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# Comparison of morphine and hydromorphone analysis on reversed phase columns with different diameters

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**Abstract**

A comparison of a reversed phase high-performance liquid chromatographic (HPLC) method performed on columns with different internal diameters is reported for the quantitative routine determination of morphine·HCl and hydromorphone·HCl in solutions used for intramuscular injection. The method is based on the ion-pairing properties of sodium dodecyl sulphate (SDS) with alkaloids on a reversed phase LiChrospher RP-18 packing material and UV-detection at 230 nm. The mobile phase consisted of an acetonitrile: water mixture 35:65 (v/v) containing 0.5% (w/v) SDS and 0.4% (v/v) acetic acid. Precision, linearity and limit of detection were compared on the 2, 3 and 4 mm i.d. × 125 mm columns. A robustness test for the determination of hydromorphone·HCl was also evaluated.


*Keywords:* Morphine hydrochloride; Hydromorphone hydrochloride; Reversed phase column; Narrow-bore columns; Pharmaceutical analysis; Robustness testing

## 1. Introduction

When developing new high-performance liquid chromatographic (HPLC) methods, suitable for quantitative routine applications, analysts often tend to use conventional columns with standard internal diameter, e.g. 4.6 mm, generally providing good separations. Especially in validated systems, performed since many years on standard HPLC columns, the high solvent consumption leads to important environmental contaminations. From this point of view, analytical laboratories are driven towards miniaturisation of existing liquid chromatographic systems [1–7]. Narrow-bore and micro-column technology nowadays unquestionably provide both economic and ecologic advantages.

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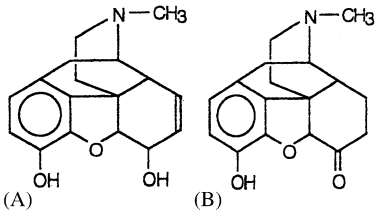


Fig. 1. Structures of morphine (A) and hydromorphone (B).

Morphine (Fig. 1), the most important opium alkaloid, is used to control moderate to severe pain. Hydromorphone (Fig. 1) is a semi-synthetic derivative and valence isomer of morphine with similar action and uses but greater analgesic potency. It is prepared by hydrogenation of the 7,8-double bond of morphine and subsequent oxidation of the hydroxyl group.

In order to encourage analytical scientists to switch over to lower internal diameter-columns [8,9], the present study provides some guidelines on how to easily change from a conventional to a narrow-bore column, illustrated by the routine analysis of morphine hydrochloride (MH) and hydromorphone hydrochloride (HMH) in vials and ampoules, respectively. The HPLC method was developed based on the publication by Menon et al. from 1989 [10] and intentionally slightly modified by our group in terms of the given problem [11]. Furthermore, it compares qualitative and quantitave information obtained from the experiments and from statistical evaluation of calibration datasets of columns with different diameters (2 and 4 mm). In addition, this method was evaluated on a 3 mm-column packed with the same, but endcapped material yielding improved separation quality when compared with both other columns.

## 2. Experimental

### 2.1. Reagents and chemicals

HPLC-grade acetonitrile (ACN) was obtained from Panreac-Química (Spain), as was sodium dodecyl sulphate (SDS), used as ion-pairing agent. Acetic acid was provided by Acros (Belgium). The studied drugs MH and HMH were obtained from Belgopia (Belgium). All other chemicals used were of analytical grade. Deionised water was used throughout.

### 2.2. Instrumentation

All chromatographic separations were performed employing a Varian 9010 SDS pump (Varian Associates Inc., USA) using a Rheodyne 7125 injector provided with a 20 μl sample loop. Detection was performed with a Hewlett Packard series 1050 diode array detector (flow-cell 5 μl) (Hewlett Packard, Germany). All connections were made with PEEK tubing (0.005 in. i.d. × 1/16 in. o.d.). The column eluate was measured at 230 nm, a suitable wavelength for the studied opiates (response time was set at 1 s for both columns). Integration was performed with the Hewlett Packard Software Package (CHEMSTATION).

### 2.3. Chromatographic conditions

The opiates were separated on a 125 mm × 3 mm, 5 μm particle, LiChrospher EcoCART RP-18 column (glass cartridge) provided with a 4–4 mm guard column with the same packing (Merck Eurolab, Germany). The mobile phase consisted of an ACN: water mixture 35:65 (v/v) containing 0.5% (w/v) SDS and 0.4% (v/v) acetic acid resulting in a pH of 3.5. The mobile phase was pumped at a flow-rate of 0.7 ml $min^{-1}$. Chromatography was performed at 40 °C. The obtained separation parameters were compared with a 2 mm (flow-rate of 0.32 ml $min^{-1}$) and a 4 mm (flow-rate of 0.7 ml $min^{-1}$) i.d. column of the same length (both stainless steel cartridges), filled with LiChrospher RP-18 packing material. The same 4–4 guard column (stainless steel) was used for the three column types.

