*. The mobile phase was maintained at pH_{app} 4.5 by addition of formic acid.

By using a 25 m*M* triethylammonium formate buffer and adjusting the pH_{app} to 4.5 (by adding 200 μl formic acid per litre of solvent) an optimal separation with minimal silanophilic effect was achieved [33]. Higher concentrations, and thus increasing ionic strength of the mobile phase, had no further positive effect, so we concluded that saturation was obtained with 25 m*M* triethylammonium formate.

3.7. Final conventional method

After making the above adjustments an optimised method on the conventional phenyl-type column (150×4.6 mm I.D., particle size 5 μm) was obtained. For detailed gradient and measurement conditions, see Section 2.4. A representative chromatogram of the separation of the opiate mixture on the conventional phenyl column can be seen in Fig. 2a.

3.8. Fast method

After developing the method for the separation of the 18 opiates on the conventional column, we adjusted the flow and gradient steepness (see Fig. 1) to achieve a similar separation, under the exact solvent and detector conditions, on the ''high-speed'' column albeit with an approximately 55% analysis time reduction. Also, the wash and equilibration periods were substantially shortened resulting in a shorter total analysis time and consequently in a reduced solvent consumption. Fig. 2b shows a typical separation of the opiate mixture on the ''high-speed'' column.

3.9. Data analysis

3.9.1. Validation

Peak height, area and their ratios to the internal standard (butorphanol) were calculated for all compounds on both columns, as well as the corresponding RSDs for six successive injections (Table 1).

3.9.2. Limit of detection (LOD)

The LOD, calculated according to the method described by the European Pharmacopoeia [20], was defined as the concentration of each component for which a signal-to-noise ratio of 3 is obtained (injected volume: 50 μl). The results, obtained for each compound of the opiate mixture on the conventional and the ''high-speed'' column, are equal and also shown in Table 1.

3.9.3. Evaluation of column performance

The resolution of successive components in the opiates mixture was determined for each of the two columns. The mean values and their corresponding RSDs are presented in Table 2.

In the case of the conventional column, the smallest mean resolution was obtained between acetylcodeine and heroin, i.e., 1.78. Currently, a resolution of at least 1.5 is considered as a good separation between two consecutive peaks. With the help of the Student *t*-test we determined that the resolution obtained for acetylcodeine and heroin is higher than 1.5, with a statistical significance of 97.5%. In the case of the ''high-speed'' column, the same situation was encountered for thebacone and acetylcodeine, and the *t*-test yielded the same result. For the rest of the compounds, the resolution, obtained on both columns, is obviously meeting the criterium used to asses a good chromatographic separation.

As such, we can conclude that for both columns all peaks were separated with a R_s (statistically significant) higher than 1.78 and therefore no significant difference in separation performance exists between the conventional and ''high-speed'' column.

Although for both columns the RSDs of the mean resolution were well below 5%, we noticed that the RSD values obtained on the ''high-speed'' column were lower for most of the separated compounds. We

Table 1
Results obtained on the ''high-speed'' column (t_R=retention time, RRT=relative retention time, PA=peak area, RPA=relative peak area and LOD=limit of detection)[a]

