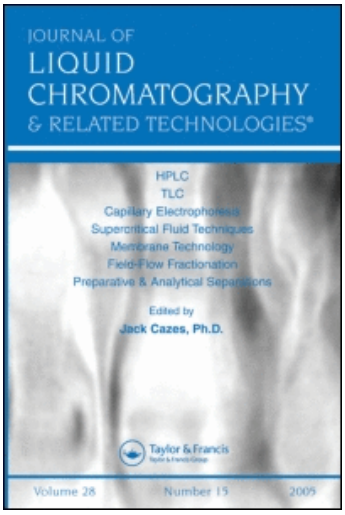

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MORPHINE DETERMINATION BY HPLC WITH IMPROVED CHEMILUMINESCENCE DETECTION USING A CONVENTIONAL SILICA BASED COLUMN

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ABSTRACT

A new high performance liquid chromatography-chemiluminescence analysis system for morphine is described. This system has an on-column limit of detection of 20 pg for morphine, allowing morphine determination down to 1 ng/mL, with a 20 μL injection size, without any concentration during sample preparation. Simultaneous determination of monoacetylmorphine to 15 ng/mL (300 pg on-column) is also achieved. The internal standard for both compounds is nalorphine and total analysis time is under 5 minutes.

INTRODUCTION

Morphine determination is of interest both in forensic cases as an indicator of heroin usage[1] and in routine pharmacokinetic studies.[2] The commonly used detection methods for morphine following high performance

liquid chromatography (HPLC) are ultraviolet (UV)[3] and electrochemical (EC).[4] UV detection lacks the sensitivity required for pharmacokinetic and trace forensic analysis and EC detection frequently requires extensive sample preparation for trace analysis.

Chemiluminescence (CL) detection, utilising the chemiluminescent reaction between morphine and acidic potassium permanganate was proposed as an ideal solution to these problems.[5] CL detection offers the sensitivity required, with a limit of detection (LOD) of 0.7 pg with flow injection analysis (FIA) and should offer enhanced selectivity as the CL reaction is non-universal. However when coupled with HPLC[6] the resulting on-column detection limits, 5 ng, fell short of those required for trace analysis. Attempts to improve upon this using electrogenerated CL met with mixed results.[7]

This disparity between the FIA LOD and the HPLC LOD was due to the problems of coupling the highly acidic conditions required for the CL reaction with the conditions required for HPLC analysis. In addition to a high LOD the previous HPLC-CL method used a specially synthesised compound as internal standard (IS) and was unable to determine monoacetylmorphine, a necessary requirement in forensic analyses.

By the use of an acidic permanganate solution the requirements for a highly acidic mobile phase are reduced and new HPLC conditions can be utilised. This paper presents a new HPLC method for morphine and monoacetylmorphine for coupling with CL. The on-column LOD has been reduced by almost three orders of magnitude over previous work, a commercially available compound is used as the IS and the simultaneous determination of monoacetylmorphine is now feasible, with a total analysis time of under 5 minutes.

EXPERIMENTAL

Equipment

The chromatographic system consisted of dual model LC-10AS solvent delivery modules (Shimadzu) and a Rheodyne 7725I injection valve with a 5 or 20 μL sample loop size. The UV detector was a SPD-10AV UV-Vis (Shimadzu), the CL detector a Shodex CL-2 (JM Science). The oxidant was mixed post-column at a stainless steel T-piece pumped through Teflon tubing (0.3 mm id x 1.58 mm od. Supelco) with a Minipuls 3 peristaltic pump (Gilson) using black/black silicone tubing (Elkay). Chromatograms were

recorded using EZChrom Chromatography software (version 6.5, Scientific Software) running on a 486DX-2 computer (Gateway 2000). HPLC columns used were Supelcosil ABZ+ (Supelco), Hypersil ODS (Alltech) and Inertsil ODS-2 and ODS-3 (Metachem).

Chemicals

Methanol (Aldrich) and tetrahydrofuran (Mallinckdrodt) were HPLC grade, water was obtained from a purification system (Millipore). Potassium permanganate (EM Science), polyphosphoric acid (Aldrich), sodium dihydrogen phosphate, disodium dihydrogen pyrophosphate, sodium hexametaphosphate (Fluka), and orthophosphoric acid (Fisher) were all reagent grade. All glassware for opiate solutions was treated with Surfsil siliconizing fluid (Pierce) prior to use. Buffer pH values were adjusted with orthophosphoric acid.

