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SIMULTANEOUS QUANTIFICATION OF ETHYLMORPHINE O-DEETHYLASE AND N-DEMETHYLASE ACTIVITY BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The N-demethylation and O-deethylation of ethylmorphine by cytochrome P-450 are simultaneously measured using high-performance liquid chromatography. All the metabolites and the substrate are extracted from the enzymatic incubation mixture with isopropanol—methylene chloride (20:80) containing 6.0 μg/ml codeine sulfate as an internal standard. Separation of the compounds is achieved on a C_{18} reversed-phase column using a mobile phase of 1% acetic acid—acetonitrile (85:15) with 1-hexanesulfonic acid as a counter-ion. Total run time is 12 min at a flow-rate of 2.0 ml/min and 144 bar. Assay of ethylmorphine N-demethylase and O-deethylase activities in rat liver microsomes revealed close agreement between this method and conventional ones. N-Demethylation was found to greatly exceed O-deethylation in liver microsomes from either control or phenobarbital-treated rats confirming results from other laboratories. This method can also be used to measure the N- and O-demethylation of codeine.

INTRODUCTION

Ethylmorphine (EM) is widely employed as a substrate for in vitro studies of microsomal mixed function oxygenase (MFO) activity. The MFO enzymes mediate both N-demethylation and O-deethylation of EM but usually only the N-demethylation activity is measured when EM is used as the substrate. Apparently N-demethylase activity predominates in EM metabolism by hepatic microsomes taken from control and phenobarbital-treated rats and mice [1, 2].

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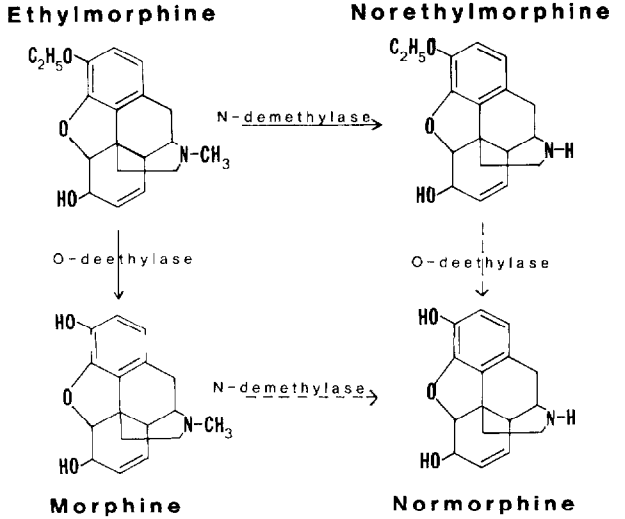

Fig. 1. Possible products of N-demethylase and O-deethylase activity on ethylmorphine. Only norethylmorphine and morphine are detected experimentally.

Ordinarily this activity is quantified by the colorimetric estimation of the semicarbazide-trapped formaldehyde that is generated [3]. Significant O-deethylase activity is present, however, and it is possible that differential effects on the two activities could be exerted by drug or toxicant action [4]. Although simultaneous measurements of both activities would be advantageous, it is not possible with the standard colorimetric assays [3, 5].

The products of EM N-demethylation and O-deethylation are norethylmorphine (NEM) and morphine respectively (Fig. 1). Either product could be converted to normorphine by subsequent metabolism (Fig. 1). Duquette and Holtzman [2] have reported the simultaneous measurement of the two enzyme activities utilizing a thin-layer chromatographic (TLC) separation of these products from radiolabeled EM. This method has also provided increased sensitivity over the colorimetric procedures. High-performance liquid chromatography (HPLC) affords a convenient alternative for measurement of these products of EM metabolism and obviates the need for synthesis and handling of radiolabeled substrate. This report describes such a method and its application in the simultaneous determination of the kinetics of the two pathways.

EXPERIMENTAL

Glucose-6-phosphate (G-6-P), glucose-6-phosphate dehydrogenase (G-6-PD, EC 1.1.1.49) and nicotinamide-adenine dinucleotide phosphate (NADP) were all obtained from Calbiochem-Behring (La Jolla, CA, U.S.A.). HPLC-grade acetonitrile was obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.). The ethylmorphine and morphine were from Merck (St. Louis, MO, U.S.A.) and the normorphine (NM) standard was generously donated by the Research Technology Branch, Preclinical Research Division of the National Institute of Drug Abuse (Washington, DC, U.S.A.). Since no commercial supplier for NEM was found, the compound was synthesized from EM in a manner similar to that

described for the synthesis of norcodeine from codeine [6]. The 1-hexanesulfonic acid counter-ion (PIC B-6®) was obtained from Waters Assoc. (Millford, MA, U.S.A.). All other materials used in the enzyme reaction or for the HPLC analysis were reagent grade.

