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Quantification of the O- and N-demethylated metabolites of hydrocodone and oxycodone in human liver microsomes using liquid chromatography with ultraviolet absorbance detection ☆

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Abstract

High-performance liquid chromatographic assays for the O- and N-demethylated oxidative metabolites of hydrocodone and oxycodone formed in human liver microsomes are described. A solvent–solvent extraction/re-extraction procedure followed by reversed-phase HPLC with UV detection at 210 nm allows for the quantification of hydromorphone, norhydrocodone, oxymorphone and noroxycodone. Calibration curve concentration ranges were 0.63–400 μ*M* (0.18–114 μg/ml) and 1.25–400 μ*M* (0.36–114 μg/ml) for hydromorphone and norhydrocodone, respectively and 0.13–20 μ*M* (0.04–6.03 μg/ml) and 1–200 μ*M* (0.30–60 μg/ml) for oxymorphone and noroxycodone, respectively. Assay performance was determined by intra- and inter-assay precision and inaccuracies for quality control samples and was <15% for all metabolites at each quality control concentration. These methods provide good precision, accuracy and sensitivity for use in in vitro kinetic studies investigating the oxidative metabolism of hydrocodone and oxycodone in human liver microsomes.

Keywords: Norhydrocodone; Hydromorphone; Noroxycodone; Oxymorphone

1. Introduction

The opioids hydrocodone and oxycodone have been used for decades as analgesics (hydrocodone and oxycodone) and antitussives (hydrocodone) [1,2]. Their chemical structures are related and they share similar oxidative metabolic pathways to codeine and dihydrocodeine (Fig. 1). The major differences to codeine in their structure are that hydrocodone and oxycodone have a ketone group at C6 instead of a hydroxyl group and, a single bond between C7 and C8. Codeine's O-demethylation to morphine is important for its analgesic effect [3] whereas for hydrocodone and oxycodone, it is still uncertain whether their O-demethylated metabolites

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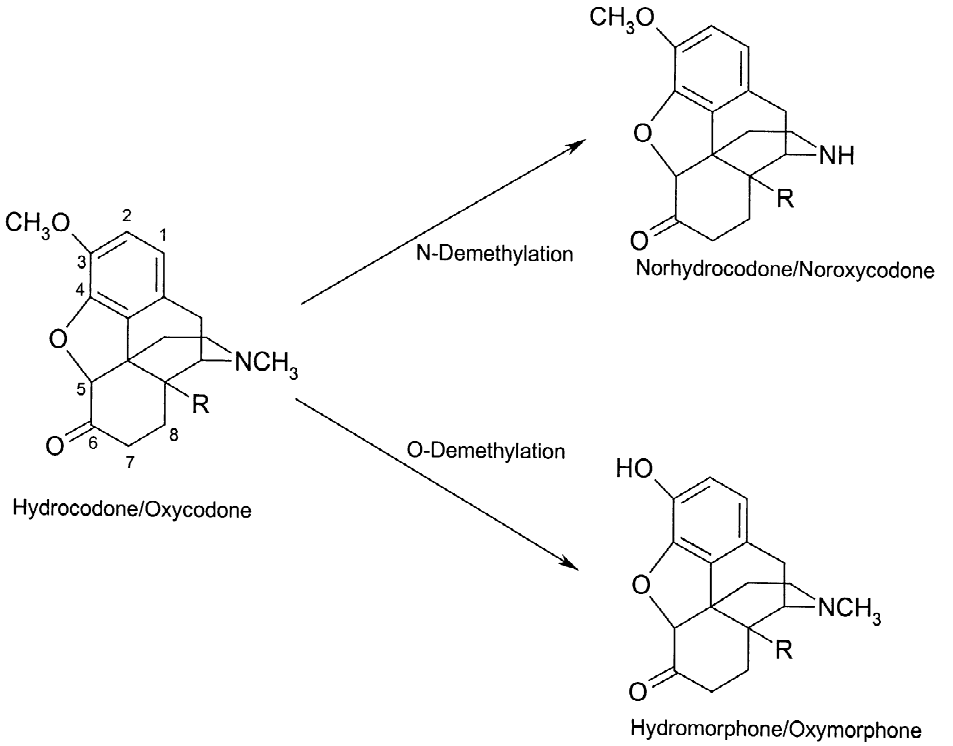

