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Short communication

# Determination of the dihydrocodeine metabolites, dihydromorphine and nordihydrocodeine, in hepatic microsomal incubations by high-performance liquid chromatography

Lynette C. Kirkwood[a,*], Roger L. Nation[a], Andrew A. Somogyi[b]

[a]*Centre for Pharmaceutical Research, School of Pharmacy and Medical Sciences, University of South Australia, North Terrace, Adelaide, SA 5000, Australia*

[b]*Department of Clinical and Experimental Pharmacology, University of Adelaide, Adelaide, SA 5005, Australia*

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**Abstract**

A high-performance liquid chromatographic assay for the oxidative metabolites of dihydrocodeine, nordihydrocodeine and dihydromorphine, formed in human liver microsomal incubations, is described. A simple solvent extraction followed by reversed-phase high-performance liquid chromatography with UV detection allows quantification of both metabolites in a single assay. Standard curve concentration ranges for dihydromorphine and nordihydrocodeine were 0.05–5 and 0.2–20 $\mu M$, respectively. Assay performance was assessed by intra- and inter-day accuracy and precision of quality control (QC) samples. The difference between the calculated and the actual concentration and the relative standard deviation were less than 15% at low QC concentrations and less than 10% at medium and high QC concentrations for both analytes. The method provides good precision, accuracy and sensitivity for use in kinetic studies of the oxidative metabolism of dihydrocodeine in human liver microsomes. © 1997 Elsevier Science B.V.

*Keywords:* Dihydrocodeine; Dihydromorphine; Nordihydrocodeine

## 1. Introduction

Dihydrocodeine (DHC) is a semisynthetic opioid that is widely used as a moderately potent analgesic and antitussive. Until recently, little was known about its metabolism and pharmacokinetics. Hufschmid et al. [1] identified DHC-6-glucuronide, nordihydrocodeine, dihydromorphine and nor-dihydromorphine as urinary metabolites in man. Current interest in DHC metabolism has focused on the potential involvement of cytochrome P4502D6 (CYP2D6), which is responsible for the sparteine/debrisoquine polymorphism, in the oxidative *O*-demethylation of DHC to the potent opioid, dihydromorphine [2].

Several methods for DHC analysis in plasma, blood or urine using radioimmunoassay, gas chromatography (GC) and high-performance liquid chromatography (HPLC) have been used for pharmacokinetic studies or forensic testing [3–10]. Fewer methods have been described for the analysis of metabolites of DHC. An HPLC method with electrochemical and UV detection was developed and applied by Ohno et al. [11] for the simultaneous

*Corresponding author.

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determination of DHC and its metabolites, nordihydrocodeine, dihydromorphine and DHC-6-glucuronide in dog plasma. Hufschmid et al. [1,12] measured the urinary metabolites of DHC in man by micellar electrokinetic capillary chromatography. The major urinary metabolite was DHC-6-glucuronide, which was excreted in similar quantities as those of the unchanged drug. Other metabolites, identified by comparison with authentic reference compounds, were nordihydrocodeine, dihydromorphine and nordihydromorphine. Hoffman et al. [13] and Fromm et al. [14] used GC–tandem mass spectrometry to quantify DHC and dihydromorphine in plasma and nordihydrocodeine and dihydromorphine in urine to determine differences in DHC metabolism following an oral dose of the drug to poor and extensive metabolisers of sparteine/debrisoquine [14].

We report here on an analytical method that involves simple solvent extraction followed by reversed-phase HPLC with UV detection for the quantification of the DHC metabolites, nordihydrocodeine and dihydromorphine, formed in human liver microsomal incubations. The method is suitable for investigating the cytochrome P450 enzymes involved in the oxidative metabolism of DHC in human liver microsomes.

