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A radiometric TLC assay of liver microsomal dextromethorphan O-demethylation

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Abstract

A simple and sensitive assay for in vitro analysis of dextromethorphan O-demethylation, a marker for P450 2D deficiency in both humans (2D6) and rats (2D1), has been devised. Commercially available [N-methyl-^{3}H]-dextromethorphan was used to develop a radiometric TLC assay for dextromethorphan O-demethylation. Hexane-triethylamine efficiently extracted dextromethorphan and metabolites from rat liver microsomes, and a solvent system of cyclohexane-toluene-diethylamine (65:15:20, v/v/v) provided sufficient separation (approximately 2 cm) between the two radioactive bands, dextromethorphan and dextrorphan, and no interference from the unlabeled N-demethylation products, 3-methoxymorphinan and 3-hydroxymorphinan. The recovery of dextrorphan from TLC plates increases with microsomal protein and incubation time. An eight-fold decrease in activity was noted in female Dark Agouti relative to the male Sprague-Dawley rats, respective models for poor and extensive P450 2D metabolizers. The assay, even with an approximately 100-fold dilution of radiolabeled substrate, had an approximate limit of detection of 100 pmol. Within- and between-run imprecision was 12.4% and 7.2%, respectively. The radiometric TLC assay for dextromethorphan O-demethylation was sensitive and easy, and used readily available equipment.

Keywords: CYP2D assay; N-demethylation; Dextromethorphan; P450 2D assay; Radiometric assay; TLC

1. Introduction

Genetic variation has been shown to be an important cause of differences between individuals in drug metabolism, and a major determinant of toxic and therapeutic responses to a variety of clinically used drugs. A multitude of cytochrome P-450 (P450) isozymes that metabolize drugs, carcinogens, other xenobiotics and many endogenous lipids have been described [1–3]. Some have been expressed as non-functional forms within human populations. The debrisoquine-sparteine type, referred to in humans as P450 2D6 and in rats as 2D1, has been widely established as a genetic polymorphism in P450s [4–6]. It has been found to be deficient in approximately 8% of Caucasian populations, to be inherited according to an autosomal recessive pattern, and to be responsible for the metabolism of approximately 30 commonly used medications [4–6]. Dextromethorphan, a widely used antitussive drug, has been found to be rapidly metabolized to dextrorphan, 3-methoxymorphinan 3-hydroxymorphinan, and conjugates at the 3-hydroxyl group position (Fig. 1). In humans, the O-demethylation of dextromethorphan to dex-

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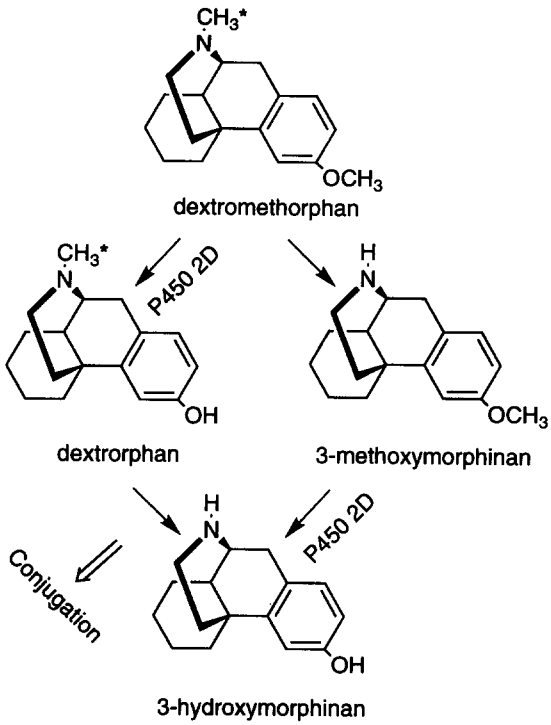

Fig. 1. Metabolic pathway of dextromethorphan. The position of the radiolabel is indicated by the asterisk. Under the in vitro conditions employed for this assay, oxidative metabolism was favoured by inclusion of the cofactor NADPH, while conjugation was prohibited by exclusion of the cofactor UDP-glucuronide.

trorphan and of 3-methoxymorphinan to 3-hydroxymorphinan have been shown to be under genetic control that co-segregated with the debrisoquine oxidation activity [7–9]. It has been demonstrated that dextromethorphan O-demethylation is a suitable in vivo and in vitro probe for studying P450 2D deficiency [8–10].

