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# Simple chromatography method for simultaneous determination of dextromethorphan and its main metabolites in human plasma with fluorimetric detection

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**Abstract**

Dextromethorphan, the innocuous non-narcotic antitussive agent, is the most widely used probe drug to assess CYP2D6 function both in vivo and in vitro. For this reason a simple and selective high performance liquid chromatography method with fluorimetric detection for simultaneous quantitation of dextromethorphan, and its main metabolites in human plasma was developed and validated. The method involved a simple and rapid protein precipitation protocol, using a mixture of $ZnSO_4$ and methanol. The analysis was performed on a 3 μm, $C_{18}$ Tracer Excel 15 cm × 0.4 cm i.d. column by gradient elution in which Mobile phase A consisted of potassium dihydrogen phosphate buffer (pH = 3, 0.01 M):methanol:tetrahydrofuran (68.5:31:0.5), and mobile phase B consisted of methanol:tetrahydrofuran (93.25:6.75). Linear calibration curves were obtained in the range of 10–500 ng/ml for dextromethorphan, dextrorphan and hydroxymorphinan. The limit of quantitation (LOQ) was 10 ng/ml for each compound. The maximum within and between days precisions were 7.4 and 7.8%, respectively. The accuracies at four different concentration levels ranged from 88.2 to 111.5%. The recoveries were between 88.0 and 108.6%. The assay method was successfully applied to determine dextromethorphan metabolic ratio after an oral dose of 30 mg of dextromethorphan hydrobromide.
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*Keywords:* Dextromethorphan; Dextrorphan; Hydroxymorphinan

## 1. Introduction

Dextromethorphan, the innocuous non-narcotic antitussive agent, is the most widely used probe drug to assess CYP2D6 function both in vivo and in vitro [1–3]. Dextrorphan, the main metabolite of dextromethorphan has been shown to possess some cough suppressant activity and is under investigation as a neuroprotective agent in the management of stroke [4].

In humans, dextromethorphan is reduced rapidly after oral absorption to dextrorphan by the polymorphic cytochrome P450 CYP2D6 enzyme. In a parallel pathway, it is *N*-demethylated to methoxymorphinan, which is catalyzed, by CYP3A4 and CYP3A5 in human liver and intestinal flora. The metabolites of *N*- and *O*-demethylation undergo further metabolism to a common secondary metabolite, 3-hydroxymorphinan. Dextrorphan and hydroxymorphinan are mainly glucuronidated and eliminated into urine (Fig. 1) [3,5].

As an in vivo index of CYP2D6 activity, the ratio of unchanged dextromethorphan to dextrorphan in a 8–12 h urine or 3 h plasma sample, following oral administration of dextromethorphan is used [1,6–9]. An excellent correlation between the log transformed ratios of dextromethorphan to dextrorphan and of dextromethorphan to the sum of molar concentrations of dextrorphan and hydroxymorphinan both in urine and plasma has also been reported [8]. Between 0 and 12% of the subjects depending on the race studied have a severely impaired capacity to express the CYP2D6 isoenzyme and are considered as poor metabolizers. The rest are so-called extensive metabolizers [10].

Several papers have dealt with analytical methods for the quantitative determination of dextromethorphan and its metabolites in biological fluids which employ a variety of

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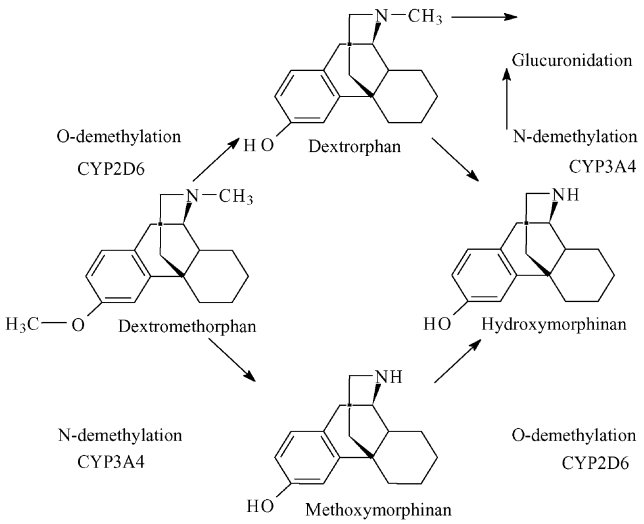


Fig. 1. Metabolic pathway of dextromethorphan.

techniques, including LC–MS/MS [11], high performance liquid chromatography (HPLC) with UV [12] or fluorescence detection [13–17]. Few of them enable the simultaneous detection of dextromethorphan and its main metabolites in human plasma [7,18–21] or in rat serum [22]. In most of them phenyl [7,22] and cyano [18,20] stationary phases are used. The major drawbacks of described methods are using polluting solvents [20], labor intensive liquid–liquid extraction as sample preparation methods [7,19,20,22]. Moreover, some of these methods suffer from poor extraction recoveries for dextromethorphan [20] or its metabolites [7,20].

