JPP Journal of Pharmacy and Pharmacology

JPP 2002, 54: 87–98

Received January 3, 2001
Accepted August 14, 2001
ISSN 0022-3573

Impurity profiling of pholcodine by liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS)

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

Abstract

Previously, a dimorpholinoethyl pholcodine manufacturing impurity was reported to be present in some samples of pholcodine. Apart from this impurity and morphine, other unknown impurities were detected in all the samples analysed by HPLC and micellar electrokinetic capillary chromatography. In this study, liquid chromatography mass spectrometry (LC-MS) analysis of samples of pholcodine showed that two of the previously unidentified compounds had mass spectra with molecular ions which differed from pholcodine by 16 amu. From this observation and other experimental data it was concluded that they are hydroxy derivatives of pholcodine. 10-*S*-hydroxy-pholcodine, which was synthesized by the oxidation of pholcodine with chromic acid, had the same chromatographic properties as one of these compounds. An early eluting compound in the LC-MS chromatograms of pholcodine was identified as pholcodine-*N*-oxide by matching chromatographic and mass spectral data of a synthesized pholcodine-*N*-oxide standard. The reaction of pholcodine with *m*-chloroperoxybenzoic acid not only produced the mono *N*-oxide, but also pholcodine-di-*N*,*N*′-oxide.

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

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

Correspondence: G. G. Skellern, Department of Pharmaceutical Sciences, University of Strathclyde, Strathclyde Institute of Biomedical Sciences, 27 Taylor Street, Glasgow G4 0NR, Scotland, UK. E-mail: g.g.skellern@strath.ac.uk

Acknowledgements: The authors thank Macfarlan Smith Ltd, Edinburgh, for providing funds for this project and for the personal support of O. M. Denk, and The Wellcome Trust for funds for the purchase of an LC-MS system. We would like to thank Dr H. A. S. Payne, Macfarlan Smith Ltd, for providing the samples and standards and for his extremely helpful advice.

Introduction

The current paper describes the identification of manufacturing impurities in pholcodine, an opioid cough suppressant. The USP (1990) describes an impurity as "any component of a drug substance (excluding water) that is not the chemical entity defined as the drug substance". Impurities may be owing to the synthetic route, source and quality of the starting materials, reagents and solvents and the subsequent purification steps (Goeroeg et al 1997). Even when the drug substance is in its final formulation, impurities can be formed owing to degradation. In view of the synthetic route to pholcodine, there is the possibility that the starting materials used for its synthesis, such as morphine and chloroethyl morpholine (Figure 1) and other side reaction products may be present in the final product. In earlier work, three previously unreported manufacturing impurities (impurities A, B and C) were isolated, by preparative reversed-phase high-performance liquid chromatography (HPLC), from the residue liquor remaining after recrystallization of a production batch of pholcodine (Denk et al 2000a). These impurities were identified by NMR and MS spectroscopy as derivatives of pholcodine possessing second 2-morpholinoethyl substituents at various positions (Figure 1). The presence and quantitation of these impurities in different samples of pholcodine has also been investigated (Denk et al 2000b) by HPLC and micellar electrokinetic capillary chromatography (MEKC).

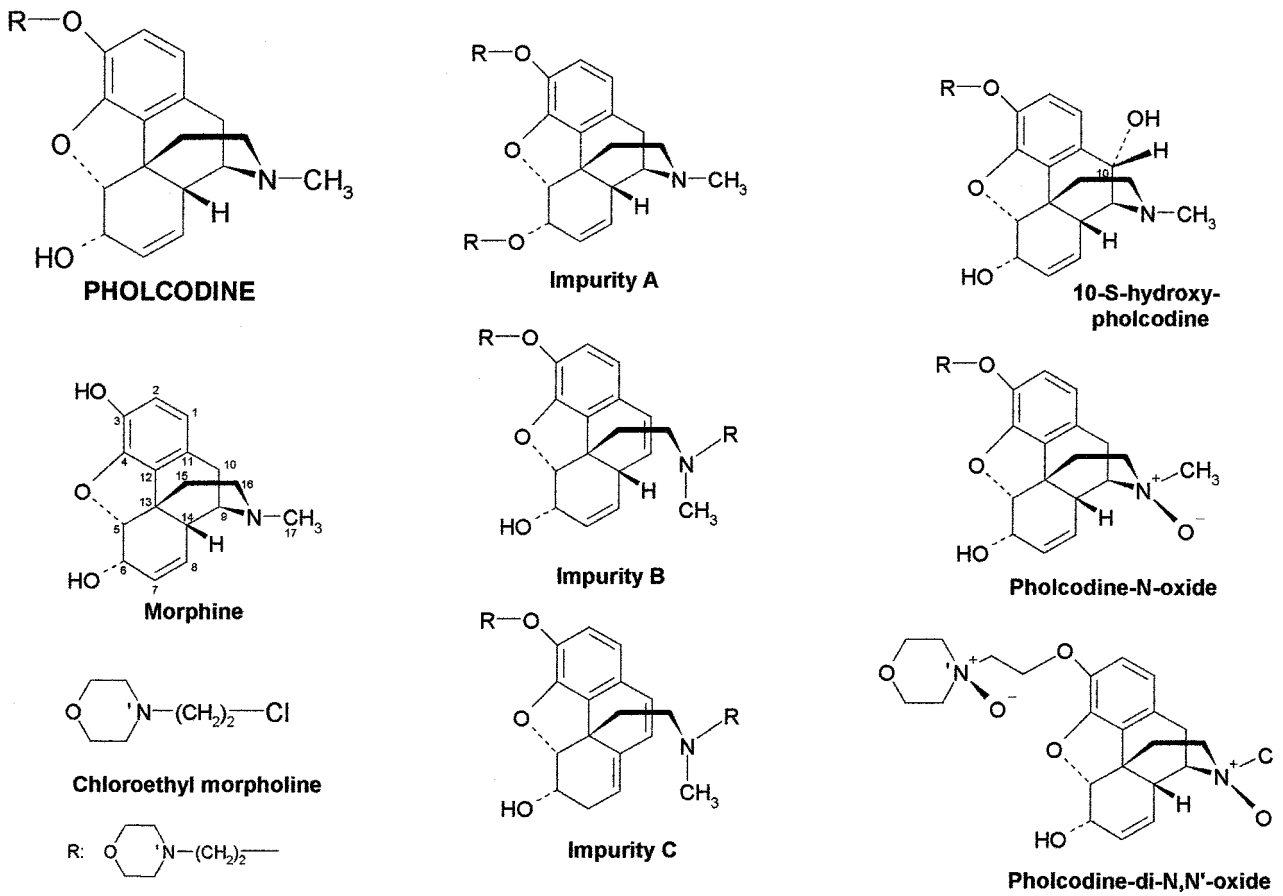

Figure 1 Structural formulae of pholcodine, morphine, chloroethyl morpholine, the synthesized compounds (10-*S*-hydroxy-pholcodine, pholcodine-*N*-oxide and pholcodine-di-*N*,*N*′-oxide) and the isolated impurities (A, B and C).