For the detection of dextromethorphan and dextrorphan in urine and plasma, as used for in vivo phenotyping, a number of analytical approaches including TLC [11], LC [12], GC/MS [13], capillary zone electrophoresis [14], and ELISA [15] have been reported. In vitro assays of dextromethorphan O-demethylation required more sensitive analytical techniques. To date, only LC with fluorescence detection has been used for this assay [10,16–18]. Studies using this probe for 2D deficiency would be facilitated by additional simple and sensitive assays to measure the drug and its metabolite, particularly assays with alternative equipment requirements. A radiometric TLC assay for the O-demethylation of dextromethorphan has been developed. It has proved to be convenient, rapid, relatively inexpensive, and used readily available equipment.

2. Experimental

2.1. Materials

[N-Methyl-^{3}H]-dextromethorphan (82.7 Ci mmol^{-1}) was purchased from NEN Products (Boston, MA). Dextromethorphan hydrobromide, NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were from Sigma (St. Louis, MO). Dextrorphan tartrate, 3-methoxymorphinan, and 3-hydroxymorphinan were kindly provided by Dr. Peter Sorter, Hoffman-La Roche Inc. (Nutley, NJ). ProLab animal diet (Rat, Mouse and Hamster 3000) was purchased from Agway, Inc. (Syracuse, NY). Silica Gel 60A plates (20 cm × 20 cm, 250 μm layer) were from Whatman (Clifton, NJ). Opti-Fluor scintillation cocktail was purchased from Packard Instrument Co., Inc. (Meriden, CT). All other chemicals used were reagent grade.

2.2. Microsome preparation

Liver microsomes were prepared from male Sprague-Dawley (SD) (mean body weight 187 g) and female Dark-Agouti (DA) rats (mean body weight 133 g) purchased from BK Universal (Freemont, CA). The rats were acclimatized for a week to the temperature- and humidity-controlled quarters, and had free access to water and food. The rats were fasted overnight, and then sacrificed under anesthesia induced by CO_2. Microsomes were prepared from liver homogenates by differential centrifugation as previously described [19]. Protein was determined as described by Lowry et al. [20].

2.3. O-Demethylation assay

^{3}H-Dextromethorphan for the assay was prepared by diluting 10 μl of the commercial material in 1 ml of cold dextromethorphan (1 mM). This provides a substrate at approximately 10 μCi μmol^{-1}, with 50–60 thousand cpm being added per assay. The dextromethorphan O-demethylation assay was performed with Tris–HCl (0.15 M; pH 7.4), $MgCl_2$ (5 mM), NADP (1 mM), glucose-6-phosphate dehydrogenase (0.4 units), glucose-6-phosphate (2.5 mM), dextromethorphan (50 μM, prepared with the labeled substrate at 10 μCi μmol^{-1}), and, unless specified otherwise, 100 μg of microsomal protein. The reaction, in a final volume of 200 μl, was initiated by the addition of

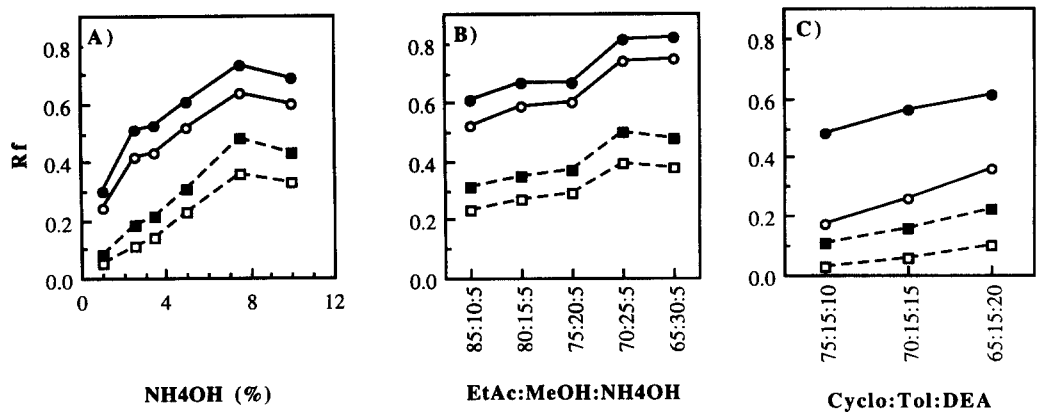

Fig. 2. Effect of variation of solvent composition of R_f values of dextromethorphan (●) and its metabolites: dextrorphan (○), 3-methoxymorphinan (■) and 3-hydroxymorphinan (□). (A) The percentage NH_4OH was varied while ethyl acetate–methanol was held at an 85:10 (v/v) ratio; (B) the ratio of ethyl acetate to methanol (EtAc:MeOH, v/v) was varied while NH_4OH was held at 5%; (C) the relative contents of cyclohexane (Cyclo), toluene (Tol), and diethylamine (DEA) were varied.