Fig. 1. Primary oxidative metabolic pathway for hydrocodone (R = H) and oxycodone (R = OH). Codeine (R = H) and dihydrocodeine (R = H) have an hydroxyl group at C6, instead of a ketone. Codeine also has a double bond between C7 and C8.

are important for activity. The cytochrome P450 (CYP) isoforms involved in the oxidative metabolism of hydrocodone and oxycodone in vitro have not been well characterised, especially the N-demethylated pathway, mainly due to the lack of suitable analytical methods. In contrast, there is considerable in vivo evidence that CYP2D6 is involved in their O-demethylation [4–6].

Several different techniques have been used to quantify hydrocodone, oxycodone and in some cases, their O- and N-demethylated metabolites in biological media, with the vast majority being for plasma concentrations of parent drug only. These include radioimmunoassay [7], gas–liquid chromatography (GLC) [8], thin-layer chromatography [9], capillary GLC [10] and various GC–MS assays [11–13]. The GC–MS assays offer good selectivity and sensitivity, but use expensive equipment which may not be readily available in some laboratories. There are also a number of urine screening and forensic assays for hydrocodone, oxycodone and other common opiates in hair, sweat and nail samples [14–18]. HPLC methods have been described and involve electrochemical detection (ECD) with liquid–liquid or solid-phase extraction for oxycodone in plasma [19,20]. Otton et al. [4,21] described methods for the determination of the O-demethylated metabolites of hydrocodone (hydromorphone) and oxycodone (oxymorphone) in human liver microsomes using HPLC with ECD. These two HPLC–ECD methods used ion-pairing agents in the mobile phase to achieve adequate separation. ECD can be technically difficult and is not as commonly available as UV instrumentation. In addition, ECD is mainly used to detect the O-demethylated metabolites but not the N-demethylated metabolites since the methoxyl group is more difficult to oxidise. In addition, the use of ion-pairing agents in the mobile phase usually requires long stabilisation times. Currently there are no published methods for the quantification of the N-demethylated metabolites of hydrocodone (norhydrocodone) and oxycodone (noroxycodone) in

human liver microsomes. These metabolites are likely to be the quantitatively major oxidative metabolites of these two opioids in humans.

We describe an analytical method that involves a solvent–solvent extraction/re-extraction procedure followed by reversed-phase HPLC with UV detection to quantify norhydrocodone, hydromorphone, noroxycodone and oxymorphone in human liver microsomes. The method is suitable for investigating the in vitro enzyme kinetics and cytochrome P450 enzymes involved in the formation of the metabolites in human liver microsomes.

2. Experimental

2.1. Chemicals

Hydrocodone base, norhydrocodone base and norhydromorphone base were obtained from Dr. S. Hosztafi (ICN Alkaloida, Tiszavasvári, Hungary). Noroxycodone and oxymorphone hydrochloride were obtained from Du Pont (Wilmington, DE, USA). Oxycodone hydrochloride, hydrocodone bitartrate, noroxymorphone base, hydromorphone hydrochloride, sodium perchlorate ($NaClO_4$), D,L-isocitric acid (trisodium salt) and isocitrate dehydrogenase (NADP, type IV) were purchased from Sigma (St. Louis, MO, USA). Nicotinamide adenine dinucleotide phosphate disodium salt (NADP-Na_2) was obtained from Merck (Darmstadt, Germany). Sodium hydroxide (NaOH), orthophosphoric acid, hydrochloric acid (HCl) and magnesium chloride ($MgCl_2$) were obtained from Ajax (Auburn, Australia). Sodium carbonate (Na_2CO_3), disodium hydrogen orthophosphate (Na_2HPO_4) and potassium dihydrogen orthophosphate (KH_2PO_4) were purchased from Merck (Kilsyth, Australia). Acetonitrile, dichloromethane (HiPerSolv for HPLC™) and methanol were obtained from BDH (Poole, UK). Milli-Q (Millipore, Bedford, MA, USA) water was used in all buffer and mobile phase preparations. The following specific CYP chemical inhibitors were used to assess the selectivity of the method: sulphaphenazole, troleandomycin, diethyldithiocarbamate, coumarin, chlorzoxazone (Sigma), furafylline, S-mephenytoin (Ultrafine Chemicals, Manchester, UK), ketoconazole (Janssen, Beerse, Belgium), dextromethorphan (Roche, Sydney, Australia), quinidine sulphate (Merck) and omeprazole (donated by Dr. Michael Ching, Department of Medicine, Repatriation Hospital, Melbourne, Australia). All chemicals and solvents were of analytical or HPLC grade, as appropriate.