## 2. Experimental

### 2.1. Chemicals

Dihydrocodeine tartrate was a gift from 3M Pharmaceuticals (Thornleigh, NSW, Australia). Traces of contaminants were removed by extracting DHC from an aqueous solution that had been adjusted to pH 13.6 with potassium hydroxide into diethyl ether. Pooled ether extracts of DHC were evaporated under nitrogen to produce a glassy residue, which was dissolved in phosphoric acid (0.002%). This produced chromatographically pure DHC. This solution was then diluted with 0.1 *M* phosphate buffer, pH 7.4, to suitable concentrations for addition to microsomal incubations. Dihydromorphine hydrochloride and nordihydrocodeine were synthesised and their structures authenticated by nuclear magnetic resonance spectroscopy as described previously [15]. Other materials were obtained from the following sources: cimetidine from Smith Kline and French Laboratories (Philadelphia, PA, USA); DL-isocitric acid (trisodium salt), isocitrate dehydrogenase type IV (EC 1.1.1.42), nicotinamide adenine dinucleotide phosphate (NADP) (potassium salt), L-cysteine hydrochloride (anhydrous), haemoglobin (Beef Type I) and haemin chloride (Equine Type III) from Sigma (St. Louis, MO, USA); sodium dihydrogen phosphate, potassium hydroxide, potassium chloride and magnesium chloride from Ajax Chemicals (Auburn, NSW, Australia); dichloromethane, 1-propanol, acetonitrile 190, methanol, ethylene diamine triacetic acid and sodium carbonate anhydrous from BDH (Poole, UK); phosphoric acid from Fluka (Buchs, Switzerland) and diethyl ether (anhydrous) from Rhône-Poulenc (Clayton South, Victoria, Australia). All chemicals and solvents were of analytical or HPLC grade, as appropriate.

### 2.2. Instrumentation

The analytical liquid chromatograph consisted of a Model LC-6A solvent delivery module (Shimadzu, Kyoto, Japan), a Model 712 WISP autoinjector (Waters, Milford, MA, USA), a Model SPD-6A variable wavelength UV absorbance detector (Shimadzu) and a Model C-R6A chromatopac integrator (Shimadzu).

A μBondapak $C_{18}$ guard column was positioned ahead of the 4 μm Nova-Pak phenyl 100×5 mm Radial-Pak cartridge (Waters). The cartridge was compressed in a Waters RCM 10×8 radial compression module and operated at ambient temperature (22°C). The single-pass mobile phase (acetonitrile–methanol–0.05% phosphoric acid in water (pH 2.2–2.4) 10:5:85, v/v) was pumped at 1 ml/min. This solvent was degassed immediately before use and sparged with ultra-high purity helium gas (CIG, St. Leonards, NSW, Australia) during operation. Compounds were detected at a wavelength of 210 nm.

### 2.3. Microsomal incubation

Microsomal fractions were prepared by differential centrifugation of human liver homogenates and aliquots were stored at −70°C until used [16]. Ethical approval was obtained from the Royal Adelaide Hospital Research Ethics Committee. The

oxidative metabolism of DHC in vitro was studied by incubation of the substrate (DHC) at concentrations of 50 to 20 000 or 24 000 μ*M* with microsomal protein (0.25 mg/ml) in a final volume of 200 μl containing an NADPH generating system (1 m*M* NADP, 1 unit/ml isocitrate dehydrogenase, 5 m*M* isocitric acid, 5 m*M* magnesium chloride) and 0.1 *M* sodium phosphate buffer (pH 7.4). The wide range of substrate concentrations was required in incubated samples to define the $K_m$ and $V_{max}$ values for both metabolic pathways. The mixture was incubated at 37°C for 30 min and the reaction was stopped by adding 250 μl of a 20% aqueous solution of sodium carbonate, vortex-mixing and placing the tubes on ice.

### *2.4. Sample preparation*

A 25-μl volume of internal standard solution (cimetidine, 15 mg/l in water), 50 μl of cysteine hydrochloride (200 μ*M*) and 3 ml of extracting solvent (1-propanol–dichloromethane, 1:9, v/v) were added to the 10 ml polypropylene sample tube containing the incubation mixture and the sodium carbonate solution. Cysteine reduced the non-enzymatic formation of nordihydrocodeine from DHC to a minimal and consistent background (see Section 3). Each sample was mixed on a mechanical vortex for 5 min and then centrifuged for 3 min (4000 *g*). The aqueous phase was aspirated and then the organic layer was transferred to a clean glass tube and evaporated to dryness at 50°C under vacuum (Buchler Instruments, Kansas City, MO, USA). The dried residue was reconstituted in 200 μl of 0.05% phosphoric acid and 2–120 μl were injected onto the HPLC column. The injection volume was varied to obtain adequate resolution of the substrate peak from metabolite and internal standard peaks over the wide substrate concentration range required in incubated samples.

