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Micellar electrokinetic and high-performance liquid chromatographic determination of potential manufacturing impurities in pholcodine

Oliver M. Denk, David G. Watson, Graham G. Skellern*

Department of Pharmaceutical Sciences, University of Strathclyde, Strathclyde Institute of Biomedical Sciences, 27 Taylor Street, Glasgow G4 0NR, Scotland, UK

Abstract

Quantitative high-performance liquid chromatographic (HPLC) and micellar electrokinetic chromatographic (MEKC) methods have been developed for the determination of four structurally related potential manufacturing impurities, including morphine, of the opiate derivative pholcodine. Pholcodine and the four impurities were separated by MEKC in less than 14 min using a 70 cm×75 μm I.D. uncoated fused-silica capillary (25 kV at 30°C) and a running buffer consisting of 10% acetonitrile (v/v) in 20 m*M* borate–phosphate buffer pH 8.0 containing 40 m*M* sodium dodecyl sulphate (SDS). The MEKC method was compared to a HPLC method using a 5 μm Luna phenyl–hexyl column (150×4.6 mm I.D.) eluted with a mobile phase consisting of a mixture of 10% (v/v) acetonitrile, 7% (v/v) tetrahydrofuran in 20 m*M* phosphate buffer pH 8.0. Both methods were fully validated and a comparison was made regarding selectivity, linearity, precision, robustness and limits of detection and quantitation. The presence of the impurities in different samples of pholcodine drug substance was investigated using both methods.

Keywords: Validation; Pholcodine; Morphine

1. Introduction

Pholcodine [(5*R*, 6*S*)-4,5-epoxy-9α-methyl-3-(morpholinoethoxy)-morphin-7-en-6-ol] (Fig. 1) is a semi-synthetic opium alkaloid, which is widely used as an anti-tussive agent. It is synthesised [1] by treating an aqueous solution of morphine with an equivalent amount of sodium hydroxide, before the addition of a solution of chloroethylmorpholine hydrochloride, previously neutralised with sodium hydroxide.

Of the few reported HPLC studies involving pholcodine it has been used either as an internal standard [2,3] or as a principle analyte in one bioanalytical study [4]. More recently capillary electrophoresis (CE) methods have been reported for analysing mixtures of opiates containing pholcodine. Micellar electrokinetic chromatography (MEKC) was used to determine nine alkaloids in crude morphine, poppy straw and opium preparations [5] with pholcodine as the internal standard. In parallel with a previously reported HPLC study [4] a CE method was developed to separate pholcodine, 6-monoacetylmorphine, morphine, heroin, codeine and dihydrocodeine in urine [6].

To date, it is only the national pharmacopoeias [7,8] that describe semi-quantitative methods for the determination of related substances in pholcodine. Since pholcodine is a semi-synthetic alkaloid, impurities in pholcodine may arise from impurities

*Corresponding author. Tel.: +44-141-548-2263; fax: +44-141-552-6443.

E-mail address: g.g.skellern@strath.ac.uk (G.G. Skellern).

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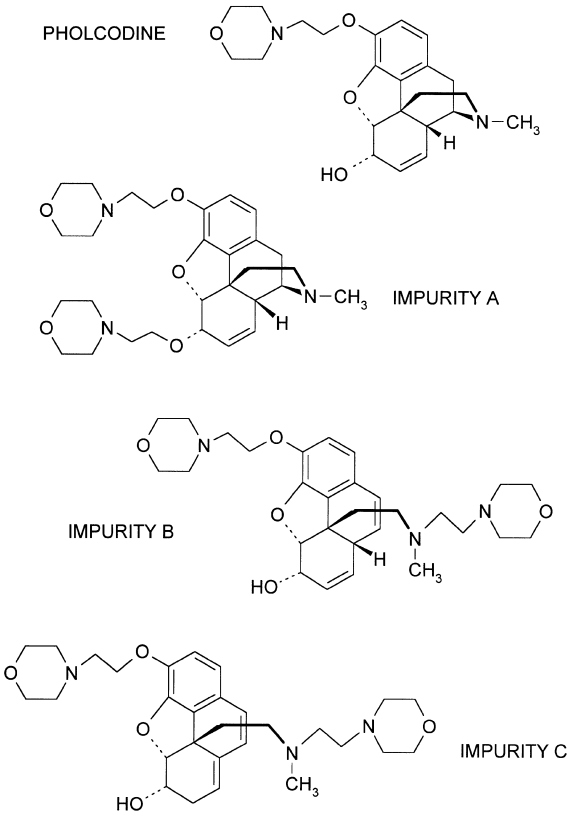