Seven different samples of pholcodine were analysed by these methods and, although there was no evidence for the presence of impurity B, morphine and impurity A (Figure 1) were detected in some of the samples. The content of impurity A was above 0.1% (w/w) in five samples. Furthermore, peaks owing to unidentified compounds were observed in the HPLC chromatograms and MEKC electropherograms of pholcodine. Since the area percentages of one of the unknown compounds eluting shortly before pholcodine was above 0.1% in four samples, the identity of this compound and the other unidentified constituents was further investigated by liquid chromatography mass spectrometry (LC-MS).

Materials and Methods

Materials

Anhydrous ammonium acetate (NH_4Ac, AnalaR), concentrated aqueous ammonium solution (35%, w/w), acetic anhydride (AnalaR), pyridine (AnalaR), 1-propanol, absolute ethanol and concentrated sulphuric acid (AnalaR) were obtained from BDH Laboratory Supplies (Poole, UK). Chromium trioxide and *m*-chloroperoxybenzoic acid (57%, w/w; m-CPBA) were obtained from Sigma-Aldrich Chemical Co. (Poole, UK). Samples of pholcodine BP (n = 7) and morphine HCl were supplied by Macfarlan Smith Ltd (Edinburgh, UK). HPLC-grade acetonitrile was obtained from Merck (Poole, Dorset, UK). Water was glass-distilled and filtered through 0.45-μm nylon filters (Whatman, Kent, UK). Pholcodine impurities A, B and C have previously been isolated (Denk et al 2000a).

Synthesis of 10-hydroxy-pholcodine (Rapoport & Masamune 1955)

Pholcodine (0.5 g) was dissolved in 1 M sulphuric acid (100 mL) and the mixture cooled to 0–3°C in an ice-bath. At this temperature, CrO_3 (0.192 g) dissolved in 10 M H_2SO_4 (20 mL) was added slowly over a period of

2 h during which the reaction solution changed from yellow to greenish-blue colour. The reaction mixture was stirred at 0–4°C for a further 1 h before adding Na_2SO_3 to stop the reaction. The solution was allowed to stand overnight at room temperature.

After transferring the reaction solution into a large beaker, the pH of the solution was adjusted to 9.0 by adding Na_2CO_3(s). The aqueous solution was extracted three times with 200-mL vols chloroform. The organic extracts were combined, dried over $CaCl_2$ and the organic solvent removed under reduced pressure with a rotary evaporator, to yield 0.108 g of a yellowish residue.

Synthesis of pholcodine-*N*-oxides (Phillipson et al 1976)

A three-neck flask (100 mL) equipped with a pressure equalizing addition funnel and a magnetic stirrer was charged with pholcodine (200 mg) dissolved in chloroform (10 mL). The reaction mixture was cooled in an ice-bath and m-CPBA (200 mg) dissolved in chloroform (20 mL) was added slowly. The mixture was then stirred for 2 h at 5–10°C, after which it was brought to room temperature and diluted to 100 mL with chloroform.

The chloroform layer was separated and washed once with 10 mL of a saturated $NaHCO_3$ solution and then with a further volume (10 mL) of distilled water. The aqueous layers were combined and acidified with 5 M HCl until no further CO_2 evolution was observed. The precipitated chlorobenzoic acid was filtered off and the filtrate taken to dryness with a rotary evaporator. The resultant residue, which had been dried in an oven at 70°C overnight, still contained large amounts of NaCl. This material was dispersed in 1-propanol (25 mL) and sonicated for 15 min. Undissolved NaCl was filtered off and 1-propanol removed under reduced pressure. A brownish residue (approx. 150 mg) was obtained and is referred to hereafter as "reaction product".

Isolation and purification of 10-hydroxy-pholcodine and the pholcodine-*N*-oxides by preparative HPLC

Preparative HPLC separations were carried out with a gradient HPLC system comprising two Gilson pumps (Gilson-305, master pump/Gilson-307, slave pump) fitted with a manometric module (Gilson 806) and a 23-mL dynamic mixer (Gilson 811C). The system was equipped with a Rheodyne Model 7125 manual injector fitted with a 2-mL injection loop. The compounds were detected at 220 nm using a Gilson 118 UV/vis detector and data were collected using a Hewlett Packard HP3395 integrator.

10-Hydroxy-pholcodine

For the purification of the reaction product from the synthesis of 10-hydroxy-pholcodine, all of the product (approx. 100 mg) was dissolved in 8 mL 10% (v/v) acetonitrile in 50 mM NH_4Ac buffer, pH 8.0. Replicate injections (n = 4) were made onto a Hypersil C-18 BDS column (5 μm; 250 × 21.2 mm i.d.). The mobile phase, delivered at a flow-rate of 16 mL min^{-1}, was a mixture of acetonitrile (solvent B) and 50 mM ammonium acetate buffer adjusted to pH 8.0 with a concentrated ammonium solution. Separation was achieved with the following gradient based on mixing A and B: A:B (85:15) for 2 min then increasing to A:B (80:20) at 5 min and held at this composition until 30 min. Four fractions were collected manually in conical flasks and for each fraction the mobile phase was removed under reduced pressure. The resultant residues were dissolved in chloroform (50 mL), which were washed with a saturated solution of $NaHCO_3$ (25 mL) to remove a residue of acetic acid, and then dried over anhydrous $CaCl_2$. The calcium chloride was filtered off and the chloroform was evaporated to a small volume under reduced pressure. All residues were transferred into tared vials.

Pholcodine-*N*-oxides

The reaction product of the synthesis of pholcodine-*N*-oxides was purified by preparative HPLC by dissolving all of the reaction product (approx. 150 mg) in 4 mL 10% (v/v) acetonitrile in 50 mM ammonium acetate buffer, pH 8.0. Replicate injections (n = 2) were carried out using the same column with an initial mobile phase composition as used for the purification of 10-hydroxy-pholcodine. The gradient used in the separation, expressed as the percentage of organic solvent B, was 15% (v/v) isocratic until 8 min after the injection, then an increase in B to 20% (v/v) at 10 min and isocratic at 20% (v/v) B until 25 min. Two fractions were collected and the mobile phase was removed under reduced pressure. The resulting residues were dissolved in absolute ethanol (50 mL) and the solvent removed under reduced pressure. This procedure was repeated four times to remove water and ammonium acetate, after which the residues were transferred into tared vials.

Thin-layer chromatography (TLC) of pholcodine-*N*-oxides

TLC was performed on silica gel 60 F_{254} polyester sheets (20 × 20 cm) obtained from E.Merck (Darmstadt, Germany). Portions (10 μL) of standard solutions of each

analyte (1.0 mg mL^{-1} in methanol) were applied onto the plates using capillary tubes. The TLC tank was lined with filter paper and the mobile phase (chloroform/*n*-butanol/methanol/concentrated ammonium solution, 40/30/30/2 by vol.) was allowed to equilibrate for 30 min. The TLC plates were developed until the solvent had travelled a distance of approximately 15 cm. The chromatograms were dried before viewing under UV light at 254 nm and subsequent spraying with Dragendorff reagent.

NMR spectroscopy

^{1}H and ^{13}C NMR spectra were recorded on a Bruker DPX400 NMR spectrometer at 400 MHz. Two-dimensional homopolar correlation spectra (^{1}H-^{1}H-COSY), heteronuclear multiple quantum coherence (HMQC) spectra, heteronuclear multiple bond correlation (HMBC) spectra and nuclear Overhauser effect spectroscopy (NOESY) spectra were obtained for 10-hydroxy-pholcodine and pholcodine. The samples were prepared for analysis by dissolving approximately 15 mg of each compound in 1 mL deuterated chloroform. Signals were referenced to the solvent chloroform at δ_H 7.27 and δ_C 77.00.