Aqueous morphine and monoacetylmorphine solutions were prepared from 1 mg/mL and 100 μg/mL methanol standards (Radian) respectively or from morphine sulphate (Sigma). Aqueous nalorphine solutions were prepared from a 1 mg/mL methanol standard (Alltech).

RESULTS

HPLC Column

Starting chromatographic conditions were based on the results of previous studies.[8] An original mobile phase of 90:10 sodium dihydrogen phosphate (0.01 M, pH = 3.8):methanol was used to compare 3 chromatographic columns for morphine peak shape and elution times. Results showed that the Supelcosil ABZ+ and Inertsil ODS-2 columns gave symmetrical peaks under these conditions and these columns were used in further studies.

Column Diameter Reduction

Reducing column diameter should result in narrower taller peaks and hence a decreased LOD. A comparison was made between three Supelcosil ABZ+ columns with ids of 4.6 mm, 2.1 mm and 1.0 mm using the mobile phase as described previously pumped at 1.0, 0.8 and 0.2 mL/min

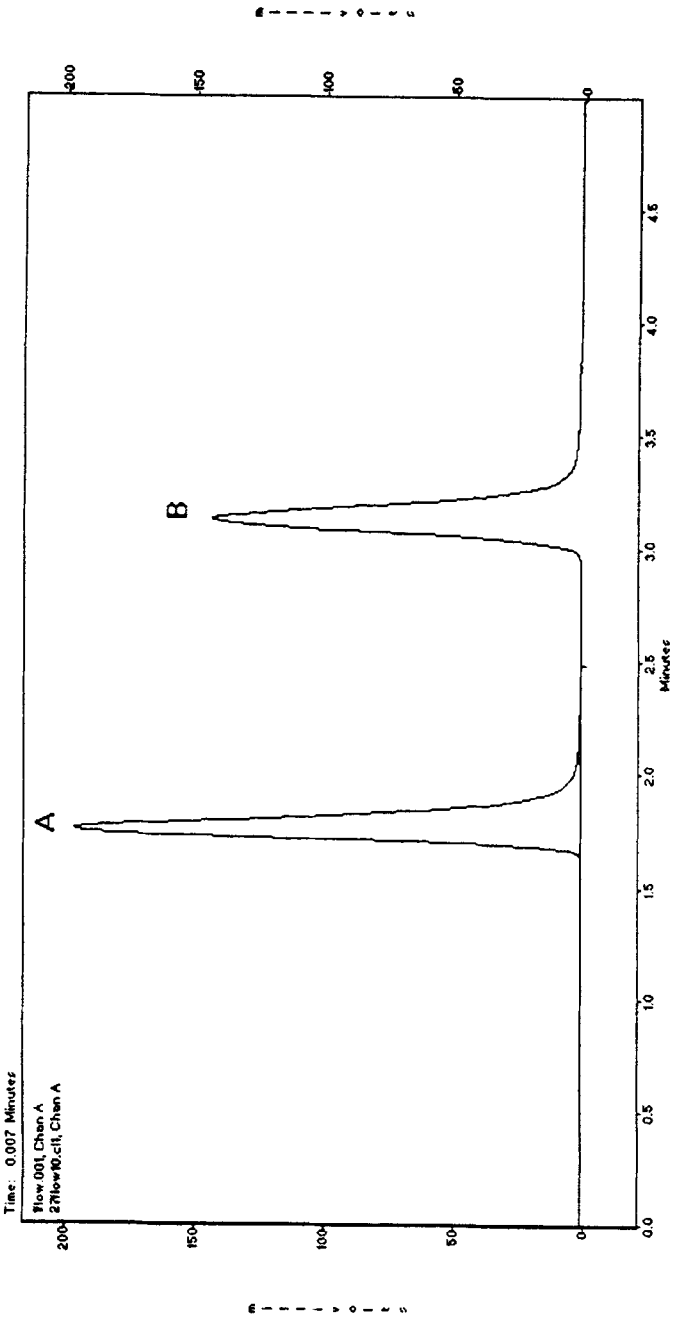

Figure 1. Comparison of two Inertsil ODS-2 columns with morphine. Both 15 cm length, column A = 2.1 mm id. and column B = 4.6 mm id. Mobile phase 90:10 buffer:MeOH.