Fig. 1. Structural formulae of pholcodine and the isolated putative impurities (A, B and C).

already present in the starting materials, one of which is morphine, or from the manufacturing process. However because impurities in morphine are controlled [9], then only morphine remains as a potential starting material impurity for which a limit is set [8]. Potential manufacturing impurities may arise from side reactions of chloroethylmorpholine with other positions in the morphine molecule. We recently reported the isolation and identification of three so far unreported manufacturing impurities, from a chloroform extract of a mother liquor remaining after the recrystallisation of pholcodine [10]. These impurities are analogues of pholcodine possessing second ethylmorpholine moieties at various positions (Fig. 1).

In this paper we report the development of a quantitative MEKC method as well as an HPLC method for the determination of these potential manufacturing impurities and the starting material morphine in samples of pholcodine.

2. Experimental

2.1. Chemicals

Anhydrous Na_2HPO_4 (AnalaR), $NaH_2PO_4 \cdot H_2O$ (AnalaR), $Na_2B_4O_7 \cdot 10\ H_2O$ (AnalaR) and sodium dodecyl sulphate (SDS) were obtained from BDH (Poole, UK). Samples of pholcodine BP, morphine hydrochloride and chloroethylmorpholine (CEM) hydrochloride were obtained from Macfarlan Smith (Edinburgh, UK). Compounds A, B and C (potential impurities) were isolated from pholcodine aqueous mother liquor as reported previously [10]. Water was glass distilled and filtered through 0.45-μm nylon filters (Whatman, Kent, UK). Acetonitrile (ACN) and tetrahydrofuran (THF) were of HPLC grade and obtained from BDH.

2.2. MEKC buffer

$Na_2B_4O_7 \cdot 10\ H_2O$ (28.396 g) and $NaH_2PO_4 \cdot H_2O$ (27.598 g) were dissolved separately in 1 l of water to give stock solutions of 25 m*M* and 0.2 *M* of each buffer salt, respectively. A portion (400 ml) of 25 m*M* $Na_2B_4O_7 \cdot 10\ H_2O$ was mixed with a volume (50 ml) of 0.2 *M* $NaH_2PO_4 \cdot H_2O$ and the mixture diluted to 1 l with water (Buffer A) to give a final concentration of 20 m*M*. Before making up the volume, the pH of the solution was adjusted to 8.0 with 1 *M* HCl. A quantity (2.3072 g) of SDS was dissolved in Buffer A (200 ml) to produce a final SDS concentration of 40 m*M*. The buffer solution was filtered through a 0.45-μm membrane filter. A volume of ACN (10 ml) was diluted to 100 ml with 40 m*M* SDS–20 m*M* phosphate–borate buffer pH 8.0. The final running buffer (10% (v/v) ACN in 40 m*M* SDS–20 m*M* borate–phosphate buffer pH 8.0) was degassed for 15 min in an ultrasonic bath before use.