The fractions obtained from the purification of pholcodine-*N*-oxides were prepared for analysis by dissolving approximately 10 mg of each sample in 1 mL CD_3OD. Signals were referenced to the deuterated methanol at δ_H 3.35 and δ_C 49.0. The atomic numbering scheme used was that of Grewe (1947) for unsubstituted morphine extended to include the morpholinoethyl substituent at various positions (Figure 1).

MS

MS was performed on each compound using a Jeol AX505 instrument. The samples were directly inserted into the mass spectrometer. The mode of ionization was electron impact (EI) at 70 eV for pholcodine and the synthesized compounds. Elemental analyses of these compounds were performed on the same instrument using the high-resolution mode. High-resolution spectra were calibrated against perfluorokerosin (PFK).

10-Hydroxy-pholcodine: calculated for $C_{23}H_{30}N_2O_5$, 414.2154; found: 414.2155 (Δ +0.3 ppm). Pholcodine-*N*-oxide: calculated for $C_{23}H_{30}N_2O_5$, 414.2154; found: 414.2164 (Δ+2.3 ppm). Pholcodine-*N*,*N*′-oxide: calculated for $C_{23}H_{30}N_2O_6$, 430.2104; found: 430.2097 (Δ−1.6 ppm).

Standard mixture solution

Separate aqueous stock solutions (1.0 mg mL^{-1}) of pholcodine, morphine HCl and impurities A, B and C were prepared. Volumes (0.5 mL) of each stock solution were transferred into a 5-mL volumetric flask and diluted with mobile phase (20%, v/v, acetonitrile in 20 mM NH_4Ac, pH 8.0) to obtain a concentration of 100 μg mL^{-1} for each solute.

Sample preparation of pholcodine samples

Stock solutions of seven different samples of pholcodine were prepared by dissolving approximately 50 mg in 10 mL of 20% (v/v) in 20 mM NH_4Ac pH 8.0 (mobile phase). Volumes (2.0 mL) of each stock solution of pholcodine were separately diluted to 5 mL with mobile phase.

Acetylation of a sample of pholcodine

Pholcodine (P3; 2 mg) was dissolved in acetic anhydride (50 μL) and pyridine (5 μL). The reaction mixture was heated for 15 min at 80°C, after which the solvent was removed under a stream of nitrogen and the residue dissolved in 1 mL mobile phase. A reagent blank was prepared in the same way.

Sample preparation for the LC-MS analysis of pholcodine-*N*-oxides

An amount (12.4 mg) of reaction product was dissolved in 10 mL 20% (v/v) acetonitrile in 20 mM ammonium acetate buffer adjusted to pH 8.0 with concentrated ammonium solution. The sample (10 μL) was analysed by LC-PDA-MS.

LC-MS instrumentation

LC-MS analyses were performed with a ThermoQuest Finnigan Automass multi LC-GC-MS system with a quadropole mass analyser. The LC was fitted with a Phenomenex Luna phenyl-hexyl column (75×4.6 mm i.d.; particle size 3 μm). Samples (10 μL) were injected using a Finnigan Spectra System AS 3000 autosampler. The mobile phase consisted of 20 mM NH_4Ac adjusted to pH 8.0 with concentrated ammonium solution (A) and acetonitrile (B). All samples were analysed using an isocratic run of 20% v/v B for 3 min after injection followed by a linear gradient from 20% (v/v) to 40% (v/v) B for 12 min (total analysis time: 15 min). In between analyses, the column was allowed to re-equi-

librate for 10 min. The mobile phase was introduced into the Aqua source without splitting at a flow-rate of 0.5 mL min^{-1}. The atmospheric pressure ionization (API) interface was operated in positive ion electrospray mode with a cone voltage of +40 V. The drying gas temperature was set at 400°C. UV detection was with a Finnigan Spectra System UV 6000 LP photodiode array detector (PDA) with a scan range of 190–350 nm.

Results and Discussion

Method development

The starting point for the development of a suitable LC-MS method for the analysis of seven samples of pholcodine was a previously developed analytical HPLC method (Denk et al 2000b), which uses a mixture consisting of acetonitrile and 20 mM phosphate buffer, pH 8.0, delivered at a flow-rate of 2.0 mL min^{-1}. The phosphate buffer salt was replaced with ammonium acetate, a volatile buffer, to avoid blockage of the ion source. This salt also has buffer capacity at the desired pH range of 8.0 (Ermer 1998).

It is known that HPLC separations can be faster with shorter columns. For example, a 10-cm column with a particle size of 3 μm of the stationary phase can achieve a similar separation to a 15-cm column with 5-μm particles, but with a 70% reduction in analysis time (Kromidas 1997). With a Luna phenyl-hexyl column half the length (75 mm) of the one used for analytical HPLC (150 mm; Denk et al 2000b) and with a reduced particle size of 3 μm, a flow-rate of 0.5 mL min^{-1} was possible, which maintained the separation of the analytes within 15 min (Figure 2).

With the conditions described, all three impurities (A, B and C) as well as morphine (Figure 1) were separated from pholcodine (Figure 2). Morphine has good detector response with UV detection, but the response is poor when it is detected by MS. This is probably owing to its lower surface activity. The electron ionization spray (ESI) process is promoted by fission of aqueous droplets, the formation of which are favoured by low surface tension (Cole 2000). In contrast, the detector response for impurity A is better with MS than with UV detection (Figure 2), but the MS response factors of the dimorpholino compounds are all similar. Protonated molecular ions $[M+H]^+$ of morphine, pholcodine and impurities A, B and C were present in the mass spectra of these compounds. Also, the UV spectra associated with each peak in the extracted ion current (EIC) chromatograms were identical to previously recorded spectra (Denk et al 2000a).

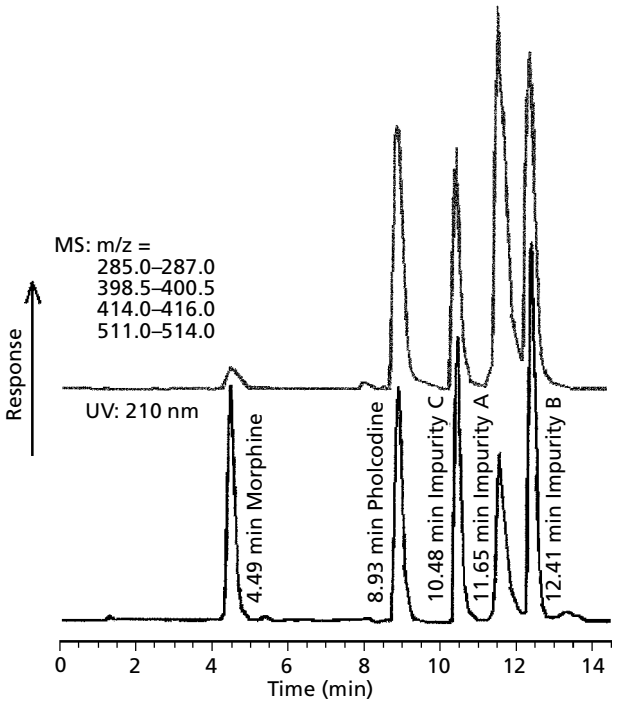

Figure 2 UV (190–350 nm) and MS-EIC (a summation of all the ion ranges listed) chromatograms of an injection of a standard mixture containing morphine HCl (m/z 285–287), pholcodine (m/z 398.5–400.5) and impurities A, B and C (m/z 511–514). Conditions: Phenomenex Luna phenyl-hexyl column (3 μm; 75×4.6 mm i.d.). The mobile phase consisted of acetonitrile and 20 mM ammonium acetate buffer (pH 8.0) delivered at a flow-rate of 0.5 mL min^{-1} using two-step linear gradient elution.