Table 1

Effect of Three Phosphate Compounds as the Buffer on the CL Signal*

Buffer	% Relative Peak Area
disodium dihydrogen pyrophospate	75.4
sodium hexametaphosphate	100.0
sodum dihydrogen phosphate	64.9

* Results obtained using a 90:10 buffer:methanol mobile phase at 0.8 mL/min on a 2.0 mm Supelcosil column with standard oxidant conditions. Buffer (0.01 M) was pH adjusted to 3.8, 20 μL injections of 5×10^{-6} M morphine.

respectively and CL detection. It was found that reducing the column diameter from 4.6 mm to 2.1 mm increased peak height by 29.7 percent, but a further reduction to 1.0 mm caused a peak height decrease of 78.5 percent from the signal seen with a 4.6 mm id column. Figure 1 shows the increase seen in reducing column diameter from 4.6 mm to 2.1 mm. The decrease upon reduction to 1 mm is due to the fact that with this smaller bore column the dead volume between the T-piece and the detector cell becomes important. The lower column flow rate means that most CL emission occurs before the detector cell. A flow design as proposed by Danielson *et al.*[9] would eliminate this problem, however such a design is not commercially available and so is not feasible for most routine analysis laboratories. Columns with an id of 2.0 mm were used in all further studies.

Mobile Phase Buffer

Phosphates have been found to enhance the CL signal seen from the morphine-permanganate reaction. Two other phosphates were investigated to see if any provided enhanced CL signal over the currently used sodium dihydrogen phosphate buffer. Table 1 shows the results of a comparison of the 3 phosphate compounds.

Sodium hexametaphosphate resulted in the largest CL signal. This correlates with recent work by Barnett *et al.*[10] who used hexametaphosphate as a replacement for polyphosphoric acid.

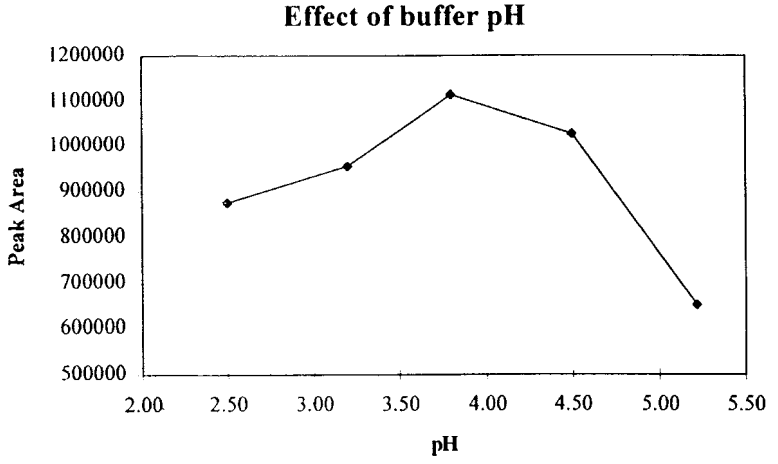

Figure 2. The effect of buffer pH upon CL signal seen.

Presumably the hexametaphosphate prolongs the emission so that more CL is generated in the detector cell. In this case the sodium hexametaphosphate is not acting as a pH buffer, but as a CL reaction enhancer.

Buffer pH

As pH is known to have a significant impact upon the CL signal, the pH of the buffer was varied over the range 5.2 to 2.5. Results are shown in Figure 2. The pH was not evaluated below 2.5 as a significant decrease in CL signal was already being seen. This is due to the fact that the pH affects the rate of the reaction that generates the CL. At pH values below 3.8 the CL reaction occurs prior to the sample entering the flow cell, at higher pH values other non-CL pathways begin to dominate and the emission is reduced. The use of this higher pH suits the analysis system well, as although the HPLC columns used are able to tolerate lower pH values than previous columns they are still limited to a pH of about 2 for extended use

Choice of IS

The requirements for the IS for this analysis system go beyond the standard need for chromatographic separation and commercial availability. The IS must also be a chemiluminescent compound otherwise it will not give

Table 2

Comparison of Capacity Factors on 3 Different Columns and 2 Mobile Phases*

	Mobile Phase					
	------ 90:9:1 ------			------ 94:5:1 ------		
Compound	**ABZ+**	**ODS-2**	**ODS-3**	**ABZ+**	**ODS-2**	**ODS-3**
morphine	0.92	0.34	0.60	1.74	1.26	1.05
nalorphine	1.82	1.30	2.21	4.55	5.08	3.66
MAM	4.26	2.95	4.48	10.89	13.01	8.42