2.2. Instrumentation

The reversed-phase HPLC system comprised a pump (LC-6A), autoinjector (SIL-9A) and integrator (C-R6A) all from Shimadzu (Kyoto, Japan) and a UV spectrophotometer (UVIDEC-100-V) Jasco (Tokyo, Japan). The stationary phase consisted of a precolumn cartridge (7.5×4.6 mm, 5 μm, Alltima™ C_{18}, Alltech, Deerfield, IL, USA) positioned ahead of a Platinum EPS™ C_{18} (53×7 mm, 3 μm) Rocket™ column (Alltech). Separation of the compounds of interest was achieved with a mobile phase of 1.4% acetonitrile (hydrocodone metabolites) or 1.0% acetonitrile (oxycodone metabolites), 20 m*M* KH_2PO_4 and 5 m*M* $NaClO_4$ adjusted to pH 3.0 with orthophosphoric acid. The one-pass mobile phase was filtered and subsequently sonicated for 15 min. The flow-rate was 3.0 ml/min with the absorbance measured at 210 nm at room temperature.

2.3. Microsomal incubations

Microsomal fractions were prepared by differential centrifugation of human liver homogenates [22] and stored at −80 °C until used. Ethics approval was obtained from the Royal Adelaide Hospital Ethics Committee and patients undergoing partial hepatectomy gave written consent for hepatic tissue to be removed.

Each microsomal incubation sample contained 175 μl of hydrocodone or oxycodone substrate made up in incubation buffer (0.1 *M* Na_2HPO_4, pH 7.4), 50 μl microsomal protein (200 μg of microsomal protein for hydrocodone substrate conditions, 125 μg for oxycodone substrate conditions) and 25 μl nicotinamide adenine dinucleotide phosphate (NADPH) regenerating system (1 m*M* NADP, 1 unit/ml isocitrate dehydrogenase, 5 m*M* D,L-isocitric acid, 5 m*M* magnesium chloride; all as final concentrations in incubation sample). Microsomal samples were incubated at 37 °C in a shaking water bath (hydrocodone

substrate conditions, 45 min; oxycodone substrate conditions, 60 min). These incubation times and microsomal protein amounts were validated to ensure linearity of metabolites' formation and are the subject of a further communication. Incubations were carried out in 10-ml flat-bottom polypropylene tubes (Techno-plas, Adelaide, Australia). The reaction was stopped by the addition of sodium carbonate (300 μl, 20% Na_2CO_3).

2.4. Sample preparation

For the hydrocodone metabolites assay, the internal standard (I.S.), noroxycodone, was contained in the sodium carbonate stop solution at a concentration of 5.5 μ*M* (1.67 μg/ml). For the oxycodone metabolites assay, 70 μl of the I.S. hydromorphone (35 μ*M*; 10 μg/ml) was added to the incubation mixture after the stop solution. Dichloromethane (4 ml) was added to the tubes followed by rotary mixing (10 min) and centrifugation (10 min, 2000 *g*). The aqueous layer was aspirated to waste and the organic phase was transferred to clean 10-ml tubes containing 0.1 *M* HCl (200 μl). The samples were rotary mixed and centrifuged as above and a 15–100-μl volume of the upper aqueous phase was injected onto the HPLC system.

2.5. Preparation of calibration and quality control samples

Calibration standards and quality control incubations contained aqueous solutions of the hydrocodone or oxycodone metabolites (50 μl), nonfunctional microsomal protein (50 μl) and incubation buffer (0.1 *M* Na_2HPO_4, pH 7.4) to a final incubation volume of 250 μl. NADPH regenerating system was omitted from standards and quality control samples to avoid any possible metabolism of the analytes. Calibration curves consisted of ten standards for hydromorphone (0.63–400 μ*M* final concentrations) and nine standards for norhydrocodone (1.25–400 μ*M*) for the hydrocodone metabolites assay. For the oxycodone metabolites assay, the calibration curve consisted of nine standards for oxymorphone (0.13–20 μ*M*) and noroxycodone (1–200 μ*M*).