LC-MS analysis of seven samples of pholcodine

In a previous study using MEKC (Denk et al 2000b), impurity C was present in three samples of pholcodine at levels above 0.1% (w/w). This result was in contrast to that observed with HPLC, where no impurity C was detected. Although the identity of the peaks was verified by "spiking" samples of pholcodine with impurity C, there was considerable doubt about its presence since the validation procedures gave similar LOD values for both the MEKC and HPLC methods (Denk et al 2000b).

In this study, seven samples of pholcodine were analysed by LC-MS. Injection of a standard mixture of the analytes demonstrated that the LC-MS method developed is capable of baseline separation of impurity C from pholcodine (Figure 2). Analysis of the mass data showed that impurity C forms a protonated molecular ion $[M+H]^+$ at approximately m/z 512 (the instrument was operated at a resolution of 500 between ions so for the molecules under study, which contain quite a number of hydrogens and are mass excessive, there is tendency

Table 1 Comparison of the percentages of impurities by HPLC[a], MEKC[a] and LC-MS in batches of pholcodine. (Determination using LC-MS can only be considered to be semi-quantitative.)

Sample	Morphine HCl Weight % (w/w)			Impurity A Weight % (w/w)			Unknown 1 Area %[b]			Unknown 2 Area %[b]			Unknown 3 Area %[b]
	MEKC t_M = 6.4	HPLC t_R = 4.0	LC-MS t_R = 4.60	MEKC t_M = 12.3	HPLC t_R = 14.4	LC-MS t_R = 12.0	MEKC t_M = 5.1	HPLC t_R = 2.7	LC-MS t_R = 5.0	MEKC t_M = 8.2	HPLC t_R = 6.4	LC-MS t_R = 7.2	LC-MS t_R = 7.2
P1	–	0.02	0.04	0.30	0.29	0.4	0.07	0.02	0.10	0.01	0.10	0.2	0.2
P2	0.05	0.07	0.08	–	–	0.04	0.07	0.02	0.10	0.01	0.07	0.2	0.10
P3	0.06	0.07	0.06	–	–	0.04	0.04	0.02	0.1	0.01	0.07	0.2	0.07
P4	–	–	–	0.95	1.09	1.0	0.04	0.01	0.1	0.12	0.05	0.4	0.08
P5	0.04	0.06	0.09	0.43	0.52	0.6	0.08	0.03	0.2	0.22	0.13	0.3	0.2
P6	0.03	0.01	–	0.11	0.36	0.2	0.23	0.02	0.02	0.04	0.10	0.1	–
P7	0.02	0.01	–	–	0.11	0.01	0.27	0.03	0.03	0.02	0.12	0.1	–

[a]Data from Denk et al 2000b. [b]Peak area percentages of the PDA chromatograms of the LC-MS experiments. t_M, migration time (min); t_R, retention time (min).

Table 2 NMR chemical shift data of pholcodine, the synthesized 10-*S*-hydroxy-pholcodine and pholcodine-*N*-oxides.

C- No.	Pholcodine		10-*S*-Hydroxy-pholcodine		Pholcodine-*N*-oxide		Pholcodine-di-*N*,*N*′-oxide	
	^{1}H NMR δ_H (ppm)	^{13}C NMR δ_C (ppm)	^{1}H NMR δ_H (ppm)	^{13}C NMR δ_C (ppm)	^{1}H NMR δ_H (ppm)	^{13}C NMR δ_C (ppm)	^{1}H NMR δ_H (ppm)	^{13}C NMR δ_C (ppm)
1	6.51 (d)	119.7	6.86 (d)	119.6	6.59 (d)	121.1	6.62 (d)	121.2
2	6.63 (d)	115.7	6.74 (d)	116.6	6.77 (d)	119.1	6.82 (d)	119.4
3	–	141.0	–	141.9	–	142.8	–	142.2
4	–	147.1	–	146.7	–	149.5	–	149.6
5	4.84 (d)	91.7	4.84 (d)	91.4	4.92 (d)	92.7	4.95 (d)	92.8
6	4.14 (m)	66.6	4.13 (m)	66.6	4.26 (m)	67.9	4.29 (m)	67.8
7	5.25 (td)	128.5	5.50 (td)	128.8	5.33 (td)	127.6	5.33 (td)	127.4
8	5.65 (d)	133.5	5.61 (d)	132.7	5.72 (d)	135.1	5.73 (d)	135.2
9	3.32 (m)	58.9	3.38 (d)	67.5	3.89 (dd)	76.0	3.94 (dd)	76.0
10	3.01 (d); 2.26 (dd)	20.6	4.79 (d)	61.5	3.32 (d) 2.95 (dd)	26.9	3.33 (d) 2.97 (dd)	26.9
11	–	128.0	–	130.7	–	125.7	–	126.1
12	–	131.6	–	131.2	–	131.5	–	131.6
13	–	43.0	–	43.4	–	42.9	–	42.9
14	2.64 (m)	40.9	2.66 (m)	39.6	3.95 (m)	35.0	3.94 (m)	35.0
15	2.03 (dt); 1.84 (d)	35.9	2.03 (dt); 1.81 (d)	35.5	2.71 (dt); 1.83 (d)	32.2	2.78 (dt); 1.85 (dt)	32.1
16	2.37 (dt); 2.54 (t)	46.5	2.31 (dt); 2.60 (m)	47.2	3.21 (dt); 3.38 (t)	60.5	3.22 (dt); 3.38 (m)	60.5
17	2.40 (s)	43.2	2.58 (s)	43.3	3.41 (s)	58.7	3.44 (s)	58.5
18	4.23 (ddd); 4.09 (ddd)	67.2	4.27 (ddd); 4.11 (ddd)	67.5	4.33 (ddd); 4.19 (ddd)	68.4	4.76 (m); 4.55 (m)	66.3
19	2.72 (t)	57.9	2.73 (t)	57.8	2.86 (t)	59.2	3.66 (t)	71.3
20, 23	2.52 (m)	54.0	2.53 (m)	54.3	2.71 (m)	55.0	$H_{20,23}(\beta)$: 3.70 (dt) $H_{20,23}(\alpha)$: 3.32 (td)	62.7
21, 22	3.71 (t)	66.8	3.69 (t)	66.9	3.75 (t)	67.3	$H_{21,22}(\beta)$: 4.22 (td) $H_{21,22}(\alpha)$: 3.82 (dt)	66.2
6-OH	2.90 (s)	–	2.90 (s)	–	3.00 (s)	–	2.90 (s)	–
10-OH			1.70 (s)	–	–	–	–	–

s, Singlet; dd, doublet of doublet; ddd, doublet of doublet of doublet; dt, doublet of triplet; td, triplet of doublet; t, triplet; and m, multiplet.