### 2.4. Preparation of solutions

Stock standard solutions were prepared in 0.9% (w/v) sodium chloride solution. The latter was also used for dilutions of the stock standard solutions to the working standard solutions and for the

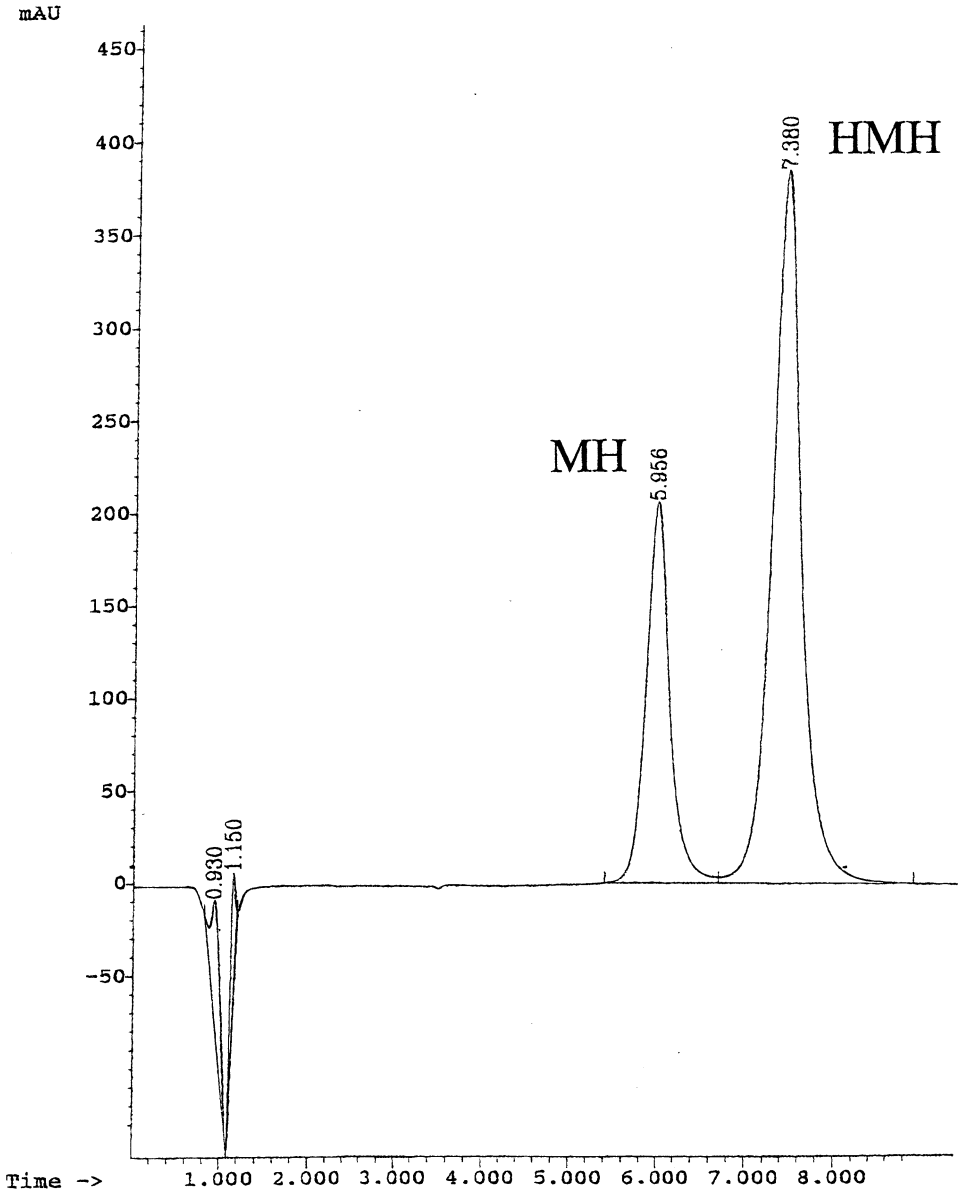


Fig. 2. Chromatogram obtained after injection of the 150% standard solution of HMH on the 3 mm EcoCART column; IS = MH; flow-rate 0.70 ml $min^{-1}$.

dilution of unknown samples (vials or ampoules). Dilutions were made to provide solutions containing between 60 and 180 μg $ml^{-1}$ MH or HMH, with the internal standard (IS, respectively, HMH or HM), at a concentration of about 120 μg $ml^{-1}$.