Quality control samples were prepared from independent weighings of the metabolites from those used for the calibration standards. Incubation concentrations were 4, 40 and 160 μ*M* for the low, medium and high quality controls, respectively, for the hydrocodone metabolites assay. For the oxycodone metabolites assay, they were 0.25, 1 and 5 μ*M* for oxymorphone and 2.5, 10 and 50 μ*M* for noroxycodone. Quality control samples were used in duplicate with each assay. After the addition of sodium carbonate stop solution, the standards and quality controls were extracted as described above. All experimental samples were assayed with calibration standards and duplicate quality control samples.

2.6. Assay performance

To determine intra-day accuracy and precision of the assays, six replicates of each quality control sample and the lowest calibration standard were analysed in one assay. Inter-day accuracy, precision and limit of quantification were determined by analysis of duplicates of each quality control sample and one low calibration standard on 6 different assay days. The lower limit of quantification (LOQ) was determined as the lowest calibration standard that produced a precision of $<20\%$ and an inaccuracy of $<\pm 20\%$.

Extraction efficiencies were assessed for all metabolites and internal standards at all three quality control concentrations (hydrocodone metabolites, $n=4$; oxycodone metabolites, $n=6$). Peak heights obtained from the extracted quality controls (adjusted for volume of solvent transfer and injection volume) were compared to those produced by direct injection of solutions of these compounds in 0.1 *M* HCl.

2.7. Selectivity

Assay selectivity was tested by determining whether chromatographic interference was obtained in microsomal incubations containing the NADPH regenerating system and the following commonly used CYP isoform-specific chemical inhibitors: sulphaphenazole (CYP2C9), troleandomycin (CYP3A4), diethyldithiocarbamate (CYP2E1), coumarin (CYP2A6), chlorzoxazone (CYP2E1), furafylline (CYP1A2), S-mephenytoin (CYP2C19), keto-

conazole (CYP3A4), dextromethorphan (CYP2D6), quinidine (CYP2D6) and omeprazole (CYP2C19). The secondary metabolites, norhydromorphone and noroxymorphone, were also tested for formation and possible chromatographic interference in microsomal incubations.

2.8. Data analysis

Peak height ratios were calculated for each of the metabolites by dividing each peak height obtained from the chromatographic integrator by that of the internal standard. The equation to the line of best fit and coefficient of determination (r^2) for each calibration curve was calculated using linear regression analysis (weighting $1/y^2$) of peak height ratios plotted against nominal concentrations (GRAPHPAD PRISM V3.02, GraphPad Software, San Diego, CA, USA). Accuracy (as %) was determined as [(calculated concentration)/(nominal concentration)]$\times$100, for each calibration and quality control sample and inaccuracy determined as accuracy$-$100. The coeffi-