2.3. HPLC mobile phase

Anhydrous Na_2HPO_4 (28.396 g) and $NaH_2PO_4 \cdot H_2O$ (27.598 g) were dissolved separately in 1 l of water to obtain 0.2 *M* stock solutions of each buffer

salt. A portion (47.35 ml) of 0.2 *M* Na_2HPO_4 buffer was mixed with a volume (2.65 ml) of 0.2 *M* $NaH_2PO_4 \cdot H_2O$ and the mixture diluted to 500 ml with water to give a 20 m*M* phosphate buffer with a measured pH of 8.0. The final buffer solution was filtered through a 0.45 μm membrane filter.

Volumes of ACN (50 ml) and THF (35 ml) were transferred into a 500 ml volumetric flask and diluted with the 20 m*M* phosphate buffer. The final mobile phase [10% (v/v) ACN–7% (v/v) THF–20 m*M* phosphate buffer (pH 8.0)] was degassed (15 min) in an ultrasonic bath before use.

2.4. Apparatus

2.4.1. MEKC

CE separations were carried out using a Spectraphoresis ULTRA CE system equipped with PC 1000 software and a Spectraphoresis UV 3000 scanning detector (Thermo Separation Products, San Jose, USA). The capillary (375 μm O.D.×75 μm I.D.) obtained from Composite Metal Services (Hallow, UK) was cut to a total length of 70 cm. The polyimide coating on the capillary was burnt off 6 cm from the cathodic end to provide a window at 64 cm (l_{eff}) of the capillary length. Detection was by on-capillary UV absorbance at 210 nm with a rise time of 0.5 s. The capillary was maintained at 30°C. Injections were made by applying a pressure (1 p.s.i.) for 10 s to the anodic end of the capillary (1 p.s.i.=6894.76 Pa). Initially, the capillary was conditioned by flushing it for 5 min with 0.1 *M* NaOH at 50°C prior to sequential washing with water and the running buffer at the analysis temperature. The first two injections to equilibrate the capillary were water using normal CE conditions. Between injections the capillary was washed with the running buffer. An automated buffer replenishment was carried out for each sample. At the end of a sample run, the capillary was flushed with water and then dried by blowing air through it. Separations were performed at a voltage of 25 kV for 20 min, which resulted in a current of 85 μA.

2.4.2. HPLC

All LC separations were carried out on an isocratic HPLC system using a ThermoQuest isocratic pump SpectraSeries P100 with a mobile phase flow-rate of 2 ml/min. Injections were made automatically with a ThermoQuest autosampler SpectraSeries AS300 fitted with a 100 μl loop and set to an injection volume of 20 μl. Separation of the compounds was achieved on a Phenomenex Luna phenyl–hexyl column (150×4.6 mm I.D.) with a stationary phase particle size of 5 μm. A guard column (30×4.6 mm I.D.) was used containing the same stationary phase. Initially the column was equilibrated with the mobile phase [10% (v/v) ACN–7% (v/v) THF–20 m*M* phosphate buffer pH 8.0] for 45 min. Before storage, the column was washed for 30 min with a mixture of ACN–water (50:50, v/v) and then with ACN. The compounds were detected at two wavelengths using two ThermoQuest SpectraSeries UV100 detectors in series. The first detector was set at 314 nm and the data collected with a Fisons DP700 integrator. The second detector which was connected to a Hewlett-Packard HP 3395 integrator and was set at 238 nm. For both integrators the rise time was 0.3 s with a range (aufs) of 0.01.

2.5. Method validation

2.5.1. MEKC

Using the conditions previously described, which gave the optimum separation of the analytes, the method was validated with respect to the following parameters.