### *2.5. Calibration, precision, accuracy and extraction efficiency*

Standards containing dihydromorphine and nordihydrocodeine were prepared by adding all of the constituents of the incubation mixture (excluding the substrate) to tubes containing 25 μl of internal standard solution, 50 μl of cysteine hydrochloride (200 μ*M*), 250 μl of sodium carbonate (20% aqueous solution) and aliquots of solutions in 0.1 *M* sodium phosphate buffer (pH 7.4) of dihydromorphine and nordihydrocodeine, such that concentrations of 0.05–5 and 0.2–20 μ*M* were achieved, respectively. Sample preparation of standards was the same as for incubated samples. Calibration curves were constructed for each of the compounds as the ratio of the peak area of the compound to that of the internal standard, and linear least-squares regression analysis weighted according to the reciprocal of peak area ratio squared was performed to determine the slope, intercept and coefficient of determination (Blackwell Regression, Blackwell Scientific Publications, Osney Mead, Oxford, UK).

Quality control samples were prepared using solutions of dihydromorphine and nordihydrocodeine in 0.1 *M* sodium phosphate buffer (pH 7.4) from weighings independent of those used for preparing the calibration standards. Final concentrations of low, medium and high quality control samples were 0.2, 1 and 4 μ*M* for dihydromorphine and for nordihydrocodeine they were 0.8, 4 and 16 μ*M*, respectively. These samples were prepared on the day of analysis in the same way as calibration standards. The performance of the method was assessed by analysis of eighteen quality control samples (six each of low, medium and high concentrations) on a single assay day to determine the intra-day accuracy and precision and analysis of six quality control samples (two each of low, medium and high concentrations) on each of eighteen consecutive assay days to determine inter-day accuracy and precision.

Extraction efficiency was assessed at each of the concentrations of the quality control samples. The peak areas (adjusted for solvent volume contraction, volume of solvent transfer and injection volume) of nordihydrocodeine, dihydromorphine and internal standard extracted from the unincubated microsomal mixture were compared to those produced by direct injections of the solutions of these compounds in 0.05% phosphoric acid.

### *2.6. Selectivity*

The selectivity of the method was tested by determining if interfering chromatographic peaks were present in human liver microsomes from nine

donors and in microsomal incubations of ten cytochrome P450 chemical inhibitors; troleandomycin, erythromycin, furafylline, α-naphthoflavone, sulfaphenazole, *S*-mephenytoin, quinine, quinidine, dithiocarb (diethyldithiocarbamate) and coumarin.

### *2.7. Confirmation of metabolite peak purity by photodiode array detection*

The UV spectra of authentic samples of dihydromorphine and nordihydrocodeine were compared to spectra of metabolite peaks produced in the microsomal incubation mixture using photodiode array detection (Model SPD-M10A, Shimadzu) with SPD-MXA data processing (Shimadzu). This analysis was also performed on a sample of the extracted unincubated microsomal mixture that had been spiked with DHC, in which a peak at the retention time of nordihydrocodeine was detected.

## 3. Results and discussion

Human liver microsomes were incubated with DHC in the presence of an NADPH-generating system and, after solvent extraction, the reaction mixture was analysed by reversed-phase HPLC. Chromatograms resulting from the analysis of a substrate-free unincubated microsomal mixture (blank), an unincubated microsomal mixture spiked with 1000 μ*M* DHC, an unincubated microsomal mixture spiked with 1 μ*M* dihydromorphine, 4 μ*M* nordihydrocodeine and internal standard (cimetidine), and a microsomal incubation of 1000 μ*M* DHC with internal standard added post-incubation are shown in Fig. 1. The microsomal reaction produced two major peaks that were detected by HPLC with UV detection with identical retention times to those of authentic samples of nordihydrocodeine and dihydromorphine. Using photodiode array detection, peak purity indices of the metabolites were greater than 0.998 and the UV spectra second derivatives had a high degree of similarity to those of the corresponding authentic reference samples. Under the chromatographic conditions described, the retention times for dihydromorphine, cimetidine (internal standard), nordihydrocodeine and DHC were 3.4, 4.0, 4.9 and 5.9 min, respectively. The total chromatography run time was 15 min.