* Mobile phase given as sodium hexametaphosphate (0.01 M, pH = 3.8):MeOH:THF @ 0.8 mL/min, except for ODS-2 run at 0.4 mL/min.

rise to a signal in the detector. Three commercially available compounds, nalorphine, naloxone, or hydromorphone are all chemiluminescent to some degree and have been used as the IS in other morphine determination methods.[4,11] Of these nalorphine is the most strongly chemiluminescent and has been used as an IS in other morphine analysis methods.[12] Preliminary studies showed that nalorphine was chromatographically resolved from both morphine and monoacetylmorphine and gave rise to a large CL signal. So nalorphine was used as the IS in all future work.

Column Type

As organic solvents such as methanol have been found to inhibit CL emission in this reaction, a mobile phase that contains a low an organic content as possible would be advantageous. To achieve a further reduction in organic content a Supelcosil ABZ+, Inertsil ODS-2 and a newly available Inertsil ODS-3 column were compared using two mobile phases. As tetrahydrofuran has been found to inhibit the CL reaction to a lesser extent than methanol experiments were conducted replacing the methanol with tetrahydrofuran. A comparison of the capacity factors for morphine, monoacetylmorphine and nalorphine with two mobile phases is shown in Table 2. One mobile phase replaces 1% of the methanol with an equal volume of tetrahydrofuran and the other a mobile phase replaces 5% of the methanol with 1% tetrahydrofuran and 4% buffer.

Whilst all columns baseline resolved all three compounds with both mobile phases, the ODS-3 column using the lower methanol content mobile phase offered the best combination of resolution and speed of elution. The elution time for monoacetylmorphine on the other two columns was considerably longer than that seen with the ODS-3 column, resulting in a significant amount of dead time in the chromatogram. It should be noted that the back pressure with the ODS-2 column was significantly higher than with the other columns and the maximum flow rate before going over the pump pressure limits (300 kgf/cm^2) was 0.4 mL/min.

Sample Injection Size

A comparison was made between the 5 and 20 µL sample loops. The morphine concentration was adjusted so that the on-column amount remained the same for both loop sizes. The 5 µL loop gave a 91.5 percent larger peak size. However the 20 µL loop was chosen as this allows four times as much sample to be injected onto the column and sample size is not expected to be a problem.

Oxidant Flow Rate and Concentration

The final parameters for optimisation were the oxidant flow rate and concentration. These need to be adjusted so that the CL detector receives the maximum amount light from the CL reaction. Results of these studies are shown in Figure 3. In each case the permanganate was prepared in a polyphosphoric acid solution ($1x10^{-4}$ M). The mobile phase was 94:5:1 buffer:methanol:tetrahydrofuran with a buffer concentration of 0.01 M, pH adjusted to 3.8 and the morphine concentration was 500 ng/mL.

Although a maximum signal was seen with a permanganate concentration of $1x10^{-4}$ M at a pump speed of 22 rpm, in order to conserve oxidant so that large volumes of solution need not be prepared and disposed of, a concentration of $6x10^{-4}$ M at a pump speed of 6 rpm was used to construct the calibration graph. This pump speed corresponds to a flow rate of 0.3 mL/min.

Calibration Graph

The final analysis conditions for morphine were an Inertsil ODS-3 column (15 cm x 2.1 mm id), mobile phase of 94:5:1 sodium hexametaphosphate (0.01 M, pH = 3.80):methanol:THF at a flow rate of 0.8