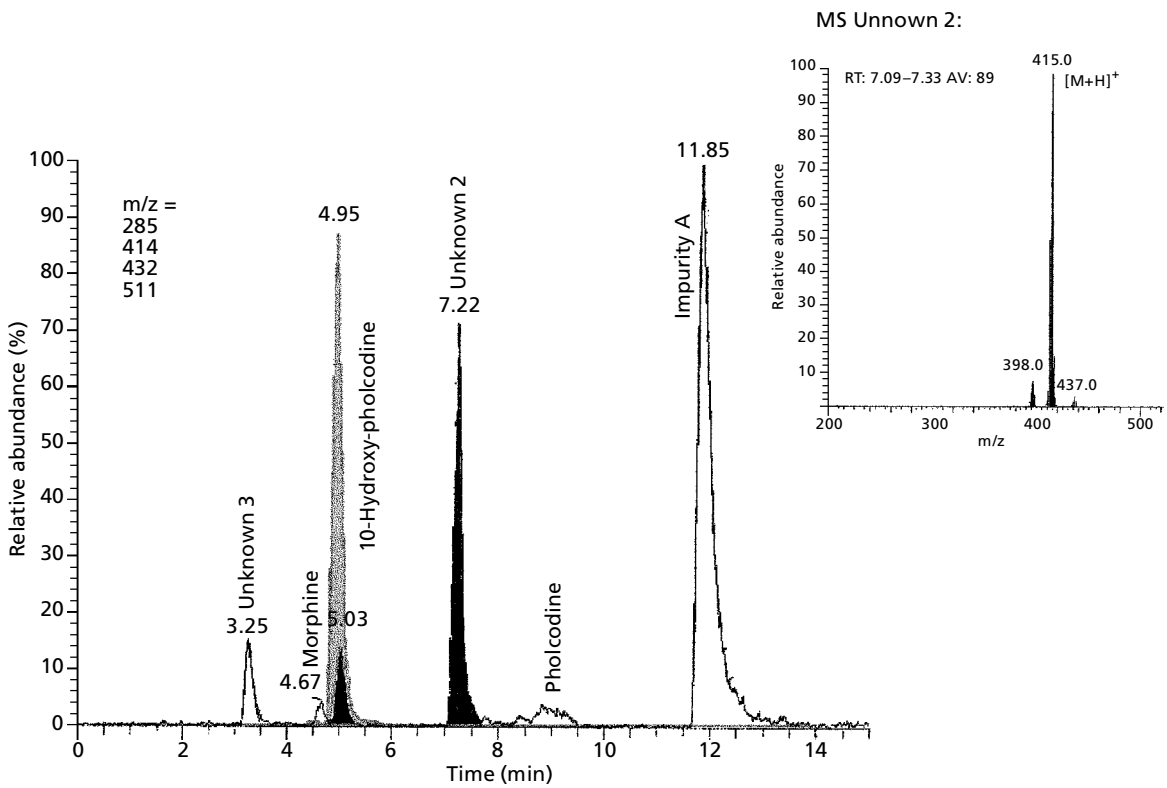

Figure 3 Superimposed LC-ESI-MS chromatograms of pholcodine (P3) and the synthesized 10-*S*-hydroxy-pholcodine using the experimental conditions given in Figure 2. The insert shows the mass spectrum of 'unknown 2'.

for masses to be rounded up rather than down, thus m/z 512.3 may be rounded up to m/z 513 rather than down to m/z 512). However, a peak corresponding to C with a retention time of approximately 10.5 min having a protonated molecular ion at m/z 512 was not present in any of the chromatograms even when the extracted ion current was displayed. This confirmed that the putative compound C identified previously by MEKC (Denk et al 2000b) was not present in the batches of pholcodine.

A semi-quantitative estimate of the weight percentages of the identified impurities were calculated based on a single point calibration using the 100 μg mL^{-1} standard mixture solution and results were compared with those obtained by HPLC and MEKC analysis (Denk et al 2000b). Morphine was detected in samples P1, P2, P3 and P5 by all three methods. However, samples P6 and P7, which were found to contain very low levels of morphine when analysed by HPLC and CE, did not contain any, as judged from the LC-MS results. This was owing to the poor MS detector response for morphine. In contrast, the UV chromatograms confirmed the presence of morphine in these samples. The weight percentages of morphine calculated from UV detection confirmed the results of the previous investigation (Table 1).

Impurity A was detected in all the samples at various concentrations with a signal-to-noise ratio, assessed using Excalibur software, of >3. The higher sensitivity of the mass spectrometer (note the relative responses to A by MS and HPLC shown in Figure 2) enabled the detection of impurity A in samples P2 and P3, which was not possible with HPLC and MEKC (Table 2). The levels of impurity A detected in all samples were always greater than those found with HPLC and MEKC. However, in general, there is good agreement between the three techniques (Table 1).

Identification of unknown impurities

A typical LC-MS EIC (extracted ion chromatogram) trace (Figure 3) of a solution of pholcodine (P5) shows that, in this mode, a peak owing to pholcodine is of very low intensity, although three unknown impurities were detected in some of the samples of pholcodine (retention times 5.1, 7.2 min), which confirmed the HPLC and MEKC results. Two of these compounds (1 and 2)

produced similar mass spectra (Figure 3) with a molecular ion (m/z 415) sixteen mass units greater than that for the molecular ion of pholcodine, which indicates the presence of an additional oxygen. The possibility that these compounds are pholcodine-*N*-oxides was thought unlikely since it would be predicted that they would elute earlier than these unknown compounds owing to their much greater polarity. Hence, it was proposed that the peaks were owing to the incorporation of a further hydroxyl group into pholcodine. Since hydroxyl groups can be readily acetylated by reaction with acetic anhydride using pyridine as a catalyst, a sample of pholcodine (P3) was acetylated and the product obtained when analysed by LC-MS had two peaks in the chromatogram with molecular ions of m/z 499. An acetylated derivative of hydroxy-pholcodine would have a molecular ion of 499, a mass shift of 84 as expected for a di-hydroxy compound. Moreover, the peak ratio of these acetylated derivatives was identical to the ratio of the two underivatized unknown compounds in the original sample. Also the longer retention times (16.2 min and 17.1 min) agreed with the expected greater lipophilicity of the ester derivatives. Therefore, the experimental evidence strongly suggested that the two unknown compounds present in the samples were hydroxy derivatives of pholcodine.

10-Hydroxy morphine is a known impurity (Proksa 1999) present in morphine. Since morphine is one of the starting materials in the synthesis of pholcodine (Stern 1955), it is very likely that any 10-hydroxy morphine present would react with chloroethyl morpholine to form 10-hydroxy-pholcodine. However, the mass spectra of the unknown impurities provided no information on the position of the additional hydroxyl groups, because spectra obtained by API-MS show very little fragmentation owing to the soft mode of ionization. Therefore, only the synthesis of the putative compounds and their subsequent comparison with the unknown impurities in the samples of pholcodine could give final confirmation of identity. Since Rapoport & Masamune (1955) had previously reported that the oxidation of morphine with cold chromic acid in sulphuric acid produces 10-hydroxy morphine, it was decided to attempt the synthesis of 10-hydroxy-pholcodine by this method.