Male Sprague–Dawley rats weighing 250–300 g were housed five per cage (44.5 × 25.4 × 17.8 cm) in a 12-h light–dark cycle. Food and water were provided ad libitum. For induction studies groups of animals were dosed with phenobarbital (80 mg/kg intraperitoneally) dissolved in 0.9% saline. These animals were compared to animals receiving the saline vehicle only. Liver microsomes were obtained from these animals 24 h after the last dose.

Rats were sacrificed by cervical dislocation and the livers were immediately removed and placed on 0.25 *M* sucrose. The livers were minced and washed three times with fresh 0.25 *M* sucrose and then a 20% homogenate was prepared using an ice-cold Potter-Elvehjem homogenizer. Microsomal pellets were obtained by standard differential centrifugation techniques [7]. The microsomal pellets were resuspended in the Tris–KCl buffer (0.1 *M* tris-(hydroxymethyl)aminomethane, 1.15% potassium chloride, pH 7.4) to approx. 5 mg/ml protein. Protein was determined by the method of Lowry et al. [8]. All microsomal suspensions were stored on ice and used within 12 h of isolation.

Spectral determinations were performed on an Aminco DW-2a UV–Vis spectrophotometer. Cytochrome P-450 was determined by the method of Omura and Sato [9, 10]. An extinction coefficient of $E_{450-490}$ = 91 m*M*/cm was used. For comparison with HPLC results, N-demethylase activity was measured by formaldehyde production as described by Nash [3].

The enzymatic reaction employed was a modification of the procedure outlined by Duquette and Holtzman [2]. All incubations were carried out in 25-ml Erlenmeyer flasks. The incubation mixture contained 10 m*M* magnesium chloride, 0.2 m*M* nicotinamide, 0.8 U/ml G-6-PD, 4 m*M* G-6-P, 1 mg/ml microsomal protein and varying amounts of EM substrate (median concentration 0.015 *M*) and Tris–KCl buffer in a total volume of 2.5 ml. Flasks were preincubated for 5 min at 37°C in a shaking water bath in air. The enzyme reaction was initiated by the addition of 0.4 m*M* NADP and allowed to proceed under the same conditions for 7 min. The reaction was terminated by placing the flasks on ice, adjusting the pH and adding the organic phase. All samples were run in duplicate. Standards were prepared by adding known concentrations of NM, morphine and NEM to flasks and adding the cofactors as described. Blanks were run using a boiled microsome suspension as the source of protein. Controls were run using active proteins but no added EM.

Extractions were carried out by an adaptation of the methods of Predmore et al. [11] and Siek [12]. All samples and standards were transferred to 30-ml extraction tubes, and 1.5 ml of 0.5 *M* sodium phosphate (pH 8.7) was added. The pH of each tube was checked and adjusted if necessary to pH 8.7 using dilute acid or base as required. A 15-μl volume of isopropanol–methylene chloride (20:80), which contained 6.0 μg/ml codeine sulfate as an internal standard, was added to each tube. The tubes were capped and mixed by inversion for 5 min and then the phases were separated by centrifugation for 10 min at 1400 *g*. The aqueous layer (upper) was aspirated and discarded and the

organic phase evaporated at 60°C under a gentle stream of nitrogen. After evaporation, the sides of the tube were rinsed with 1.0 ml of 95% ethanol and the rinse was evaporated as previously described. The residue was dissolved in 0.15 ml of HPLC mobile phase (described below), vortexed and transferred to injector vials for HPLC analysis.

The HPLC analysis was performed using a Waters Assoc. Model 6000A pump to deliver the mobile phase of 1.0% acetic acid- acetonitrile (85:15) containing 0.005 *M* hexanesulfonic acid. Samples were injected (0.01 ml) using a Waters Intelligent Sample Processor (WISP) Model 710 onto a μBondapak C_{18} reversed-phase column (30 cm × 3.9 mm, 10 μm particle size). Column effluent was monitored at 254 nm on a Waters 440 fixed-wavelength absorbance detector (sensitivity 0.1 a.u.f.s.) and data recorded on a linear strip chart recorder (10 cm/h). The flow-rate was 2.0 ml/min at 144 bar. Total run time was 12 min. Results were quantified by comparing peak-height ratios of spiked standards to that of microsomal incubates.