### 2.5. Calibration

Calibration graphs constructed by plotting MH/IS peak-area ratios against MH concentration for MH standard solutions or by plotting HMS/IS

Table 1
Separation quality parameters for HMH

| i.d. column (mm) | Capacity factor k′ | Retention time (min) | Resolution HMH/MH Rs | Peak asymmetry T |
|---|---|---|---|---|
| 2 | 6.9 | 7.26 | 2.26 | 1.75 |
| 3 | 9.6 | 6.64 | 4.13 | 1.05 |
| 4 | 4.9 | 7.34 | 2.57 | 1.40 |

peak-area ratios against HMH concentration for HMH standard solutions, were analysed by least-squares regression. Statgraphics® was used for the statistical data analysis.

## 3. Results and discussion

### *3.1. Switching to a narrow-bore column: qualitative aspects*

Due to the availability nowadays of high quality HPLC columns with a 2 and 3 mm i.d., the method transfer from normal-bore to narrow-bore and method development directly on narrow-bore columns can be performed. Moreover, standard instrumentation in general is certainly suitable for narrow-bore column work especially for the 3 mm i.d. columns. The isocratic reversed phase method was already applied by our group for 1 year in the routine analysis studying the stability of MH and HMH in pharmaceutical preparations, using the 3 mm EcoCART column, after complete method validation. In the present report, the same method was tested (identical mobile phase, detector cell, injection sample loop), only differing in flow-rate, on a column with a different internal diameter. It is obvious that also the volume of the detector cell and the injection loop play an important role in the separations achieved on miniaturised columns. By switching to narrow-bore columns, whilst using the same injection loop, detector cell and mobile phases, one may follow two pathways: (1) keeping the same flow-rate, if resolution is not the limiting factor or (2) reducing the flow-rate in order to obtain the same retention times of the analytes, hence maintaining the same linear velocity.

If resolution is high enough, the flow may be kept constant while switching to a smaller column i.d.. This would save more time depending on the column diameter, but on the contrary cause a resolution decrease. When assessing complete validation, specificity needs to be checked by observing possible interferences between both opiates, excipients, structurally related compounds and degradation products. When these parameters are controlled, validation of the method on the column can be initiated. In the present example, when going from a 4 to a 2 mm i.d. column, it was not possible to separate both components keeping a constant flow-rate. Therefore, a reduction of flow-rate appeared logic and necessary. The required flow-rate to obtain the same linear velocity can be calculated according to the following formula:

$$\frac{\text{flowrate}_1}{\text{flowrate}_2} = \frac{d_1^2}{d_2^2}$$

in which $d_1$ and $d_2$ represent the internal diameter of column 1 and 2, respectively. Flow-rate$_1$ and flow-rate$_2$ belong to columns 1 and 2, respectively. As the 3 mm i.d. column was operated at a flow-rate of 0.70 ml min$^{-1}$, 0.32 ml min$^{-1}$ was used as flow-rate for the 2 mm i.d. column. A typical chromatogram after a 20 μl injection on the 3 mm columns can be seen in Fig. 2.

As can be noticed (Table 1), more or less similar retention is obtained for HMH when compared with MH on the columns tested. The pay-off for the diameter reduction comprises resolution and peak asymmetry factor. When resolution is not the limiting factor and when applying the same injection volumes there theoretically is a four times higher sensitivity when going from a 4 mm toward a 2 mm i.d. column. The signal is less diluted by a factor of 4 in the narrow-bore column, and therefore, the concentration-sensitive UV-detector records a four times higher peak. Thus a

Table 2
Accuracy for HMH

| Column | 2 mm i.d. | 3 mm i.d. | 4 mm i.d. |
|---|---|---|---|
| Cochran test | 0.4607 | 0.3714 | 0.1352 |
| Fisher test | 0.00978 | 1.038 | 0.06596 |
| Recovery | 101.34±0.92 | 100.67±0.57 | 100.44±1.40 |

considerably higher sensitivity can be achieved using narrow-bore columns. However, a possible overload of the column must be kept in mind because there is less packing material available. To eliminate peak broadening effects when applying small i.d. columns, smaller volumes of UV-detector flow-cells must certainly be taken into consideration.