Synthesis of 10-hydroxy-pholcodine

When the fraction (1), which was obtained by the by reaction with chromic acid in cold sulphuric acid and purified by preparative HPLC, was analysed by NMR and MS spectroscopy, it was confirmed that oxidation had occurred at position 10 (Figure 1). Changes in the ^{1}H NMR chemical shift data for the protons and carbons at position 10, when compared with those for pholcodine, indicated that oxidation had occurred at this position. The ^{1}H NMR spectra, in conjunction with COSY and HMQC experiments, proved that C-10 possessed only one proton with a chemical shift of δ_H 4.79 (Table 2). Moreover, the ^{13}C signal of C-10 shifted from 20.6 to 61.5 ppm. Furthermore, CH-9 appears as a doublet (δ_H 3.38) coupling only with H-10, whereas in pholcodine, the corresponding proton couples with two protons leading to a doublet doublet. All other signals were found to be identical to those for pholcodine (Table 2).

The stereochemistry of the synthesized 10-hydroxy-pholcodine was investigated by NOESY NMR experiments. The NOESY NMR spectrum showed a correlation between the H-10 proton and the axial proton at position 16 (Figure 1). Examination of three dimensional computer models of the two possible enantiomers of 10-hydroxy-pholcodine showed that only the *S* enantiomer is capable of producing the observed NOESY signal. The attack by chromic acid is probably only possible from the side of the molecule facing away from the nitrogen bridge, which results in the stereoselective formation of 10-*S*-hydroxy-pholcodine.

The compound produced the expected molecular ion of m/z 414 with the predicted elemental composition. All the evidence (NMR and MS investigations) confirms that the isolated material is (5*R*,6*S*,10*S*)-4,5-epoxy-10-hydroxy-*N*-methyl-3-(2-morpholinoethoxy)-morphin-7-en-6-ol (10-*S*-hydroxy-pholcodine).

Figure 3 shows the superimposed LC-MS chromatograms of a sample of pholcodine (P5) with two of the unknown impurities and the purified 10-*S*-hydroxy-pholcodine. It is evident that the peak labelled 'unknown 1' has the same retention time as the synthesized 10-*S*-hydroxy-pholcodine. Moreover, both compounds (Figure 3) have similar mass spectra. The peak labelled 'unknown 2' (Figure 3) is most likely owing to the other 10-hydroxy epimer, 10-*R*-hydroxy-pholcodine. In nature, both 10-hydroxy morphine isomers might be present in morphine leading to the formation of both 10-*S*- and 10-*R*-hydroxy-pholcodine in the manufacturing process. However, the only way to prove this hypothesis would be to convert the synthesized 10-*S*-hydroxy-pholcodine into its *R* epimer.

Synthesis of pholcodine-*N*-oxides

Examination of the chromatograms of the seven different samples of pholcodine also shows the presence of a

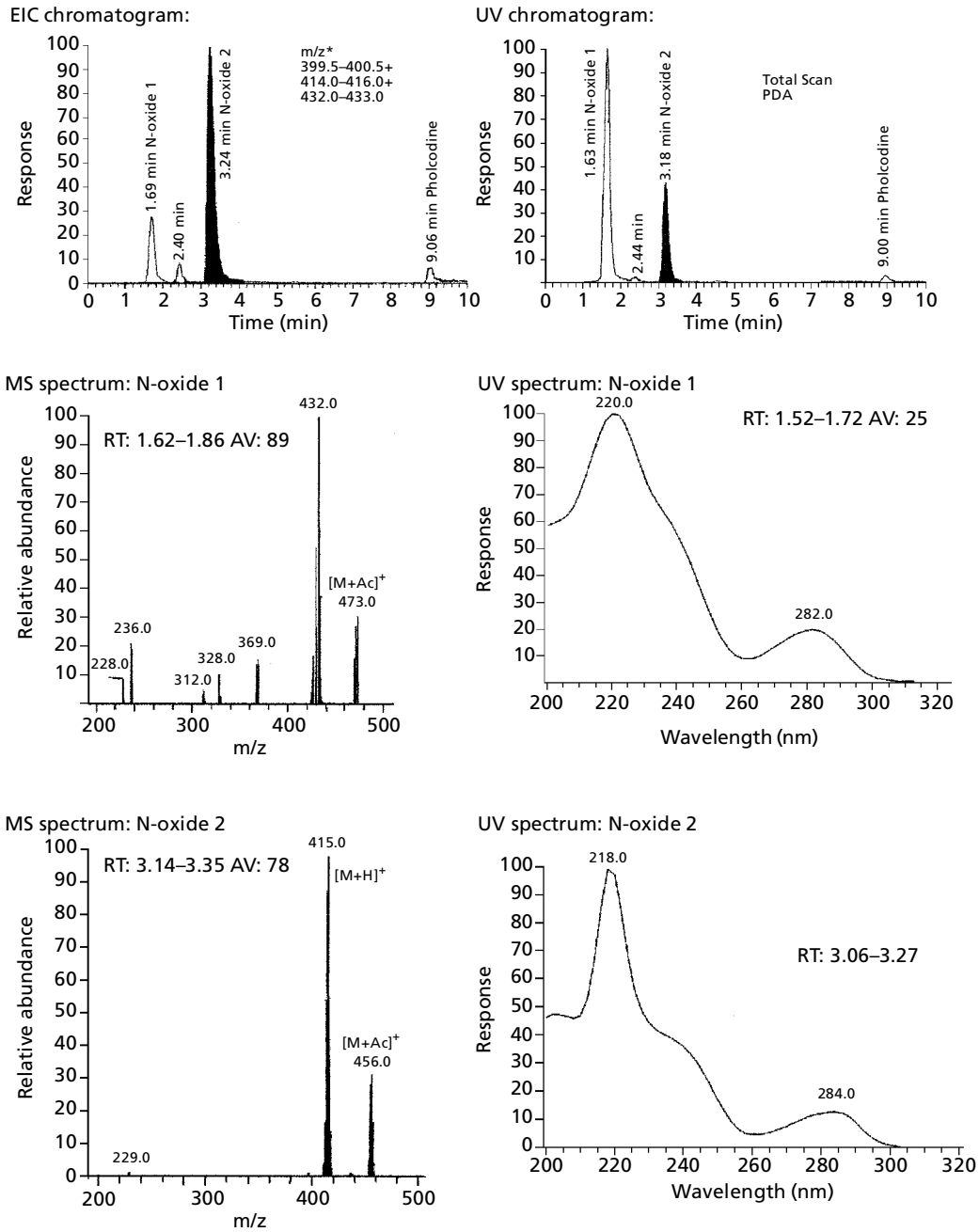

Figure 4 MS and UV chromatograms of the pholcodine-*N*-oxides with their corresponding MS and UV spectra.

very early eluting compound with a retention time of 3.25 min (Figure 3). The mass spectral data obtained for this compound indicated that it might be pholcodine-*N*-oxide. To prove the identity of the putative pholcodine-*N*-oxide, a previously reported method (Phillipson et al 1976) for the synthesis of the *N*-oxides of morphine, codeine and thebaine using m-CPBA was carried out with pholcodine.