RESULTS

Representative chromatograms of extracts from microsomal incubation mixtures are presented in Fig. 2. Retention times for the identified compounds of interest are 2.0 min for nicotinamide, 3.4 min for morphine, 5.7 min for codeine (internal standard), 8.2 min for NEM and 9.1 min for EM. The NM standard eluted at 2.8 min (Fig. 2B) but at no time was this product detected in the microsomal incubation (Fig. 2C). Extraction efficiency for spiked standards was 85% with a coefficient of variation of 10%. The analysis was

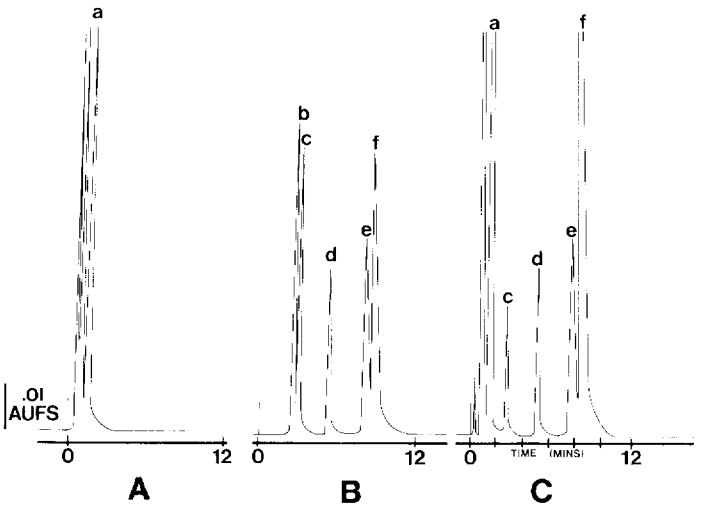

Fig. 2. Chromatograms of an HPLC determination of ethylmorphine metabolism. Conditions: flow-rate 2 ml/min using 1% acetic acid—acetonitrile containing 0.005 *M* hexanesulfonic acid, C_{18} column (30 cm × 3.9 mm), 0.1 a.u.f.s. and 254 nm. Run time 12 min. (A) Blank sample analyzed without addition of internal standard or substrate. (B) Analysis of standard mixture. (C) Typical chromatographic analysis of microsome enzyme activity. Peaks: a = nicotinamide, b = normorphine, c = morphine, d = codeine, e = norethylmorphine, f = ethylmorphine.

linear over the range 3–300 nmol for all three reaction products. Using these conditions, we were able to detect as little as 0.4 ± 0.05 μg/ml NEM (mean ± S.D.).

Because an authentic NEM standard was not available commercially it required synthesis and positive identification. The retention time for the synthesized NEM standard coincided with the peak postulated to be the N-demethylase product of EM and co-eluted with it when added to extracts of microsomal incubates. To confirm our presumptive tests, the suspected NEM peak was collected for mass spectrometric analysis. The electron-impact (EI) probe mass spectral comparison for the synthesized standard and the collected fraction is reproduced in Fig. 3. The EI base peaks are in good agreement with the known molecular mass for EM (313.38) and NEM (299.35). The synthesized product for EI mass spectral assay showed traces of unreacted EM (Fig. 3B), approx. 11% by mass. Further purification of NEM for use as an analytical standard was accomplished by preparative TLC, a modification of the TLC separation described by Duquette and Holtzman [2]. Since complete baseline separation of NEM and EM was not achieved, the collected fractions also showed some EM substrate contamination (Fig. 3C) by EI probe mass spectral analysis.

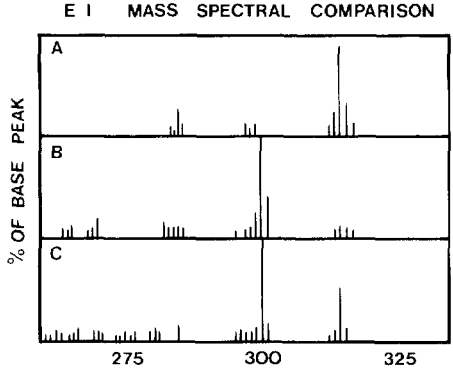

Fig. 3. (A) Gas chromatographic–mass spectrometric analysis of ethylmorphine standard [electron-impact (EI) base peak at 313 a.m.u.; known molecular mass 313.38]. (B) Norethylmorphine standard synthesized from ethylmorphine (EI base peak at 299 a.m.u.; known molecular mass 299.35). Traces of unreacted ethylmorphine at EI base 313 are present to the extent of 11% of the synthesized product. These traces were removed by preparative TLC prior to use of the material as a standard. (C) Norethylmorphine fraction collected from HPLC after microsomal metabolism of ethylmorphine. In addition to the EI base peak at 299 a.m.u., traces of the ethylmorphine parent compound are also present in these fractions (EI base peak at 313 a.m.u.) because of a lack of baseline separation.