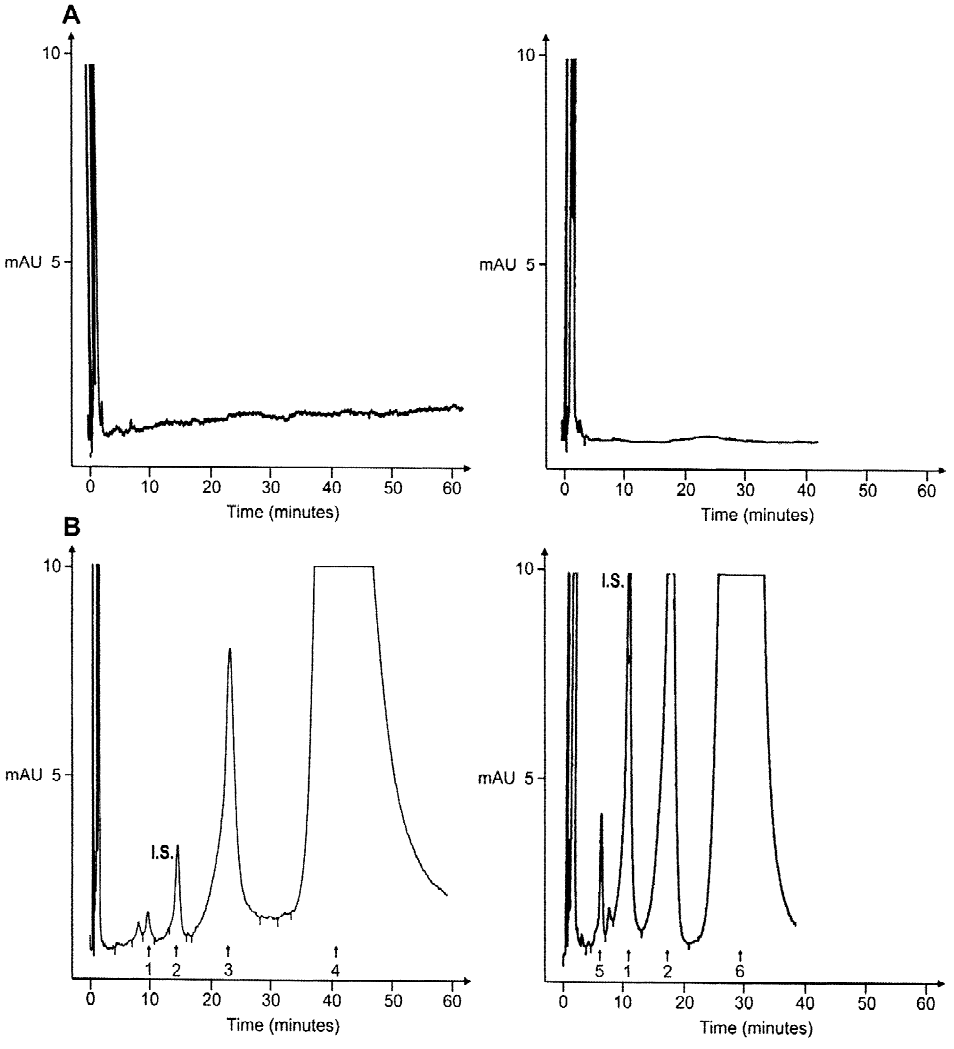

Fig. 2. (A) Chromatograms obtained from blank microsomal incubations (HC conditions, top left; OC conditions, top right). (B) Chromatograms obtained from incubations of 1.0 m*M* hydrocodone (left) and 300 μM oxycodone (right). 1 = hydromorphone, 2 = noroxycodone, 3 = norhydrocodone, 4 = hydrocodone, 5 = oxymorphone, 6 = oxycodone. I.S. = internal standard.

cient of variation, determined as [(standard deviation)/(arithmetic mean)]×100, was used as the index of precision.

3. Results and discussion

Chromatograms obtained from blank microsomal extractions are shown in Fig. 2A, and the oxidative metabolites of hydrocodone and oxycodone formed by human liver microsomes are shown in Fig. 2B. The chromatograms for hydrocodone and oxycodone incubations contained four peaks of interest: (i) O-demethylated metabolite hydromorphone (10 min) or oxymorphone (7 min); (ii) I.S. noroxycodone (15 min) or hydromorphone (12 min); (iii) N-demethylated metabolite norhydrocodone (23 min) or noroxycodone (17 min) and (iv) substrate hydrocodone (40 min) or oxycodone (30 min), respectively. All peaks were well resolved, with no interfering peaks. The run times for both methods were within 60 min.

The calibration curves for the hydrocodone and oxycodone metabolites' assays were linear over the range used. The mean slopes±standard deviation (SD) and coefficients of determination (r^2) were 0.078±0.003, 0.995; 0.136±0.004, 0.994; 0.136±0.008, 0.997 and 0.348±0.016, 0.996 ($n=6$) for norhydrocodone, hydromorphone, noroxycodone and oxymorphone, respectively. The intra- and inter-assay precision and accuracy data for the quality controls and lowest calibration standards are shown in Table 1. Precision and inaccuracies were <15% for all quality control samples and <20% for the lowest calibration standards. The limit of quantification was 0.63 μ*M* for hydromorphone, 1.25 μ*M* for norhydrocodone, 1.0 μ*M* for noroxycodone and 0.13 μ*M* for oxymorphone.