Although many literature studies deal with qualitative aspects, few studies have been devoted to the effect of column internal diameter upon quantitave aspects (linearity, detection limit, precision, ...). It could be concluded that, as expected, column dimensions and packing materials play an important role for optimising chromatographic separations. The batch of the packing material and the history of the column need to be taken into account.

### 3.2. Comparison of the quantitave information

#### 3.2.1. Precision, accuracy

Repeatability of successive injections was determined by injecting the same standard solution (100%) and the same sample solution for eight times on each column. Analysis repeatability was assessed by preparing eight new sample solutions on the same day followed by calculating the R.S.D. in between the preparations. When taking into account the use of an IS, more or less the same precision could be noticed on the 2 and 4 mm i.d. columns (R.S.D. $< 0.6\%$). Intra-day variations on the three columns were lower than 0.8%. Without the IS, however, the method appears to be more precise when using the 4 mm i.d. column. As an overall conclusion it can be stated that all columns provide acceptable precision. The accuracy was investigated on the different columns (Table 2). Best results were obtained on the 3 and 4 mm i.d. columns. From he Cochran test—homogeneity of variation—could be deduced that there is a decrease of homogeneity with decreasing i.d. From the Fisher test no conclusion could be obtained.

Table 3
Linearity for HMH

| Column | 2 mm i.d. | 3 mm i.d. | 4 mm i.d. |
|---|---|---|---|
| R | 0.9996 | 0.9998 | 0.9998 |
| | 0.9997 | 0.9998 | 1.0000 |
| | 0.9996 | 0.9996 | 0.9999 |
| Mean r | 0.9996 | 0.9997 | 0.9999 |

Table 4
Limit of detection and limit of quantification of HMH

| Column | 2 mm i.d. | 3 mm i.d. | 4 mm i.d. |
|---|---|---|---|
| Detection limit (ng $ml^{-1}$) | 508.8 | 966.4 | 240.6 |
| Quantification limit (μg $ml^{-1}$) | 3.813 | 3.624 | 4.992 |

#### 3.2.2. Linearity

For each column studied, three calibration curves were constructed, each on a different day. The calibration curve was established after injection of five concentrations of standard HMH solutions (60, 90, 120, 150, 180 μg $ml^{-1}$). From the slope values for the different columns and from the peak areas, it could be concluded that mass sensitivity is some 60% higher for the 2 mm i.d. column than for the 4 mm i.d. column. Narrow-bore LC is, therefore, very well suited for the determination of drug impurities or for the analysis of low-level samples. The correlations calculated with the IS approach are shown in Table 3. An acceptable correlation was obtained for the three columns, although a slight decrease in correlation was observed with smaller column internal diameters.

#### 3.2.3. Limit of detection and limit of quantification

For the limit of detection a signal-to-noise ratio of three was applied and for the limit of quantification a solution was injected eight times and an R.S.D. $\leq 20\%$ was applied as a criterion (Table 4).

Table 5
Relative retention of analytes, structurally related to MH

| Column | 2 mm i.d. | 3 mm i.d. | 4 mm i.d. |
|---|---|---|---|
| Morphine-*N*-oxide | 0.90 | 0.90 | 0.90 |
| MH | 1.00 | 1.00 | 1.00 |
| HMH | 1.24 | 1.24 | 1.18 |
| Codeine phosphate | 1.62 | 1.64 | 1.36 |
| Diamorphine | 4.44 | 4.56 | 2.65 |
| Pseudomorphine | 12.53 | 14.14 | 8.69 |

Table 6
Two level design

| Parameter | − Level | 0 Level | + Level |
|---|---|---|---|
| Column temperature (°C) | 35 | 40 | 45 |
| % (v/v) Acetic acid | 0.35 | 0.40 | 0.45 |
| Concentration SDS (g $l^{-1}$) | 4.5 | 5.0 | 5.5 |
| % (v/v) ACN | 33 | 35 | 37 |
| Flow-rate (ml $min^{-1}$) | 0.6 | 0.7 | 0.8 |

As can be seen from this table, a decrease of quantification limit is observed with small column diameters. Abnormalities in limits of detection and limits of quantification when comparing the values obtained on both columns may be due to differences of individual column performance as only one column was used in the experiments. Limit of detection and limit of quantification were also calculated (without and with IS) applying the ALAMIN program [12,13], leading to the same conclusion.