Component	t_R (min)	RSD (%)	RRT	RSD (%)	PA (arbitrary units)	RSD (%)	RPA	RSD (%)	LOD (ng/ml)	Amount on-column (ng/50 μl)
Normorphine	1.3	0.24	0.161	0.25	55.0	3.48	0.424	3.08	92.2	4.60
Morphine	1.8	0.23	0.217	0.24	96.3	2.24	0.732	1.39	199.7	9.98
Norcodeine	2.9	0.29	0.352	0.21	54.6	4.57	0.405	1.51	84.6	4.20
Naloxone	3.2	0.26	0.384	0.14	76.2	1.71	0.575	1.26	222.4	11.1
Codeine	3.4	0.30	0.416	0.20	103.6	1.32	0.785	0.92	205.9	10.3
Hydrocodone	4.0	0.28	0.483	0.17	52.5	3.03	0.393	1.91	200.0	10.0
6-MAM	4.4	0.28	0.532	0.18	83.4	1.89	0.629	0.73	203.3	10.2
Ethylmorphine	4.8	0.22	0.585	0.11	101.2	1.95	0.764	0.47	205.3	10.3
Acetyldihydrocodeine	6.5	0.17	0.786	0.07	77.8	2.93	0.585	0.45	206.8	10.3
Thebacone	6.8	0.17	0.824	0.07	93.8	2.23	0.707	0.24	236.6	11.8
Acetylcodeine	7.1	0.16	0.854	0	98.0	1.79	0.741	0.47	214.2	10.7
Heroin	7.3	0.15	0.884	0.06	103.6	1.95	0.783	0.22	100.4	5.02
Butorphanol (I.S.)	8.3	0.14			132.0	1.35			98.2	4.91
Papaverine	9.1	0.09	1.100	0.07	408.1	1.11	3.112	0.31	52.4	2.62
Dextromethorphan	9.5	0.12	1.146	0.07	135.3	2.15	1.021	0.17	99.3	4.97
Noscapine	10.0	0.06	1.210	0.11	101.5	3.00	0.759	0.46	99.3	4.97
Buprenorphine	10.7	0.05	1.292	0.14	67.1	4.37	0.498	1.84	390.7	19.5
Normethadone	11.1	0.07	1.345	0.11	26.1	4.36	0.194	3.10	421.2	21.0
Methadone	11.5	0.05	1.397	0.12	28.5	2.50	0.216	2.63	449.1	22.4

[a] Data are given as mean (n=6) with corresponding RSDs.

Table 2
Comparison of resolution for the opiate mixture on the conventional and ''high-speed'' phenyl column[a]

Components	Conventional column (150×4.6 mm, 5 μm)		''High-speed'' column (53×7.0 mm, 3 μm)	
	Resolution (R_s)	RSD (%)	Resolution (R_s)	RSD (%)
Normorphine+morphine	5.21	0.71	4.84	1.74
Morphine+norcodeine	10.08	3.77	9.96	1.07
Norcodeine+naloxone	1.84	3.18	2.12	1.73
Naloxone+codeine	2.40	1.37	2.19	0.82
Codeine+ hydrocodone	4.61	3.67	4.61	0.93
Hydrocodone+6-MAM	3.05	1.55	3.26	0.72
6-MAM+ethylmorphine	3.71	0.54	3.40	0.84
Ethylmorphine+acetyldihydrocodeine	13.21	0.98	12.46	0.76
Acetyldihydrodcodeine+thebacone	2.26	3.88	2.23	0.59
Thebacone+acetylcodeine	1.81	0.76	1.78	0.29
Acetylcodeine+heroin	1.78	0.62	1.81	0.60
Heroin+butorphanol	7.04	1.41	6.64	0.60
Butorphanol+papaverine	5.92	1.21	5.60	0.28
Papaverine+dextromethorphan	2.49	1.40	2.56	1.95
Dextromethorphan+noscapine	3.44	1.48	3.32	1.23
Noscapine+buprenorphine	4.25	1.21	4.31	1.90
Buprenorphine+normethadone	3.60	1.64	2.95	1.88
Normethadone+methadone	3.61	1.08	3.55	1.47

[a] Data are given as mean (n=6) with corresponding RSDs.

can therefore conclude that the use of the new ''high-speed'' column resulted in a more reliable separation, representing a more stable and reproducible method.

In addition, the method was already successfully applied on extracts of serum samples and on a number of seized street samples of heroin, in order to characterise the heroin by a fingerprint, i.e., impurity profile, and to develop a predictive model for batch membership.

4. Conclusion

A new ''high-speed''column (53×7.0 mm I.D., 3 μm particle size) with phenyl silica bonded phase has been found effective for the rapid analysis of opiate drugs.

The use of a phenyl-type column, instead of the commonly used alkyl (C_{18} and C_8) bonded phases, proved to give a higher retention and better separation for aromatic structures such as opiates.

Compared to a conventional 150×4.6 mm phenyl-type column (5 μm particle size), separations were performed in approximately half the analysis time, with no significant loss in resolution nor in sensitivity. The faster separations do not directly result in a lower solvent consumption during the elution but did allow a shorter wash and equilibration period, therefore resulting in a lower total solvent consumption for the whole of one analysis. The new column configuration was also utilised with traditional HPLC equipment without special modifications, so no extra investments are necessary.

We therefore conclude that ''high-speed'' columns show tremendous potential to replace larger conventional columns for various applications in the analytical laboratory, as demands for decreased solvent consumption and faster analysis, thus higher sample throughput, continue to increase.

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