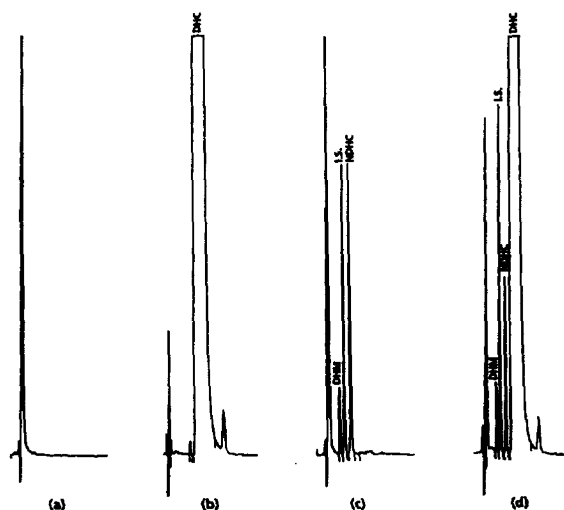


Fig. 1. Representative chromatograms from a substrate-free unincubated microsomal mixture (blank) (a); an unincubated microsomal mixture spiked with 1000 μ*M* dihydrocodeine (b); an unincubated microsomal mixture spiked with 1 μ*M* dihydromorphine, 4 μ*M* nordihydrocodeine and internal standard (cimetidine) (c) and a microsomal incubation of 1000 μ*M* dihydrocodeine containing internal standard added post-incubation and the metabolites, dihydromorphine (1.0 μ*M*) and nordihydrocodeine (2.2 μ*M*) formed during incubation (d). DHC= dihydrocodeine; DHM=dihydromorphine; NDHC= nordihydrocodeine and I.S.=internal standard.

In incubations of rat liver microsomes, a metabolite peak was tentatively identified as nordihydromorphine by spectral analysis using photodiode array detection (data not shown). This secondary metabolite eluted before dihydromorphine and was well resolved from it. It was not detected in human liver microsomal incubations that had a much lower metabolic capacity.

After extraction of the unincubated microsomal mixture spiked with DHC, a small peak (below the limit of quantification) corresponding to nordihydrocodeine was seen, as illustrated in Fig. 1b. Identification of the compound as nordihydrocodeine was supported by comparison of the UV spectrum and its second derivative with those of authentic nordihydrocodeine. Nordihydrocodeine was not detected after direct injection of DHC. The compound arose from the substrate during extraction and was

dependent on the concentration of microsomal protein added. This phenomenon was also demonstrated with the addition of haemin and haemoglobin to the unincubated mixture instead of microsomes (results not shown). Haemin has been reported to enhance catalytic activity in microsomes produced from a cultured human cell line [17]. This effect was reproduced by Geertsen et al. [18] who noted that, under certain conditions, haemin/dimethylsulfoxide produced a shift in the metabolic profile of methoxyphenamine in vitro to that of the *N*-demethylated product. Although the action of haemin/dimethylsulfoxide may be more complex in this setting, nonenzymatic catalytic activity may contribute to the "enzyme induction" proposed by these authors. In the present assay, nordihydrocodeine formation could be minimised to a consistent background by the addition of the reducing agent, cysteine, prior to extraction, but it could not be eliminated. The effect of cysteine was concentration-dependent. Its mechanism may involve the reduction of iron within the haem complex of P450 from the ferric to the ferrous state. Hence, electron transfer from the methyl tertiary amine group of DHC to the haem complex, which leads to demethylation of the substrate, is inhibited. The amount of nordihydrocodeine formed during extraction of the unincubated samples was dependent on substrate concentration and it remained below 15% of that measured in incubated samples. For enzyme kinetic analysis, the background nordihydrocodeine measured in unincubated DHC-spiked samples was subtracted from nordihydrocodeine concentrations measured in incubated samples.