Extraction efficiencies were evaluated at low, medium and high quality control concentrations for each metabolite and the internal standard (hydrocodone metabolites assay $n=4$; oxycodone metabolites assay $n=6$). For the hydrocodone metabolites assay, the overall extraction efficiencies were: hydromorphone 75±7%, norhydrocodone 73±7% and noroxycodone 82±3% ($n=12$); for the oxycodone metabolites assay, the extraction efficiencies were: oxymorphone 95±8%, noroxycodone 94±5% and hydromorphone 75±4% ($n=18$). No concentration-dependent extraction was observed.

Table 1
Validation data for the HPLC assay of O- (hydromorphone and oxymorphone) and N- (norhydrocodone and noroxycodone) demethylated metabolites of hydrocodone and oxycodone, respectively in human liver microsomes. Data are presented as % accuracy±% CV; $n=6$ for all

	LQC	MQC	HQC	LOQ
Intra-assay				
Norhydrocodone	110±6	98±2	108±5	93±6
Hydromorphone	109±4	100±1	109±5	100±2
Noroxycodone	100±4	103±3	112±5	115±7
Oxymorphone	103±14	102±4	107±6	119±8
Inter-assay				
Norhydrocodone	108 ±7	98±4	107±6	109±5
Hydromorphone	114±4	99±4	109±6	106±4
Noroxycodone	93±8	100±5	105±7	116±5
Oxymorphone	92±9	99±7	103±8	115±10

Low quality control (LQC): NHC, HM, 4.0 μ*M*; NOC, 2.5 μ*M*; OM, 0.25 μ*M*. Medium quality control (MQC): NHC, HM, 40.0 μ*M*; NOC, 10.0 μ*M*; OM, 1.0 μ*M*. High quality control (HQC): NHC, HM, 160.0 μ*M*; NOC, 50.0 μ*M*; OM, 5.0 μ*M*.
Limit of quantification (LOQ): NHC, 1.25 μ*M*; HM, 0.63 μ*M*; NOC, 1.0 μ*M*; OM, 0.13 μ*M*.
NHC, norhydrocodone; HM, hydromorphone; NOC, noroxycodone; OM, oxymorphone.

Noroxycodone's extraction efficiency was found to be dependent on the age and previous exposure to air of the extraction solvent, dichloromethane. After using a previously opened and approximately half full bottle of dichloromethane (stored at 4 °C for approximately 1 year), noroxycodone's extraction efficiency decreased in a concentration-dependent manner (100 μ*M*, 97%; 60 μ*M*, 72%; 20 μ*M*, 41% and 10 μ*M*, 20%). Studies by Cone et al. [23] with codeine revealed that the metabolite norcodeine reacts with impurities in the chloroform-based solvent used for its extraction to form drug artifacts such as norcodeine ethyl carbamate and norcodeine carbamoyl chloride. The secondary amine of norcodeine is more reactive to the solvent breakdown products than the tertiary amine of the parent drug. Factors influencing artifact formation were found to be the preservative used, age and exposure to air and light of the solvent. In some cases, complete loss of norcodeine to drug artifact was observed. It is likely that the secondary amine of noroxycodone is reacting with the impurities in dichloromethane, thus resulting in the observed concentration-dependent extraction efficiencies. The extraction efficiency was

restored to normal by washing dichloromethane in equal volumes of acid (0.1 *M* HCl), followed by base (0.1 *M* NaOH) and then Milli-Q water.

Peak fronting and tailing which are associated with phenanthrenes that have a ketone group at C6 has been the topic of previous research. Brogel et al. [24] found that changing the temperature of the column improved the peak shape. We found that the use of the ion-pairing agent, sodium perchlorate, in the mobile phase significantly improved the peak shape of these phenanthrenes without the long stabilisation times associated with such commonly used ion-pairing agents. The use of a short, large I.D. (53×7 mm) column enabled a high flow-rate (3.0 ml/min) to be used without resulting in excessive back pressure (<8.2 MPa). Such a high flow-rate made it possible for all peaks of interest, including substrate, to elute in less than 60 min. These conditions, however, resulted in up to 180 ml of mobile phase being used for a single chromatographic run. Whilst appearing excessive it equates to a maximum of 2.5 ml of acetonitrile per sample and was therefore not expensive.