### 3.2.4. Specificity

The separation of MH and HMH from other opiates and from some degradation products were investigated on the different columns (Table 5). The most critical separation, as expected, was the separation of morfine-*N*-oxide from MH. The separation, as investigated by the Kaiser peak separation index, was 0.68, 0.97 and 0.92, respec-

Table 7
Saturated 16th fraction design

| Number of experiment | Column temperature | Acetic acid | Concentrated SDS | Concentrated ACN | Flow-rate | Dummy 1 | Dummy 2 |
|---|---|---|---|---|---|---|---|
| 1 | −1 | 1 | −1 | −1 | 1 | −1 | 1 |
| 2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 3 | −1 | −1 | −1 | 1 | 1 | 1 | −1 |
| 4 | 1 | −1 | −1 | −1 | −1 | 1 | 1 |
| 5 | −1 | −1 | 1 | 1 | −1 | −1 | 1 |
| 6 | −1 | 1 | 1 | −1 | −1 | 1 | −1 |
| 7 | 1 | −1 | 1 | −1 | 1 | −1 | −1 |
| 8 | 1 | 1 | −1 | 1 | −1 | −1 | −1 |

Table 8
Results of experiments

| Number of run | k′ HMH | Ratio area HMH/MH | Resolution | Run time (min) |
|---|---|---|---|---|
| 1 | 10.33 | 1.3659 | 3.29 | 11.0 |
| 2 | 5.72 | 1.3727 | 2.39 | 6.5 |
| 3 | 6.23 | 1.3713 | 2.70 | 7.2 |
| 4 | 8.78 | 1.3599 | 3.33 | 12.0 |
| 5 | 5.39 | 1.3729 | 2.63 | 8.1 |
| 6 | 9.37 | 1.3597 | 3.37 | 12.8 |
| 7 | 8.29 | 1.3660 | 3.33 | 8.7 |
| 8 | 5.17 | 1.3705 | 2.29 | 7.8 |

Table 9
Calculation of the normalised effects of different parameters

| Influence of | k′ HMH | Peak area ratio | Resolution | Run time (min) |
|---|---|---|---|---|
| Temperature | 3.231 | 0 | −3.148 | 1.870 |
| % Acetic acid | 1.827 | 0 | 3.348 | 0.958 |
| Concentrated SDS | 1.673 | 0.2 | 0.652 | 0.867 |
| % ACN | 13.712[a] | 4.6 | 16.452[a] | 6.795 |
| Flow-rate | 1.788 | 1.5 | 0.348 | 3.329 |
| Dummy 1 | 0.885 | 1.6 | 1.348 | 1.323 |
| Dummy 2 | 1.115 | 0.4 | 0.348 | 0.502 |

[a] Significant/critical t-value 9.9.

tively, for the 2, 3 and 4 mm i.d. columns. When a 2 mm i.d. column is used in a conventional HPLC system, peaks with a low k′-value may be broad. Generally the values of resolution between the different analytes reveal that the 3 mm i.d. columns provided the best separation.

### 3.3. Robustness testing for quantitative HMH determination

A robustness test [14] for the determination of HMH was applied on the 3 mm i.d. narrow-bore column. Contrarily to an optimisation design, small variations of method parameters were applied for the robustness test. In most cases a two-level design is applied to investigate the influence of the parameters upon the method. A Placket Burman design was utilised with eight experiments (Table 6), taken into account some five variable parameters of the mobile phase. A saturated 16th fraction design (Table 7) (Statgraphics® version 3.1) with seven factors was applied and as only five parameters were studied, two dummies were introduced. The obtained results of the measurements are shown in Table 8. From the normalised effects of the different parameters (Table 9) the conclusion could be made that the effect of ACN content in the mobile phase can be considered significant in the studied range ($13.7115 > 9.9 =$ crital t-value) upon the k′-value of HMH. The same conclusion could be made for the effect of organic modifier content upon the resolution ($16.452 > 9.9$).

## 4. Conclusion

The proposed isocratic reversed phase chromatographic separation for the analysis of HM and HMH shows acceptable linearity, sensitivity and precision on all columns studied. Applying narrow-bore (2 mm i.d.) instead of the conventional (4 mm i.d.) columns results in an increase of detector response for the opiates. These 2 mm i.d. narrow-bore columns can be applied for this purpose with conventional HPLC instrumentation, however, some loss of chromatographic performance (resolution, peak asymmetry) can be observed in comparison with columns of conventional diameter, due to extra column effects. These effects were not observed when utilising the 3 mm EcoCART column.

Therefore, it may be stated that routine HPLC procedures carried out on conventional 4.6 mm i.d. columns should be discouraged in favour of lower internal diameter columns leading to a safer and more economical laboratory environment. Analysts must be encouraged for introducing miniaturised HPLC systems leading to considerable reductions in the use (and costs) of organic solvents, thus establishing greener laboratories, without loosing chromatographic performance.

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