Unlike morphine, codeine and thebaine, which only have one tertiary nitrogen, pholcodine has two tertiary nitrogen atoms (Figure 1). Therefore three products were expected, two mono-*N*-oxides as well as the di-

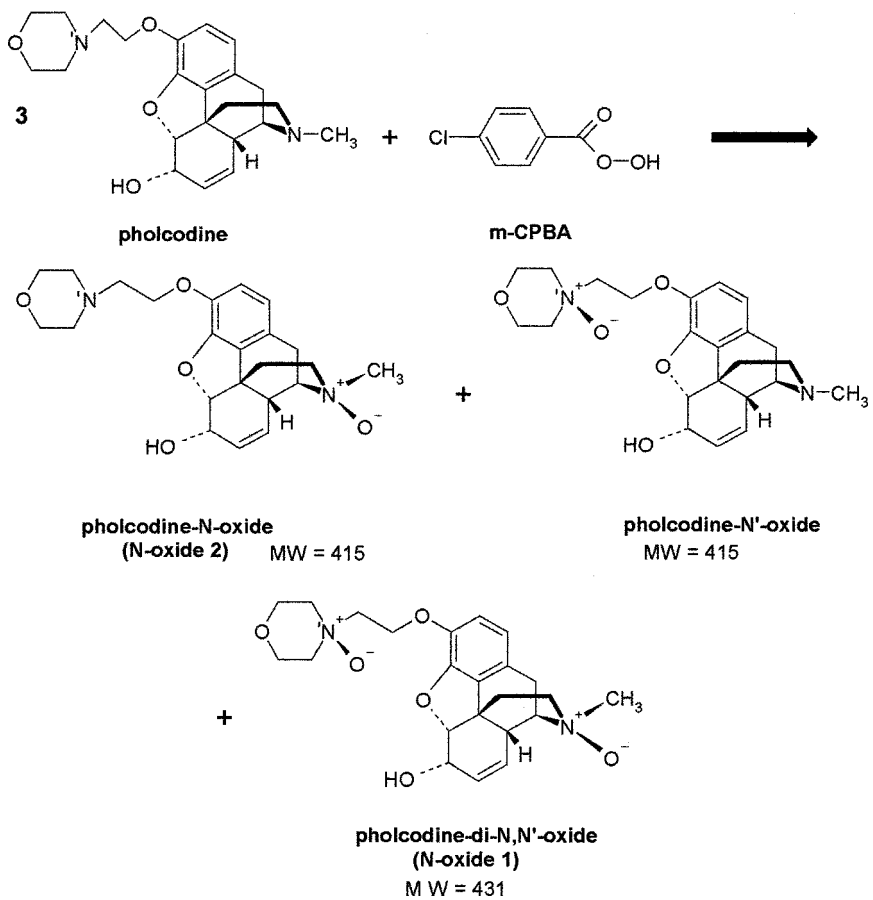

Figure 5 Possible pholcodine-*N*-oxide isomers resulting from the oxidation of pholcodine with m-CPBA.

N,*N*′-oxide (Figure 4). Not surprisingly the TLC chromatogram of the reaction mixture after 2 h showed two main product spots with R_f values of 0.20 and 0.08. During the work-up procedure, the remaining traces of pholcodine (R_f 0.58) as well as chlorobenzoic acid (R_f 0.44) were removed.

Mass and UV chromatograms of the reaction mixture showed two major peaks (Figure 4). The early eluting compound (*N*-oxide 1, grey) was thought to be the more polar di-*N*,*N*′-oxide (Figure 5), whereas the later peak (*N*-oxide 2, white) was owing to a pholcodine-mono-*N*-oxide (Figure 5). The peak owing to the mono *N*-oxide gave an expected molecular ion at m/z 415, however, the di-*N*-oxide gave an apparent molecular ion at m/z 432, which was one mass unit higher than the predicted molecular ion (Figure 4). As can be observed in Figure 4, the mass spectra of the all the molecules contained adducts formed by interaction with acetate present in the mobile phase and in the case of the di-*N*-oxide the acetate adduct has the expected ion at m/z 463. The UV spectra of both *N*-oxides were similar to the UV spectrum of pholcodine (Denk et al 2000a). Interestingly, the UV and MS detector responses for the compounds were reversed, with the peak attributed to the *N*-oxide 1, a dioxide, being much smaller in the MS chromatogram than in the HPLC chromatogram (Figure 4). This is probably owing to the compound's inability to be protonated readily since it no longer possesses a basic centre.

The retention time of the peak owing to *N*-oxide 2 was found to be equivalent to the retention times obtained for the early eluting compound in the pholcodine samples (see Figure 2). Also, the mass spectral data was the same. Therefore, it was concluded that the early eluting compound and *N*-oxide 2 were identical.

Although the LC-MS experiment confirmed that one of the impurities was an *N*-oxide, it did not distinguish at which *N*-atom oxidation had occurred (i.e. the nitrogen (N) of the morphine structure or the nitrogen (N′) of the morpholino ring. To confirm which mono-*N*-oxide was present, the mono-*N*-oxide was separated from the di-*N*,*N*′-oxide by preparative HPLC.

Amounts of *N*-oxide 1 (88.7 mg) and *N*-oxide 2

(17.5 mg) were analysed by NMR spectroscopy. Hydrogen bearing carbon atoms and the corresponding protons were identified by tracing ^{13}C-^{1}H and ^{1}H-^{1}H coupling via HMQC and COSY experiments. The quaternary carbons were assigned unambiguously from 2-dimensional long-range correlation measurements (HMBC). Complete ^{1}H and ^{13}C signal assignments for pholcodine and the two pholcodine-*N*-oxides are detailed in Table 2.

N-Oxide 2

Comparison of the ^{1}H and ^{13}C NMR spectra (Table 2) of pholcodine and *N*-oxide 2 (Figure 5) showed that all of the signals owing to pholcodine were present in the ^{1}H spectrum and the ^{13}C spectrum of *N*-oxide 2. Only changes of the chemical shift values of the hydrogens and carbons in the vicinity of the nitrogen atom of the morphine residue of the molecule (H-14, H-9, H-10, H-16 and N-CH_3) were observed (Table 2). Marked changes had occurred for the signals owing to all carbon and hydrogen atoms (H-9, H-16 and N-CH_3) directly bonded to the nitrogen of the morphine ring. For pholcodine, the 9-CH resonates at approximately δ_H 3.3 (δ_c 59.0) and the 16-CH_2 resonates at approximately δ_H 2.4/2.5 (δ_c 46.5). In contrast, for the N-oxide 2, the 9-CH signal shifts to δ_H 3.9 (δ_C 76.0) and the 16-CH_2 resonates at δ_H 3.2/3.4 (δ_C 60.5) (Table 3). Also, the signal for the 17-CH_3 protons is shifted downfield from δ_H 2.40 (δ_C 43.2) in pholcodine to δ_H 3.41 (δ_C 58.7) in the N-oxide 2.

The 14-CH proton resonates at δ_H 3.95 (δ_C 35.0) in the pholcodine-*N*-oxide in comparison with δ_H 2.64 (δ_C 40.9) in pholcodine (Table 2). Only the 14-CH proton can be strongly deshielded by the oxygen of the nitrogen-*N*-oxide if the oxygen is on the same side of the heterocyclic ring as the 14-CH proton (Figure 5). This strongly confirms the formation of a pholcodine-mono-*N*-oxide with oxidation having taken place at the nitrogen of the morphine moiety.