Linearity of response was determined with five concentrations of each of the compounds dissolved in 20 m*M* borate–phosphate buffer pH 8.0 in the following ranges: morphine HCl, 5–25 μg/ml; pholcodine BP, 50–250 μg/ml; impurity A, 20–100 μg/ml; impurity B, 20–100 μg/ml and impurity C, 25–125 μg/ml. A 20 m*M* phosphate–borate buffer (pH 8.0) was used as a blank. The analyte peak areas in the electropherograms were normalised by dividing them with their corresponding migration times [11]. Two injections were made per standard solution and regression equations determined for each compound. Relative standard deviations (RSDs) of the slopes and the intercepts were calculated. The within-day precision of the method was determined from ten replicate injections of a standard mixture solution containing morphine·HCl (20 μg/ml), pholcodine BP (200 μg/ml), compound A (80 μg/ml), compound B (80 μg/ml) and compound C (100 μg/ml)

in 20 m*M* phosphate–borate buffer pH 8.0. The peak area of each analyte was divided by the migration time and the RSDs determined. The day-to-day precision was determined in the same way over a period of 5 days using three replicate samples. Detection limits and quantification limits were estimated for morphine·HCl and compounds A, B and C using an approach reported for chromatographic methods [12].

2.5.2. HPLC

In order to achieve an optimum resolution between compound C and pholcodine, C was monitored at 314 nm, a secondary UV absorption maximum. The accuracy of the assay for the determination of compound C at this wavelength was established by adding 50 μg of this compound to a 2.5 mg/ml solution of pholcodine (P3). Linearity of response was determined using five different concentrations of each substance dissolved in 20 m*M* phosphate buffer pH 8.0 in the following ranges: morphine·HCl, 2–10 μg/ml; pholcodine BP, 40–120 μg/ml; compound A, 10–50 μg/ml; compound B, 5–25 μg/ml and compound C, 5–25 μg/ml. A 20 m*M* phosphate buffer solution (pH 8.0) was used as a blank. Chromatograms of each set of calibration standards were recorded. Two injections were made per standard solution for each compound. RSDs of the slopes and the intercepts of plots of peak area versus concentrations were calculated. The within-day precision of the method was determined from ten replicate injections of a standard mixture solution containing morphine·HCl (8 μg/ml), pholcodine (100 μg/ml), compound A (40 μg/ml), compound B (40 μg/ml) and compound C (20 μg/ml) in 20 m*M* phosphate buffer pH 8.0. The day-to-day precision was determined in the same way over a period of 5 days using three replicate samples. Detection limits and quantification limits were estimated for morphine·HCl and compounds A, B and C using a previously described method [12].

2.6. Sample preparation

2.6.1. MEKC

Stock solutions of each sample of pholcodine (P1 to P5) were prepared by dissolving ca. 50 mg of each sample in 5 ml of 20 m*M* phosphate–borate buffer pH 8.0. Duplicate dilutions of these solutions were made with the running buffer to give a final concentration of 1.0 mg/ml. The diluted samples were filtered and analysed in duplicate. The electropherograms were recorded and the areas of the peak due to the analytes normalised by dividing them by their respective migration times. The identity of the detected impurities was confirmed by spiking the sample solutions with known amounts of the putative impurity. To assist in the identification of the impurities, the ultraviolet spectrum in the range 200 to 350 nm of each compound producing a peak in the electropherogram was recorded.

2.6.2. HPLC

The same five pholcodine samples (P1 to P5) as used for the MEKC study as well as an additional sample of pholcodine (P6) was analysed by HPLC. Stock solutions of each sample were prepared by dissolving ca. 100 mg of sample in 20 ml of 20 m*M* phosphate buffer pH 8.0. Duplicate dilutions of these solutions were made with the buffer to give a final concentration of pholcodine of 2.5 mg/ml. The diluted samples were analysed in duplicate. The identity of detected impurities was confirmed by spiking the sample solutions with known amounts of the putative impurity.