The application of this method to enzymatic assays for N-demethylase and O-deethylase activities in rat liver microsomes is illustrated in Table I. N-Demethylase activity was almost 2.5 times the O-deethylase activity. This was true whether the activity was normalized to microsomal P-450 or microsomal protein content. Following phenobarbital induction N-demethylase activity was more than double that of non-induced animals but O-deethylase activity remained unaffected when normalized to microsomal protein content.

TABLE I

ETHYLMORPHINE METABOLISM IN CONTROL AND PHENOBARBITAL-INDUCED MALE SPRAGUE–DAWLEY RATS

Values represent mean ± standard error of the mean for five animals.

	P-450 (nmol/mg microsomal protein)	O-Deethylase (nmol/mg of protein per min)	N-Demethylase (nmol/mg of protein per min)
Control	0.88 ± 0.02	2.44 ± 0.45	5.89 ± 0.91
Phenobarbital*	1.91 ± 0.34	2.13 ± 0.48	14.04 ± 1.2

*80 mg/kg intraperitoneally; four consecutive days.

TABLE II

KINETIC PARAMETERS OF ETHYLMORPHINE (EM), N-DEMETHYLASE AND O-DEETHYLASE ACTIVITY IN CONTROL RAT LIVER MICROSOMES

	O-Deethylase		N-Demethylase	
	V_{max} (nmol/mg of protein per min)	K_m (μmol EM)	V_{max} (nmol/mg of protein per min)	K_m (μmol EM)
HPLC	3.03	23.8	7.07	108
Nash [3]	—	—	9.26	105
Duquett and Holtzman [2]	1.8	52	5.0	250

When activity was normalized to cytochrome P-450 content, however, the turnover number was halved for the O-deethylase enzyme as compared to microsomes from non-induced rats. In contrast, the N-demethylase turnover number was not altered.

Kinetic analysis of N-demethylase and O-deethylase activity was performed on microsomes isolated from non-induced rats only. A summary of the enzymatic parameters is presented in Table II. The differences in the kinetic parametes between the two enzyme activities are in good agreement with those reported from other laboratories in which the activities were measured separately [1, 13]. Similarly there was good agreement for the N-demethylase activity when measured by either the HPLC method or the Nash reaction [3] (Table II).

DISCUSSION

The results we have presented represent the simultaneous assay by HPLC of N-demethylase and O-deethylase activity in a single sample. A number of researchers have described HPLC methods for the separation of opioid alkaloids [14, 15], but in our hands the limitation with most of these methods proved to be the inability to separate the N-demethylated product from its parent compound. An earlier HPLC method to determine total EM metabolism

measured substrate disappearance rather than the appearance of products [4]. A key advance in the ability to separate such mixtures involved the use of an ion-pairing reagent [16], which in a buffered aqueous–organic mobile phase forms a lipophilic complex with the salt of a drug [17]. This reported HPLC method utilizes a commercial counter-ion which permits the detection and quantification of all potential products of in vitro P-450-mediated EM metabolism: normorphine, morphine and norethylmorphine. The procedure involves a one-step extraction of the enzyme reaction mixture, has a detection limit of 3 nmol and is linear over the range 3–300 nmol for all three reaction products under the described conditions. The analysis is further facilitated by lower substrate requirements than the conventional colorimetric assays, by short incubations and short analysis time.

Duquette and Holtzman [2] were the first to report the simultaneous analysis of these two enzyme activities using a radioassay. This represented a significant advance in sensitivity over colorimetric methods. The HPLC method reported herein closely approximates the sensitivity reported by Duquette and Holtzman [2] for their radioassay procedure when final protein concentrations of 1.0 mg/ml were used in their incubation media. Moreover, it eliminates the need for synthesis and use of a radiolabelled substrate and the product extraction has been simplified.

Enzymatic values obtained from this method are in good agreement with previous studies [1, 13]. The Michaelis constants (K_M) obtained with the HPLC method are lower than those reported by Duquette and Holtzman [2], but are within reasonable limits and may reflect differences in the strain of animals used. In this study the K_M obtained by the HPLC method and the Nash reaction [3] were identical.

One additional advantage to the HPLC method is that we have used it to assay N- and O-dealkylation employing codeine as a substrate (data not shown). Ethylmorphine is becoming less available within the United States and codeine represents a viable alternative substrate when coupled with this HPLC method.

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