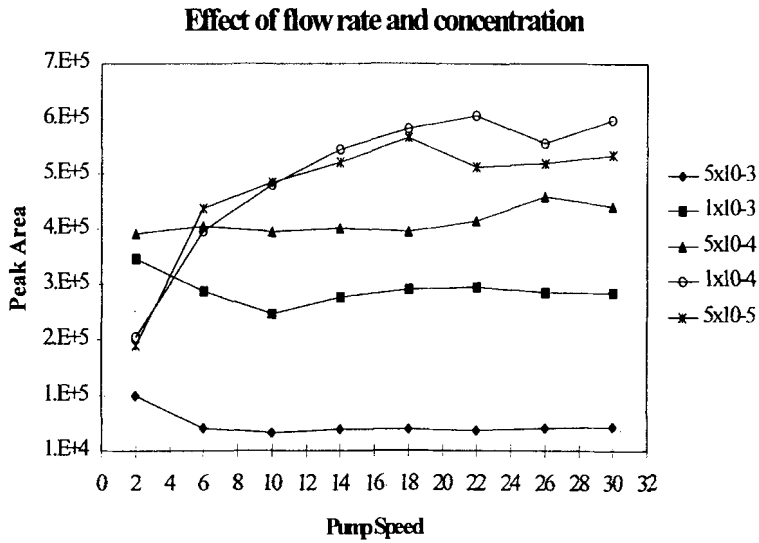

Figure 3. The effect of potassium permanganate flow rate and concentration upon CL signal seen. HPLC conditions given in the text.

mL/min. The oxidant to generate CL was potassium permanganate ($6x10^{-4}$ M) in polyphosphoric acid ($5x10^{-4}$ M) at 0.3 mL/min. Calibration graphs were obtained for both morphine and monoacetylmorphine over the concentration ranges 10 to 1000 ng/mL and 25 to 100 ng/mL respectively. Plotting peak height for morphine or monoacetylmorphine against nalorphine peak height gave a straight line relationship.

The morphine calibration graph is shown in Figure 4. Equation for the line is; $y = 0.00434x - 0.0441$ and $R^2 = 0.9996$. The calculated LOD (2 x noise) was 1 ng/mL (20 pg on-column) for morphine and 15 ng/mL for monoacetylmorphine. The linearity for monoacetylmorphine is expected to continue beyond that seen here. The concentration of starting standard prevented a larger calibration range being examined. More concentrated standards are now available and confirmatory work in this area will be undertaken shortly.

Figures 5 and 6 show chromatograms from the calibration graph runs with morphine at the 750 and 250 ng/mL levels respectively. Baseline resolution is clearly obtained with a total analysis time of under 5 minutes.

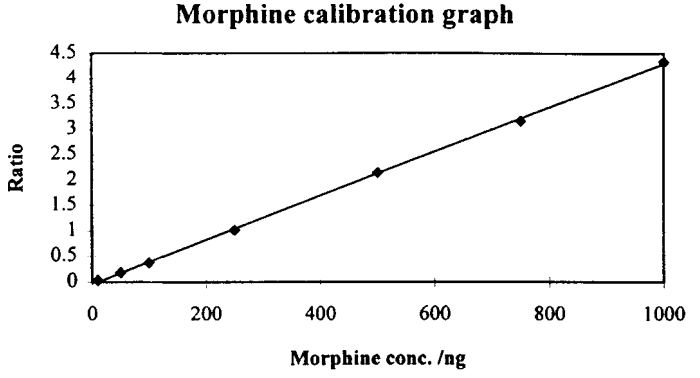

Figure 4. Calibration graph for morphine, range 10 to 1000 ng/mL for morphine, R^2 = 0.9996, equation is: y = 0.00434x - 0.0441.

Intra- and Inter-day Variability

To test the stability of the analysis system the intra- and inter-day variability was monitored. The intra-day ratio variability, given as relative standard deviation (rsd) of the ratio of morphine peak area to IS peak area was found to be 2.8% and 5.3% (n=3) and the interday ratio rsd over a week (n=5) was found to be 2.3 and 1.7% at the 500 and 50 ng/mL levels respectively.

DISCUSSION

The results seen upon reducing the column diameter demonstrate the importance of instrumental design when using CL detection. Utilising a smaller flow path from the T-piece to the detection cell should improve detection limits still further as it would then be possible to take advantage of the narrow peaks generated by 1.0 mm id columns. The importance of considering reaction kinetics and flow cell design was recently discussed by Nieman et al.[13]

The high pressure seen with the ODS-2 column and subsequent reduction upon going to the ODS-3 column is presumably due to the narrower particle size distribution of the ODS-3 column. A narrower particle size distribution means fewer numbers of small diameter particles which cause an increase in the back pressure.