3. Results and discussion

3.1. Validation

In the course of the validation studies it was found that the efficiency (*N*) calculated from the peaks for morphine and the three impurities was about 40 times greater for the MEKC method compared to the HPLC method (Table 1). As a result the resolution between adjacent peaks was in general three to four times better for all pairs of analytes with the MEKC method in comparison to the HPLC method (Table 1). Good baseline separation of pholcodine and the four potential impurities was obtained in 13 min with MEKC (Fig. 2A) whereas with the HPLC method (Fig. 3) nearly baseline separation was achieved after 20 min since compound C was not completely resolved from pholcodine (Fig. 3). However, in order to detect the small amounts of impurities present in

Table 1
Comparison of the validation data for MEKC and HPLC methods

Sample	Morphine·HCl		Pholcodine		Impurity C		Impurity A		Impurity B	
	MEKC	HPLC	MEKC	HPLC	MEKC	HPLC[a]	MEKC	HPLC	MEKC	HPLC
Migration/retention time (min) (n=10) (RSD, %)	6.43 (0.02)	3.11 (0.85)	9.74 (0.03)	5.96 (0.53)	11.10 (0.03)	7.73 (0.87)	11.96 (0.04)	11.02 (0.91)	12.50 (0.05)	14.06 (0.03)
Resolution	19.9	3.6	7.0	1.8	6.2	2.4	4.1	1.7	–	–
Efficiency of peak N ($\times 10^4$)	11.6	0.31	2.2	0.39	9.2	0.11	7.3	0.13	8.8	0.22
Linearity										
Slope ($\times 10^3$ for HPLC)	505	36.5	451	34.7	130	7.7	209	14.7	126	45.7
(RSD, %) (n=2)	(0.51)	(0.46)	(0.93)	(0.51)	(0.98)	(0.82)	(1.63)	(1.36)	(1.11)	(1.35)
Intercept ($\times 10^3$)	+0.04	−1.17	+0.32	−8.77	−0.03	−3.19	−0.11	−0.24	−0.08	+6.06
Correlation coefficient (r^2)	0.9993	0.9994	0.9981	1.0000	0.9988	0.9980	0.9978	0.9981	0.9970	0.9998
Precision										
Within day (n=10) RSD (%)[b]	1.00	7.33	0.83	1.13	0.76	1.23	0.71	0.91	0.96	1.97
Day-to-day (n=5) RSD (%)[b]	1.47	1.09	2.87	0.24	2.91	0.86	2.63	3.55	2.16	0.97
LOD (μg/ml)	0.05	0.06	–	–	0.27	0.23	0.18	0.58	0.26	0.22
LOQ (μg/ml)	0.08	0.10	–	–	0.44	0.38	0.30	0.96	0.43	0.36

[a] λ=314 nm.

[b] Conc. (μg/ml) for MEKC: morphine=20, pholcodine=200, A=80, B=80 and C=100. Conc. (μg/ml) for HPLC: morphine=8, pholcodine=100, A=40, B=40 and C=50. LOD, limit of detection; LOQ, limit of quantitation.

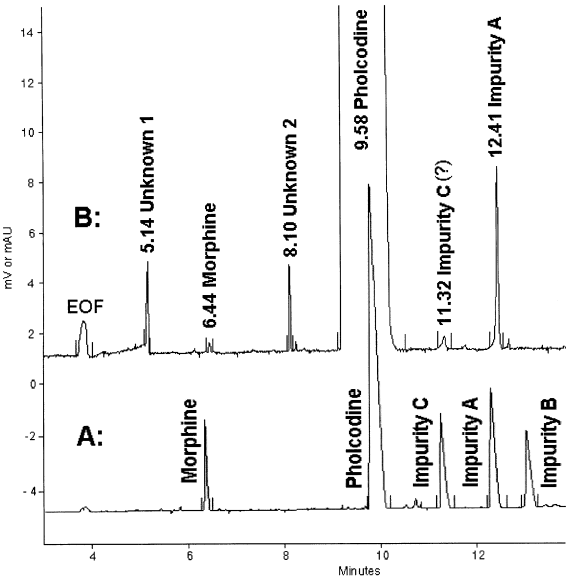

Fig. 2. (A) MEKC electropherogram of a standard mixture of morphine, pholcodine and impurities A, B and C. The running buffer was 10% (v/v) acetonitrile, 40 mM sodium dodecyl sulphate in 20 mM phosphate–borate buffer at pH 8.0, applied voltage 25 kV (30°C), hydrodynamic injection for 10 s at 1 p.s.i. and detection at 210 nm. (B) Representative MEKC electropherogram of a solution of pholcodine (P4). Experimental conditions as in (A).