The applicability of the methods was assessed by studying the oxidative metabolism of hydrocodone and oxycodone in a pilot study of one liver (CYP2D6 extensive metaboliser). The formation rate of the metabolites versus substrate concentration curves are shown in Fig. 3. The N-demethylated metabolites were formed to a substantially greater extent than the O-demethylated metabolites. Microsomal incubations with the previously stated CYP isoform-specific chemical inhibitors alone did not produce chromatographic interference with the exception of omeprazole, which interferes under the oxycodone substrate conditions only. Diethyldithiocarbamate and quinidine both elute after hydrocodone and oxycodone substrates, therefore the run time had to be increased when using these two inhibitors. A reference standard of the secondary metabolite, noroxymorphone, eluted at 5 min (oxycodone substrate conditions) and was resolved from

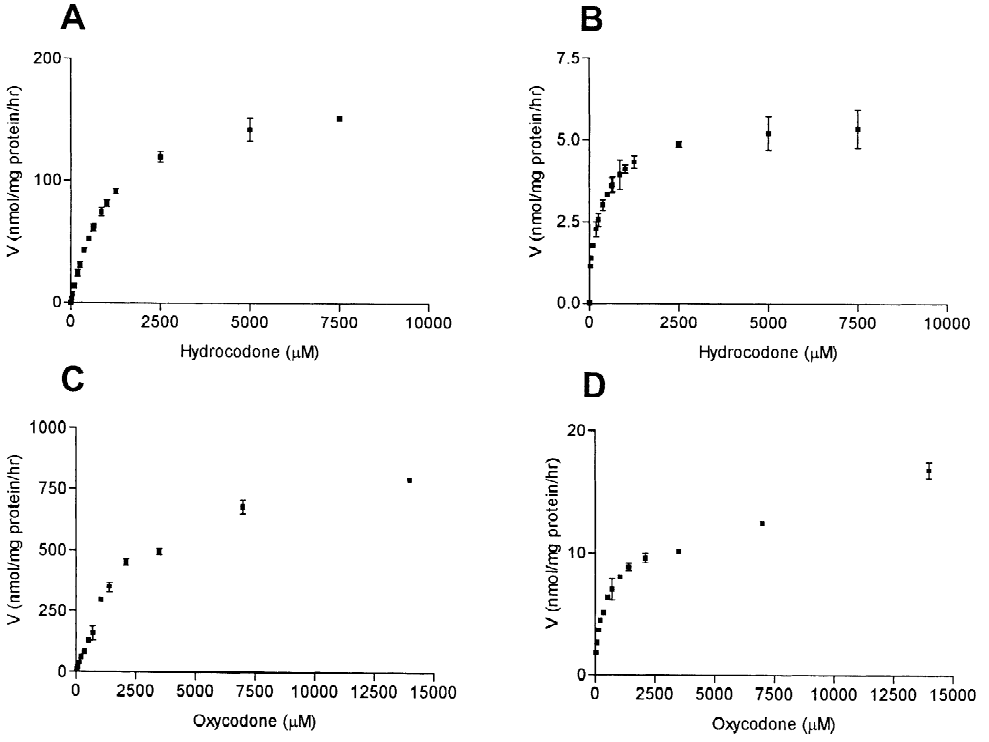

Fig. 3. Norhydrocodone (A), hydromorphone (B), noroxycodone (C) and oxymorphone (D) formation (mean±SD of duplicates) in a CYP2D6 extensive metaboliser liver, following incubation of human liver microsomes with an NADPH generating system and hydrocodone (A and B) and oxycodone (C and D). V is the formation rate of the respective metabolite.

the analytes of interest. Noroxymorphone formation was not observed in microsomal incubations with oxycodone substrates. Similarly, the secondary metabolite norhydromorphone did not interfere under hydrocodone substrate conditions, eluting at 6 min. Microsomal incubations with hydrocodone substrate yielded no secondary metabolite (norhydromorphone) formation.

We describe an original analytical method that involves a solvent–solvent extraction/re-extraction procedure followed by reversed-phase HPLC with UV detection. This method has been used to investigate the in vitro enzyme kinetics and cytochrome P450 enzymes involved in the oxidative metabolism of both hydrocodone [25] and oxycodone [26] in human liver microsomes.

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