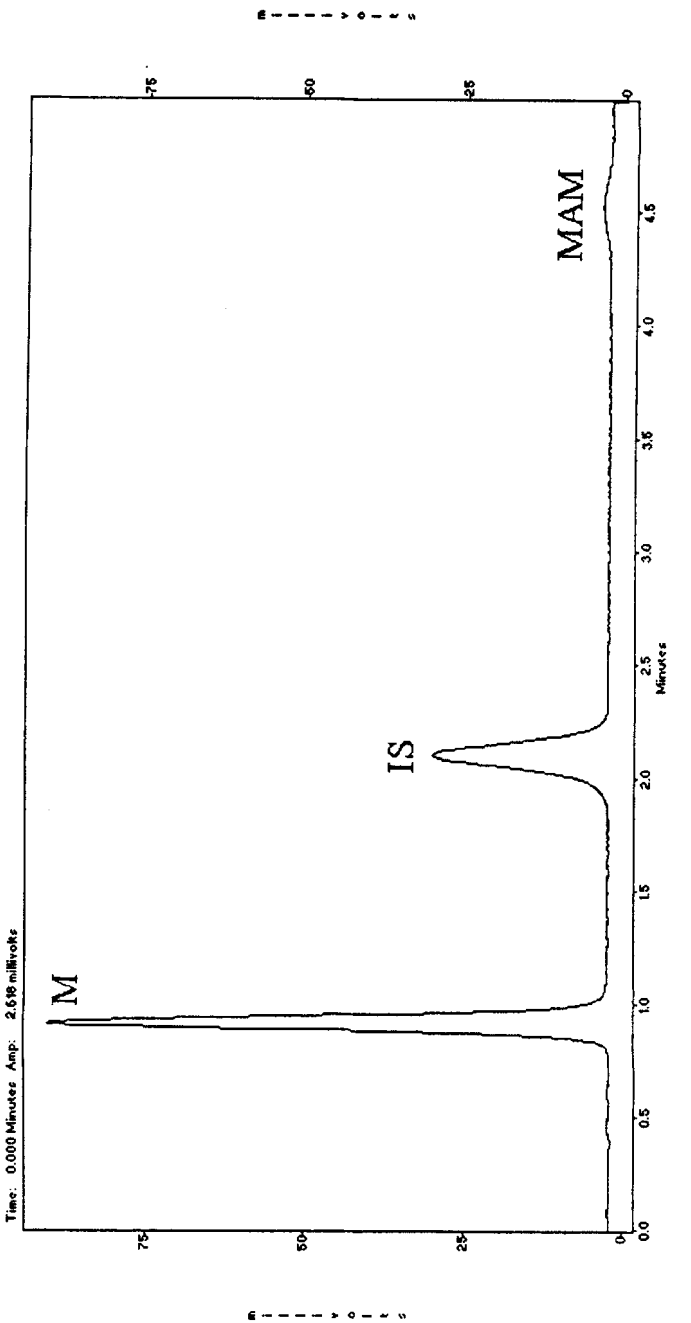

Figure 5. Chromatogram of calibration graph run, morphine (M, 750 ng/mL), nalorphine (IS, 500 ng/mL) and monoacetylmorphine (MAM, 75 ng/mL).