N-Oxide 1

Analysis of the ^{1}H and ^{13}C NMR spectra (Table 1) of *N*-oxide 1 (Figure 5) showed the same changes of chemical shift values for the protons and carbons of 9-CH, 14-CH, 16-CH_2 and 17-CH_3 as already observed for pholcodine-*N*-oxide. Additionally, it was found that the 19-CH_2 protons were more deshielded than those in pholcodine and pholcodine-*N*-oxide, causing the 19-CH_2 protons to resonate at δ_H 2.72 (δ_C 57.9) in comparison with δ_H 3.66 (δ_C 71.3) in pholcodine.

The oxidation of the morpholino nitrogen atom (Figure 6) produced significant changes with the ^{1}H NMR signals of the protons of the morpholino ring. In pholcodine, the CH_2 groups at positions 20 and 23 and positions 21 and 22 are equivalent owing to the symmetrical nature of the essentially planar morpholino ring (Figure 6). The formation of an oxygen nitrogen

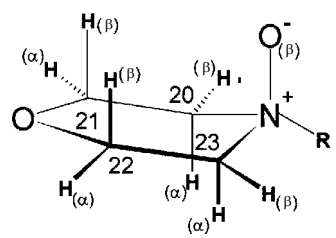

Figure 6 Chemical structure of the morpholino ring in pholcodine-di-*N*,*N*′-oxide.

Table 3 Summary of the levels of impurities found in the batches of pholcodine.

Sample No.	Morphine HCl Weight % (w/w) HPLC, MEKC, LC-MS	Impurity A Weight % (w/w) HPLC, MEKC, LC-MS	Pholcodine-*N*-oxide Area %[a] LC-MS	10-*S*-Hydroxy-pholcodine Area %[a] HPLC, MEKC, LC-MS	10-*R*-Hydroxy-pholcodine Area %[a] HPLC, MEKC, LC-MS
P1	0.02–0.04[b]	0.29–0.46	0.17	0.02–0.10	0.01–0.19
P2	0.05–0.08	0.04[c]	0.10	0.02–0.10	0.01–0.24
P3	0.06–0.07	0.04[c]	0.07	0.02–0.12	0.01–0.16
P4	–	0.95–1.39	0.08	0.01–0.05	0.12–0.37
P5	0.04–0.09	0.43–0.63	0.22	0.03–0.17	0.13–0.30
P6	0.01–0.03[d]	0.11–0.36	–	0.02–0.23	0.04–0.12
P7	0.01–0.02[d]	0.01–0.11[b]	–	0.03–0.27	0.02–0.14

[a]Peak area percentages of the PDA chromatograms of the LC-MS experiments; [b]not detected by MEKC; [c]only detected by LC-MS; [d]not detected by LC-MS.

bond abolishes this symmetry so that one face of the ring differs from the other. If the oxygen is considered to be on the β-face of the ring, then each proton can be categorized as α or β depending on its position relative to the oxygen. Protons that were on the same side as the oxygen were termed β protons, whereas protons facing away from the oxygen were called α protons (Figure 6).

The deshielding effect of the oxygen causes the signals for the β protons at C-20 and C-23 and at C-21 and C-22 to shift to δ_H 3.70 and δ_H 4.22, respectively (Table 2). Since the α protons at C-20 and C-23 and at C-21 and C-22 are less affected by the deshielding effect of the oxygen atom, their signals shifted downfield to a lesser extent than the neighbouring β protons. The δ_H value of the α protons of C-20 and C-23 was 3.32, and for the α protons of C-21 and C-22, it was 3.82 (Table 3). Thus, it would appear that the N′ atom was oxidized at the axial position forming the di-*N*,*N*′-oxide derivative of pholcodine with oxidation occurring at both tertiary nitrogen groups.

The observed changes of chemical shifts agreed with the differences in chemical shifts between the parent alkaloids and their *N*-oxides for morphine (Phillipson et al 1976). Also, the mass spectral data obtained confirmed the NMR findings.

Summary

LC-MS analysis of batches of pholcodine was able to answer some more of the questions raised by the HPLC and MEKC studies (Denk et al 2000b). The quantities of impurities found in the batches of pholcodine analysed by HPLC, MEKC and LC-MS are summarized in Table 3. Although impurities B and C (Figure 1) were not present in the samples, morphine HCl as well as impurity A were detected in some samples of pholcodine at levels up to 0.09% and 1.39% (w/w), respectively (Table 3). Furthermore, the presence of other unidentified compounds in these samples of pholcodine was confirmed.

An early eluting compound (Figure 2) in the LC-MS chromatograms was identified as pholcodine-*N*-oxide (Figure 4) by matching chromatographic and mass spectral data of a synthesized pholcodine-*N*-oxide standard. Pholcodine-*N*-oxide and pholcodine-di-*N*,*N*′-oxide standards (Figure 5) were obtained by the oxidation of pholcodine with m-CPBA.

LC-MS analysis also established that another impurity, ‘unknown 1’ was 10-*S*-hydroxy-pholcodine by matching it with synthesized 10-*S*-hydroxy-pholcodine, which was fully characterized by MS, ^{1}H and ^{13}C NMR spectrometry. It is strongly suspected that the peak eluting shortly before pholcodine (Figure 2) is owing to the *R* epimer of 10-hydroxy-pholcodine. Synthesis of the 10-*R* compound might be possible in future work.

References

Cole, R. B. (2000) Some tenets pertaining to electrospray ionisation mass spectrometry. *J. Mass Spectrom.* **35**: 763–772

Denk, O. M., Gray, A. I., Skellern, G. G., Watson, D. G. (2000a) Isolation and identification of three potential impurities of pholcodine bulk drug substance. *J. Pharm. Pharmacol.* **52**: 819–829

Denk, O. M., Watson, D. G., Skellern, G. G. (2000b) Micellar electrokinetic and high-performance liquid chromatographic determination of potential manufacturing impurities in pholcodine. *J. Chromatogr. A* **895**: 9–16

Ermer, J. (1998) The use of hyphenated LC-MS technique for characterisation of impurity profiles during drug development. *J. Pharm. Biomed. Anal.* **18**: 707–714

Goeroeg, S., Babjak, M., Balogh, G., Brlik, J., Csehi, A., Dravecz, F., Gazdag, M., Horvath, P., Lauko, A., Varga, K. (1997) Drug impurity profiling strategies. *Talanta* **44**: 1517–1526

Grewe, R. (1947) Synthetic drugs with morphine action. *Angew. Chem.* **59**: 194

Kromidas, S. (1997) *HPLC-Tips*. Verlag Hoppenstedt, Darmstadt, Germany

Phillipson, J. D., Handa, S. S., El-Dabbas, S. W. (1976) N-oxides of morphine, codeine and thebaine and their occurrence in *Papaver* species. *Phytochemistry* **15**: 1297–1301

Proksa, B. (1999) Separation of morphine and its oxidation products by capillary zone electrophoresis. *J. Pharm. Biomed. Anal.* **20**: 179–183

Rapoport, H., Masamune, S. (1955) 10-Hydroxymorphine. *J. Am. Chem. Soc.* **77**: 6359–6361

Stern, E. S. (1955) (2-Morpholinoethyl)-morphine. *British Patents* **722**: 467

USP (1990) *United States Pharmacopoeia XXII*. United States Pharmacopoeial Convention Inc., New York