pholcodine samples, it was necessary to inject large amounts (50 μg) of pholcodine onto the column, which caused excessive broadening of the peak due to pholcodine. When a concentrated sample of pholcodine containing a known amount of compound C was analysed by HPLC at 238 nm, C appeared as a shoulder on the pholcodine peak. All three compounds (A, B and C) absorb intensely at 238 nm justifying its use as the primary wavelength of detection. Since compound C absorbed at 314 nm while pholcodine has very little UV absorbance at this wavelength C was monitored at 314 nm. An HPLC chromatogram obtained at 314 nm of a sample of pholcodine spiked with C (50 μg) showed baseline separation of pholcodine and compound. Single wavelength detection was sufficient for the MEKC assay since pholcodine did not interfere in the quantitation of compound C.

Table 1 shows the main parameters for the quantitative validation of both methods. In CE, the peak area of a particular analyte is proportional to its migration time because later migrating compounds move through the detector at a slower rate than compounds with shorter migration times. This is different from HPLC where all solutes move through the column and detector at the same velocity, which

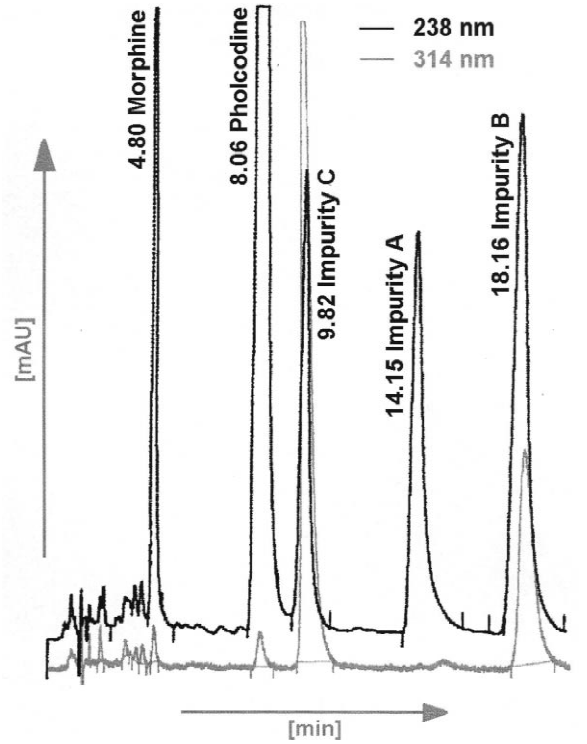

Fig. 3. HPLC chromatograms of a standard mixture of morphine, pholcodine and impurities A, B and C separated on a Luna phenyl–hexyl 5 μm column (15 cm×4.6 mm I.D.). The mobile phase consisted of 10% (v/v) acetonitrile, 7% (v/v) in 20 m*M* phosphate buffer (pH 8.0) delivered at a flow-rate of 2 ml/min. Detection: 238 and 314 nm.

is equal to the mobile phase velocity. Any variations in the peak area in CE caused by changes in analyte velocity can be compensated for by using normalised peak area (peak area divided by migration time) [13]. Hence, normalised peak area rather than peak area was used for the validation of the MEKC method.

The correlation coefficients (r^2) obtained for the regression lines of the MEKC plots of normalised peak area versus concentration and those for the HPLC method were all greater than 0.997. However, slightly better correlations were obtained with the HPLC method. The within-day precision with the MEKC method for migration times was less than 0.1% and for the normalised peak areas equal to or less than 1.0% for all analytes. In contrast, within-day precision for retention times and peak areas with the HPLC method gave higher RSDs values (Table 1). This demonstrates that it is possible to achieve an acceptable intra-day reproducibility with MEKC with careful buffer production and frequent buffer replenishment to maintain the level of buffer in the buffer vials [14].