microsomal protein. Unless specified otherwise, the mixture was incubated at 37 °C for 45 min. The reaction was stopped by the addition of 1 ml of *n*-hexane (with 1% triethylamine v/v) and kept on ice until an additional 7 ml of *n*-hexane solution had been added. The tubes were vortexed and left on a rocker for 90 min, after which the organic phase was carefully transferred to a clean tube and dried under nitrogen. 100 μl of *n*-hexane solution was added to each tube, which was then vortexed four times (10 s each). All of the reconstituted material was then carefully transferred to a silica-gel TLC plate using capillary tubes. Dextrorphan and dextromethorphan were also spotted with the samples as markers. All assays included controls, to which no microsomes, or no NADP were added, to determine the non-enzymatic and non-monooxygenase activities, respectively.

Different combinations of chromatographic solvent systems were tried, as described more thoroughly in Section 3. The most suitable system was cyclohexane–toluene–diethylamine (65:15:20, v/v/v). After elution, the plate was dried and the spots identified under UV light. 1 cm bands, corresponding to the position of dextromethorphan and dextrorphan, were then wetted with a drop of water, scraped with a spatula, placed in 3 ml of scintillation cocktail and counted. An aliquot of the radioactive substrate used for that batch was counted at the same time to determine its specific activity.

3. Results and discussion

The reaction mixture containing radiolabeled dextromethorphan, the microsomal preparation and an NADPH generating system was incubated in a water bath at 37 °C. Dextromethorphan and its metabolites were extracted with *n*-hexane containing 1% triethylamine solution. To find an optimal extraction volume, successive 1 ml extractions were performed on an incubation mixture, after each organic phase had been removed and an aliquot was taken for counting. A plateau was reached (i.e. approximately 60% of the total radioactivity added to the microsomes) after the fourth 1 ml extraction (data not shown). To ensure maximum extraction an extra 3 ml was added for each extraction procedure (total 7 ml). With this single extraction, 80–90% of the total radioactivity was recovered in the reconstituted extract following 30–90 min of mixing. Shorter extraction times were not tested, but may have provided equal recovery with a more time-efficient extraction.

Using cold reference material for dextromethorphan and its metabolites, several different solvent systems were evaluated to optimize the TLC separation of dextromethorphan and dextrorphan. A previous report [11] used ethyl acetate–methanol–NH_4OH for qualitative assessment. Although adequate for qualitative separation with visual detection, we found that the separation between dextromethorphan

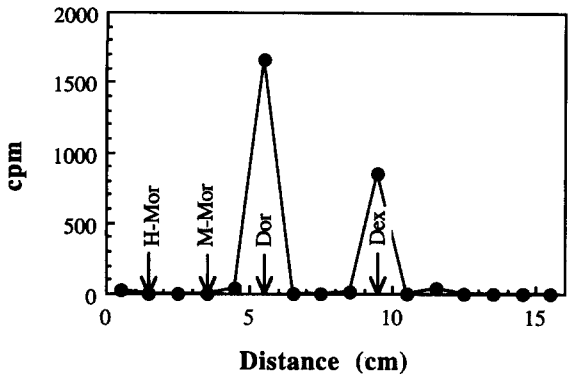

Fig. 3. The TLC separation and recovery of radioactive compounds generated from incubation of ^{3}H-dextromethorphan with rat liver microsomes. Radioactivity detected in one lane of the TLC plate which was scraped at 1 cm intervals. The migration of standards of dextromethorphan (Dex) and its metabolites, dextrorphan (Dor), 3-methoxymorphinan (M-Mor), and 3-hydroxymorphinan (H-Mor), separated during the same TLC are designated by arrowheads.

and dextrorphan was not inadequate for quantitative separation and recovery. Alterations in the percentage of NH_4OH or ratio of ethyl acetate–methanol did not improve the separation of dextrorphan from dextromethorphan (Fig. 2). Optimal separation of 3-methoxymorphinan, 3-hydroxymorphinan, dextrorphan, and dextromethorphan was obtained with a solvent system of cyclohexane–toluene–diethylamine (65:15:20, v/v/v) (Fig. 2).