Calibration curves were linear over the concentration range used for both analytes with mean $r^2$ values being greater than 0.995. The mean $y$-intercepts for dihydromorphine and nordihydrocodeine were 0.003 and 0.005, respectively, and the relative standard deviation (R.S.D.) of the slopes was less then 10% for each of the sets of eighteen calibration curves constructed on independent assay days. The accuracy and precision of quality control samples are shown in Table 1. The differences between the calculated and the actual concentration and the R.S.D. were less than 15% at low quality control (QC) concentrations and less than 10% at medium and high QC concentrations for both analytes.

Extraction efficiency was assessed at low ($n$=6), medium ($n$=6) and high ($n$=6) QC concentrations for each of the analytes and for the internal standard. There was no evidence of concentration dependence for any of the compounds. The overall mean (±S.D.) percentage extraction efficiencies ($n$=18) for dihydromorphine, nordihydrocodeine and for the internal standard were 98.6±4.5, 107.6±3.3 and 106.0±2.7, respectively.

The method was used to study the kinetics of the oxidative metabolism of DHC in human liver microsomes from donors of the CYP2D6 (sparteine/debrisoquine)-poor and extensive metaboliser genotypes. Metabolite formation rate versus substrate concentration curves for dihydromorphine and nordihydrocodeine in human liver microsomes from an

Table 1
Intra- and inter-day (eighteen consecutive assays) accuracy and precision, assessed by mean calculated concentration and R.S.D. of quality control (QC) samples

| | Actual concentration (μ*M*) | Calculated concentration (μ*M*) | R.S.D. | Actual concentration (μ*M*) | Calculated concentration (μ*M*) | R.S.D. | Actual concentration (μ*M*) | Calculated concentration (μ*M*) | R.S.D. |
|---|---|---|---|---|---|---|---|---|---|
| | Low QC intra-day ($n$=6) | | | Medium QC intra-day ($n$=6) | | | High QC intra-day ($n$=6) | | |
| DHM | 0.2 | 0.203 | 3.65 | 1 | 0.994 | 2.17 | 4 | 3.91 | 2.10 |
| NDHC | 0.8 | 0.806 | 3.48 | 4 | 3.92 | 2.20 | 16 | 15.70 | 1.81 |
| | Low QC inter-day ($n$=36) | | | Medium QC inter-day ($n$=36) | | | High QC inter-day ($n$=36) | | |
| DHM | 0.2 | 0.202 | 10.13 | 1 | 1.02 | 6.84 | 4 | 3.91 | 5.48 |
| NDHC | 0.8 | 0.837 | 9.68 | 4 | 4.04 | 4.64 | 16 | 15.41 | 7.25 |

DHM=dihydromorphine; NDHC=nordihydrocodeine; R.S.D.=100(S.D./mean calculated concentration).

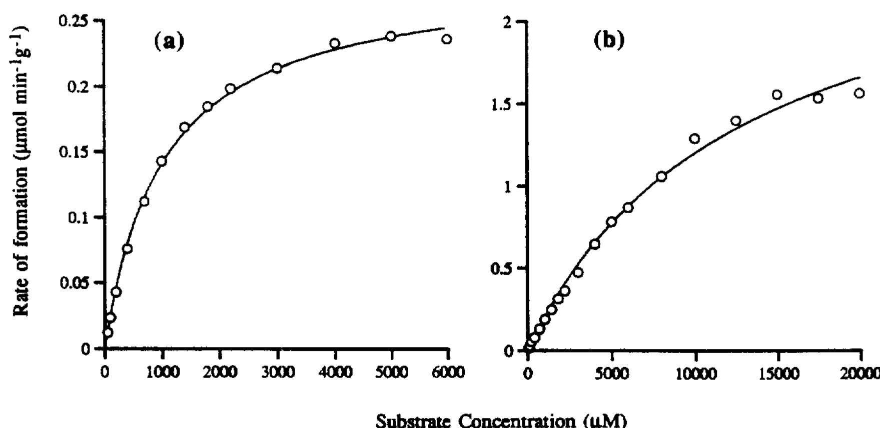


Fig. 2. Rate of formation of dihydromorphine (a) and nordihydrocodeine (b) versus substrate concentration in extensive metaboliser human liver microsomes (line represents modelled data derived from the Michaelis–Menten single enzyme equation).