The variation of the migration times for a particular analyte and hence the variation in peak areas may arise because of minor physical differences between capillaries from different batches. Consequently the extent of ionisation of the silanol groups on the inside of the capillary wall may vary between batches. When determining the inter-day precision the capillary had to be replaced twice (due to blockage or breakage). This experimental problem would explain the RSDs values of about 2.5% for the MEKC method in comparison to those of about 1.0% for the HPLC method. Of the two methods the HPLC method is regarded as the more robust but for critical separations CE is much more efficient.

There is a considerable decrease of sensitivity in CE because the pathlength of the beam is restricted to the diameter of the CE capillary (I.D. 75 μm). In order to be able to detect small amounts of impurities the less discriminative but more sensitive wavelength of 210 nm was used in the MEKC method. Limits of detection (LODs) and quantitation (LOQs) were estimated for both methods using a published method [12]. Similar LOD and LOQ values were obtained for morphine and the compounds C and B with both methods (Table 1). The MEKC method was found to be slightly more sensitive for the detection of impurity A.

3.2. Determination of impurities in samples of pholcodine

Five different samples (P1–P5) of pholcodine were analysed by both MEKC and HPLC. One sample (P6) of pholcodine was only analysed by HPLC. A representative MEKC electropherogram (Fig. 2B) and a typical HPLC chromatogram (Fig. 4) show that there was no evidence for the presence of compound B (Fig. 1) in any sample.

Morphine was detected in samples P2, P3 and P5 (Table 2) by both methods as well as in P6 by HPLC only. The values obtained were slightly lower with the MEKC method. Nevertheless, the quantities of morphine found in these samples are in good agree-

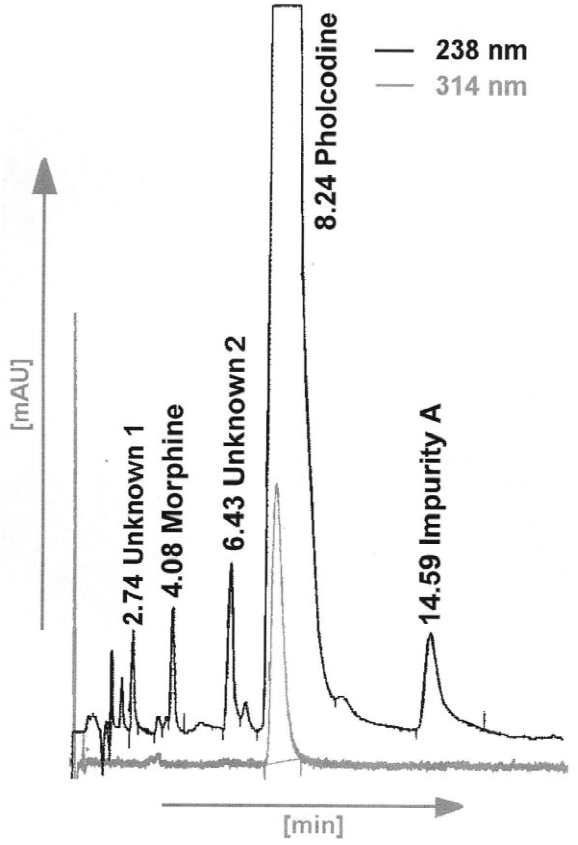

Fig. 4. Representative HPLC chromatograms of a sample solution of pholcodine (P5). Experimental conditions as given in Fig. 3.

ment between the methods. There was no evidence for the presence of morphine in sample P1 with the MEKC method. Since the amount of morphine is very small in this sample as shown by the HPLC method, it might be possible that morphine was not detected in this sample by MEKC. However morphine is present in all batches (Table 2) well below a value of 0.1% (w/w), which complies with the BP specification [8].