As the substrate available for the dextromethorphan O-demethylation assay was tritiated on the N-methyl position, only the parent compound, dextromethorphan, and the primary O-demethylation product, dextrorphan, retained the radiolabel for detection. Rat liver microsomes were incubated with ^{3}H-dextromethorphan, extracted, and separated by TLC as described in Section 2. The fully deveoped TLC plate was scraped at 1 cm intervals and counted. Only two spots corresponding to dextrorphan and dextromethorphan were identifiable (Fig. 3). In daily assays, where only the spots of interest were scraped, we routinely used UV light to visualize the location of the standards for dextromethorphan and dextrorphan. These compounds could be visualized with reagents such as Ludy Tanger's solution [11]; however, we found that the reagent caused considerable quenching (approximately 90%), and its use is not recommended during assays. Silica itself did not cause appreciable quenching. When 1 cm^2 of blank TLC plate was scraped and added to radiolabeled dextromethorphan in the scintillation cocktail, there was no reduction in cpm.

The formation of dextrorphan was measured versus protein content and incubation time using microsomal protein obtained from male SD and female DA rats (Fig. 4). The latter have been shown to be deficient in P450 2D1, including the O-demethylation of dextromethorphan [16,18,21,22]. The formation of dextrorphan increased along with increased microsomal protein from both strains of rat (Fig. 4(A)). This was near-linear for up to 100 μg of protein for the SD rat, and this was chosen as the operating amount of protein for the assay. With this amount of microsomal protein, and a substrate concentration of 50 μM, dextrorphan production was linear with time for up to 120 min (Fig. 4(B)). Preliminary experiments with 1 μM dextromethorphan demonstrated that the linearity of product formation was reduced to shorter times as the substrate concentration was lowered. The omission of either NADP or microsomes greatly reduced the formation of radiolabel migrating with dextrorphan to just above tritium background counts, demonstrating that they were suitable blanks for the assay.

The slope of the dextrorphan production versus time plots resulted in activities of 0.448 and 0.040 nmol min^{-1} per mg protein for the microsomes from male SD and female DA rats, respectively. Both male and female SD rats have been used as models of extensive metabolizers for comparison to the P450 2D deficient female DA rat [16,18,21,22]. The activity of dextromethorphan O-demethylation found in this work for the male SD rat compares favorably with that calculated for 50 μM of substrate (0.56 nmol min^{-1} per mg protein) from the kinetic values reported by Zysset et al. [16] for female SD rats, but is less than the 0.96 nmol min^{-1} per mg protein calculated from values reported by Kerry et al. [18] for female SD rats. When assayed at this single substrate concentration, we found an 11-fold difference between the dextromethorphan O-demethylation activities of the male SD and female DA rats. Kerry et al. [18] observed biphasic kinetics for the SD rats and single-phase kinetics for DA rats. In this case, the high affinity V_{max} for the female SD rats was approximately three-fold the V_{max} of the female DA rat. Zysset et al. [16] found no difference in

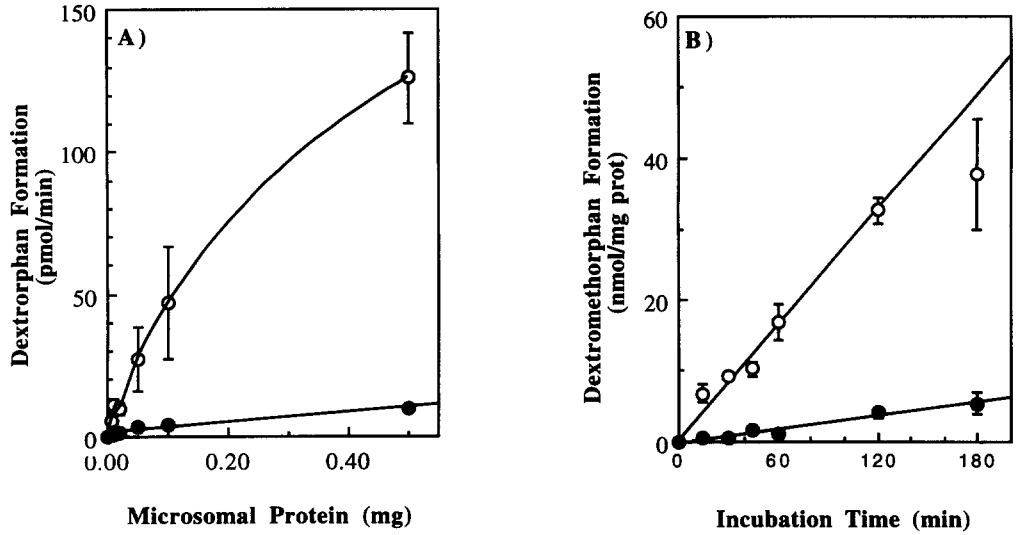

Fig. 4. Effect of (A) microsomal protein added (45 min incubation) and (B) incubation time (100 μg protein) on the generation of radiolabeled dextrorphan in microsomes from male SD (○) and female DA (●) rats. The line for SD rats in (B) represents the linear portion of the assay up to 120 min. Incubations were performed as described in the Section 2. Values are the mean ± SD of triplicate assays.