extensive metaboliser donor are illustrated in Fig. 2. Good selectivity of the method has been demonstrated. Microsomal samples from nine donors and incubations with ten cytochrome P450 chemical inhibitors produced no interfering chromatographic peaks.

In summary, we report an HPLC method for the quantification of two oxidative metabolites of DHC, nordihydrocodeine and dihydromorphine. Non-enzymatic *N*-demethylation of the substrate was observed and was minimised by the addition of cysteine prior to extraction of samples. This interesting phenomenon may occur with microsomal incubation assay methods involving solvent extraction of other methyl tertiary amines that undergo demethylation. The assay is simple, inexpensive, precise and accurate, with adequate sensitivity and selectivity for studies of DHC metabolism in human liver microsomes. It has been used to investigate the role of specific cytochrome P450s in the oxidative pathways of DHC metabolism in human liver microsomes.

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## References

[1] E. Hufschmid, R. Theurillat, U. Martin, W. Thormann, J. Chromatogr. B. 668 (1995) 159.
[2] L.C. Kirkwood, M.G. Venning, R.L. Nation, G. Reynolds, A.A. Somogyi, L.N. Sansom, Pharm. Sci. 1 (1995) 573.
[3] R.A. Seymour, SAAD Digest. 5 (1983) 172.
[4] J.N. Barnes, A.J. Williams, M.J.F. Tomson, P.A. Toseland, F.J. Goodwin, Br. Med. J. 290 (1985) 740.
[5] K.R. Bedford, P.C. White, J. Chromatogr. 347 (1985) 398.
[6] D.A. Cowan, G. Woffendin, A. Noormohammadi, J. Pharm. Sci. 77 (1988) 606.
[7] K.N. Davies, C.M. Castleden, A. McBruney, C. Jagger, Eur. J. Clin. Pharmacol. 37 (1989) 375.
[8] G.R. Nakamura, W.J. Stall, R.D. Meeks, J. Forensic Sci. 32 (1987) 535.
[9] F.J. Rowell, R.A. Seymour, M.D. Rawlins, Eur. J. Clin. Pharmacol. 25 (1983) 419.
[10] D.P. Vaughan, A.H. Beckett, J. Pharm. Pharmacol. 25 (1973) 104.
[11] M. Ohno, Y. Shiono, M. Konishi, J. Chromatogr. B. 654 (1994) 213.
[12] E. Hufschmid, R. Theurillat, C.H. Wilder-Smith, W. Thormann, J. Chromatogr. B. 678 (1996) 43.
[13] U. Hofmann, M.F. Fromm, S. Johnson, G. Mikus, J. Chromatogr. B. 663 (1995) 59.
[14] M.F. Fromm, U. Hofmann, E.U. Griese, G. Mikus, Clin. Pharmacol. Ther. 58 (1995) 374.
[15] L.C. Kirkwood, R.L. Nation, G.D. Reynolds, A.A. Somogyi, L.N. Sansom, Pharm. Sci. 2 (1996) 1.
[16] U.M. Zanger, F. Vilbois, J.P. Hardwick, U.A. Meyer, Biochemistry 27 (1988) 5447.
[17] B.W. Penman, J. Reece, T. Smith, C.S. Yang, H.V. Gelboin, F.J. Gonzalez, C.L. Crespi, Pharmacogenetics 3 (1993) 28.
[18] S. Geertsen, B.C. Foster, D.L. Wilson, T.D. Cyr, W. Casley, Xenobiotica 25 (1995) 895.