Both methods demonstrated that impurity A (Fig. 1) was present in the samples P1, P4 and P5 but absent from P2 and P3. The content of impurity A was above 0.1% (w/w) in P1 and P5 and about 1.0% (w/w) in P4. The determined quantities for A were in good agreement between the two methods (Table 2).

Interestingly although impurity C seemed to be present in some samples of pholcodine (P1, P4 and P5) when measured with the MEKC method (Fig. 2B), it was not detected with the HPLC method. This observation is difficult to explain since a comparison of the LOD and LOQ values for both methods indicated that the LOD values (Table 1) for this impurity are similar. Quantification using the peak due to the putative impurity gave a content in these samples above 0.1% (w/w), which should be detectable by the HPLC method. The tentative assignment of the peak in the electropherograms of samples of pholcodine was achieved by spiking the samples

Table 2
Summary of impurities found in the samples of pholcodine

Sample number	Morphine·HCl (%, w/w)		Impurity A (%, w/w)		Impurity C (%, w/w)		Unknown 1 (area%)		Unknown 2 (area%)	
	MEKC, t_m=6.4	HPLC, t_R=4.0	MEKC, t_m=12.3	HPLC, t_R=14.4	MEKC, t_m=11.2	HPLC[a]	MEKC, t_m=5.1	HPLC, t_R=2.7	MEKC, t_m=8.2	HPLC, t_R=6.4
P1	–	0.02	0.30	0.29	0.13	–	0.07	0.02	0.01	0.1
(RSD [%], n=2)	–	(2.57)	(2.89)	(2.72)	(1.66)	–	(2.79)	(2.46)	(2.29)	(1.70)
P2	0.05	0.07	–	–	–	–	0.07	0.02	0.01	0.07
(RSD [%], n=2)	(4.03)	(1.09)	–	–	–	–	(3.02)	(3.63)	(5.04)	(3.12)
P3	0.06	0.07	–	–	–	–	0.04	0.02	0.01	0.07
(RSD [%], n=2)	(1.94)	(0.51)	–	–	–	–	(10.5)	(2.94)	(1.94)	(1.77)
P4	–	–	0.95	1.09	0.19	–	0.04	0.01	0.12	0.05
(RSD [%], n=2)	–	–	(2.77)	(0.28)	(6.91)	–	(5.78)	(8.94)	(1.77)	(2.11)
P5	0.04	0.06	0.43	0.52	0.15	–	0.08	0.03	0.22	0.13
(RSD [%], n=2)	(7.44)	(3.79)	(2.28)	(2.60)	(0.39)	–	(1.77)	(3.14)	(3.93)	(0.99)
P6	n/a	0.03	n/a	0.32	n/a	–	n/a	0.01	n/a	0.02
(RSD [%], n=2)	n/a	(2.57)	n/a	(0.48)	n/a	–	n/a	(2.94)	n/a	(3.21)

[a] λ=314 nm; –, not detected; t_R (retention time) and t_m (migration time) are given in min.

with known amounts of impurity C. Although an increase of peak size occurred, there is still the possibility that this peak is in fact not due to impurity C. Secondly, additional peaks occurred only in electropherograms of samples of pholcodine containing impurity A. Also the ratio between impurity A and impurity C was constant. A sample with a greater content of impurity A showed also a higher amount of the potential impurity C and vice versa, a sample of pholcodine with a small quantity of A had less of compound C. Thus it could be concluded that this 'impurity C' is an artefact of impurity A co-eluting with C.

Two unknown compounds were detected by both methods (Table 2) in all samples of pholcodine. The identity of the compound labelled as 'Unknown 2' (Figs. 2B and 4) is of particular interest since the area percentage values in samples P1 and P5 are close to 0.1% (Table 2). We are presently undertaking LC–MS investigations in order to elucidate the structure of this compound and also to confirm the presence or absence of impurity C in the samples of pholcodine.

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