V_{max} for the two strains when single-phase kinetics were used for both strains. An approximate five-fold difference in specific activities was noted; however, at the same substrate concentration used in this study (50 μM), which was approximately one-third of the high affinity K_m described by Kerry et al. [18]. These differences in activity may have been due to differences in the method employed, sex variation in the case of SD rats, source of rats, and/or different methods of microsomal preparation and storage. Our current results, however, supported those of the other laboratories in demonstrating that O-demethylation of dextromethorphan was a marker of poor metabolism in the female DA rat.

The sensitivity of the assay was estimated from the lowest detectable activity (defined as at least twice the background cpm) in three different experiments. Minimal activities were clearly detected following 45 min incubations of 50 μg microsomal protein from the DA rat (Fig. 4(A)), and 15 min incubations of 100 μg microsomal protein from the DA rat (Fig. 4(B)). An additional experiment, where 1 μM dextromethorphan was incubated with 100 μg of protein for 45 min, produced 130 pmol of product. These translated to detection of 166, 130, and 92 pmol, respectively, which gave an indication of the limit of detection for the assay. While this was more than the limits of 0.04–10 pmol reported for the dextromethorphan O-demethylation assay using LC with fluorescence detection [10,16–18], the assay was sufficiently sensitive to detect activity in the deficient strain. Further, the radiolabeled substrate had been diluted 100-fold, suggesting that enhanced sensitivity was a possibility. The precision and reproducibility of the assay were determined using conventional procedures [23]. Based on the average results for separate assays over three days, and of five assays performed on a single day, the inter- and intra-assay imprecisions were found to be 7.2% and 12.4%, respectively. Additional assays on rat liver microsmes conducted by two different analysts approximately six months later (Law et al., unpublished data) and by the same analyst 12 months later resulted in similar activities.

Recently, Rodrigues et al. [24] described another approach to radiometric assay of in vitro dextromethorphan metabolism. In this specially synthesized [O-methyl-^{14}C]-dextromethorphan was used, and the release of ^{14}C-formaldehyde was measured directly. While this assay was much quicker, not requiring separation, [O-methyl-^{14}C]-dextromethorphan was not commercially available, and the assay measured the combined rates of dextromethorphan and 3-methoxymorphinan. As these two pathways have been reported to have different kinetics [18], the current assay offered advantages for any kinetic studies. The com-

mercial availability of the [*N*-methyl]-tritiated dextromethorphan was the major attraction for its use. A product with the label in a metabolically inert position would have offered additional advantages. As our TLC system was able to resolve all three of the metabolites, such a substrate would have allowed simultaneous measurement of all the oxidative pathways. Had the label been a stronger emitter, such as ^{14}C, scanning of the TLC plates may have been a feasible time-saving alternative to scraping and scintillation counting.

The results of this study demonstrated that commercially available radiolabeled dextromethorphan could be employed along with TLC separation to provide an easy, sensitive, and relatively inexpensive (less than one dollar per assay) assay for dextromethorphan O-demethylation. Previous work by Robbins and Wedlund [11] demonstrated the ability of TLC to separate dextromethorphan from dextrorphan; this, with the availability of tritiated dextromethorphan from dextrorphan; this, with the availability of tritiated dextromethorphan, set the stage for the development of a quantitative assay. We found, however, that improved separation was required for quantification, and have now extablished the mobile phases required to meet this need. This assay offers an alternative to LC assays, and would be particularly attractive to laboratories where scintillation counters are readily available, but LCs with the requisite detector are not available for the extended periods of time often devoted to routine enzyme assays. Product formation, with our assay, increased with time of incubation and amount of microsomal protein employed, and the assay was sensitive enough to detect the lower activity present in the deficient rat strain. As dextromethorphan has gained favor as a safe in vivo probe [7–9], in vitro assays for the same substrate have become particularly useful. This TLC-radiometric assay now provides an alternative method to conduct such studies.

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