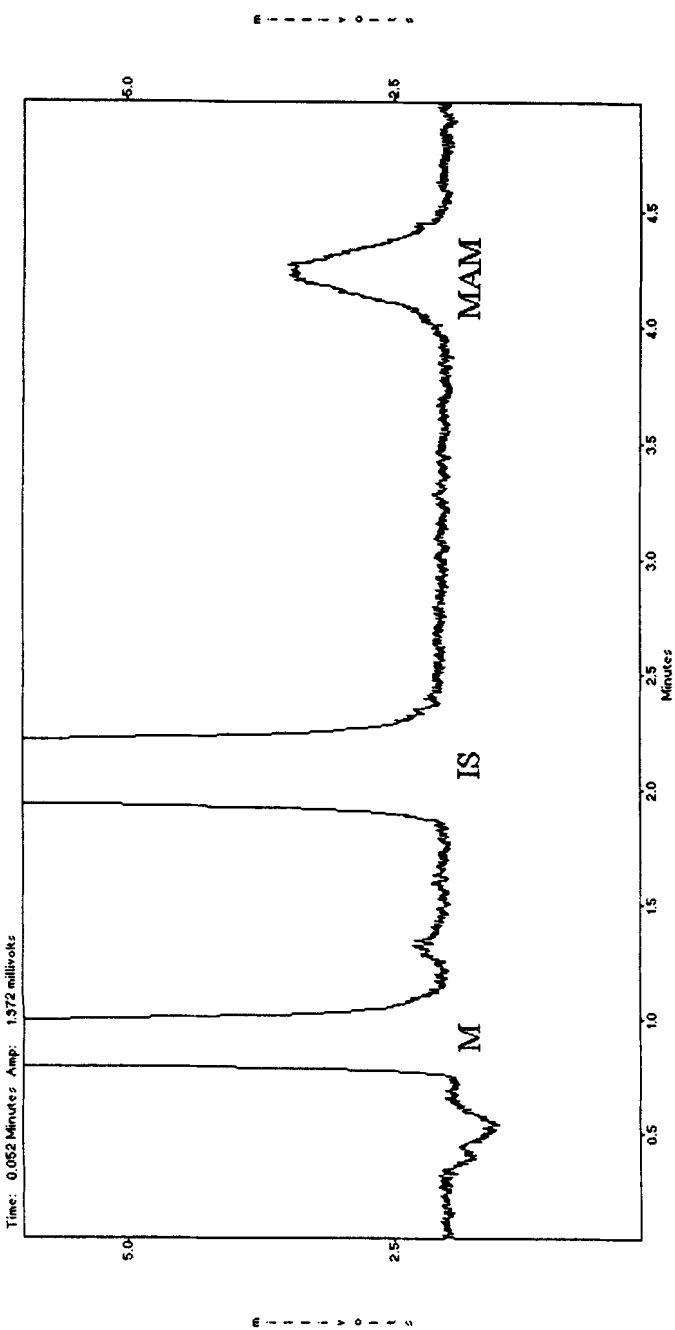

Figure 6. Chromatogram of calibration graph run, morphine (M, 250 ng/mL), nalorphine (IS, 500 ng/mL) and monoacetylmorphine (MAM, 25 ng/mL). The scale is adjusted to clearly show the MAM peak.

The decrease in capacity factor seen upon changing to the ODS-3 column also indicates the need to consider the surface coverage of the bonded phase and not just the column loading. The surface coverage can be calculated from the following equation:[14]

$$N = \frac{10^6 P_c}{\left[1200 n_c - P_c(m-1)\right] s} \quad (1)$$

Where N is the surface coverage (in micromol/M^2), P_c the percent carbon loading, n_c the number of carbon atoms in the bonded phase,[18] m the molecular weight of the silica bonded phase (331) and s the surface area of the silica (M^2/g). Using P_c and s values of 18,5, 320 and 15, 450 for ODS-2 and ODS-3 respectively $N_{ODS\text{-}2} = 37.3$ and $N_{ODS\text{-}3} = 20.0$.

These values show that although the Pc values are changed only slightly, a 18.9% reduction, the surface coverage has a 46.4% reduction. This contributes to the large capacity factor reduction (35.3% for MAM) when THF is present in the mobile phase.

The retention mechanism is more complex than simple interactions with the bonded phase because when no THF is present in the mobile phase an increase in capacity factor is seen upon changing from ODS-2 to ODS-3 columns. This may be because interactions with the silica packing play a role in the retention and a decrease in surface coverage by the bonded phase would lead to an increase in the silica surface available for these interactions.

It appears that THF helps block these interactions in some manner, reducing retention time and hence capacity factor.

The low inter-day variability indicates the stability of the acidic permanganate solutions. Although some decrease in absolute values is seen over the course of the week the use of the IS accounts for these changes. This stability coupled with the use of a low flow rate allows solutions to be prepared on a weekly instead of daily basis.

This work has successfully coupled the use of an acidic permanganate solution and newly developed HPLC columns to obtain greater than two order of magnitude reduction in the on-column LOD for morphine whilst allowing for the determination of MAM in the same chromatographic run. The determination of MAM is critical for the method to be acceptable to

laboratories undertaking forensic analyses. The improved HPLC conditions also allow for the use of a more conventional IS when compared to the IS used in previous reports.

CONCLUSIONS

The final analysis conditions allow for the simultaneous determination of morphine and MAM at 1 and 15 ng/mL levels with 20 µL injections using CL detection. This is an on-column LOD reduction of over two orders of magnitude from previously published CL detection methods for morphine after HPLC separation. These improvements made to the HPLC-CL detection system make it a viable analysis method for morphine determination in forensic, pharmacokinetic and quality control situations. Studies on developing a simple extraction method for morphine and MAM from biological fluids to complement this determination method are in progress and will be reported on shortly.

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