This paper presents a simple, rapid and reproducible protein precipitation method, followed by separation on a common $C_{18}$ column with fluorescence detection, which allows detection of dextromethorphan and its metabolites, dextrorphan and hydroxymorphinan, in a single run. The assay was applied to determine CYP2D6 activity in healthy volunteers.

## 2. Experimental

### 2.1. Chemicals and solutions

Dextromethorphan hydrobromide (99.8%) and methoxymorphinan hydrochloride were kindly provided by Toulid daru (Tehran, Iran) and Hoffmann-La Roche (Basel, Switzerland), respectively. Dextrorphan tartrate (ICN Biomedical Inc, Cat. No. 153741) was a gift from Dr. Jamali. Hydroxymorphinan hydrobromide was purchased from Sigma (Munich, Germany).

B-glucuronidase/arylsulfatase (100,000 Fishman Units of β-glucuronidase and 800,000 Roy Units of arylsulfatase) (127698) was supplied by Boehringer Mannheim (Mannheim, Germany). Acetonitrile, acetone, methanol and tetrahydrofuran were all of gradient grade purchased from Merck (Darmstadt, Germany). All other chemicals were of analytical grade and purchased from Merck (Darmstadt, Germany). Double distilled water was used throughout the study.

### 2.2. Solutions

Stock solutions (0.1 mg/ml as free base) of the analytes were prepared separately by dissolving each compound in methanol. These solutions were stable for at least 4 months at −20 °C. Working standard solutions were prepared daily by diluting the stock solutions with distilled water.

### 2.3. Apparatus and chromatographic conditions

The chromatographic apparatus included a low pressure gradient HPLC pump, a fluorescence detector type RF-10Axl, (excitation wavelength of 280 and emission wavelength of 320) and an online degasser all from Knauer (Berlin, Germany). A 65 μl volume of sample was introduced to a Rheodyne model 7725i injector. Data were analyzed by means of Urochrome chromatography software (Knauer, Berlin, Germany). The stationary phase was a Tracer excel reversed phase column ($C_{18}$, 15 cm × 0.4 cm i.d., 3 μm), (Teknokroma, Spain).

Mobile phase A consisted of potassium dihydrogen phosphate buffer (pH = 3, 0.01 M):methanol:tetrahydrofuran (68.5:31:0.5), and mobile phase B consisted of methanol: tetrahydrofuran (93.25:6.75) Gradeint elution started at 100% A for 10 min, decreased to 88% for 2 min, kept constant up to 25 min, increased to 100% for1 min, kept constant for 7 min to re-equilibrate. Total analysis time was 33 min at a flow rate of 0.7 ml/min at room temperature (25 °C). Compounds were quantified using their peak height.

### 2.4. Sample preparation

An aliquot of 100 μl of human plasma was mixed with 100 μl of β-glucuronidase solution containing 1000 U/ml in 0.2 M acetate buffer, pH 5.0. The solution was kept at a constant temperature of 37 °C for 18 h. Subsequently 20 μl of a zinc sulfate solution (35%) and 100 μl methanol were added to precipitate plasma proteins. Sample was vortexed for 1 min and centrifuged at 15,800 × *g* by a 5415C eppendorf centrifuge for 10 min. The clear supernatant was transferred into a clean tube and a volume of 65 μl was injected into HPLC column.

### 2.5. Method validation

#### 2.5.1. Preparation of calibration standards and quality control samples

Appropriate aliquots of the standard solutions were added into blank freshly prepared human plasma. Final concentrations were 0, 10, 20, 50, 75, 100, 200, 400 and 500 ng/ml for

the calibration standards and 0, 20, 50, 100, 200 ng/ml for quality control samples (QC). The resulting mixtures were subjected to the sample pretreatment described in Section 2.4 and injected into HPLC.

Values of limit of quantitation (LOQ) were calculated according to the FDA guidance for bioanalytical method validation [23] as at least five times the response compared to blank response and a precision of 20% and accuracy of 80–120%.

#### 2.5.2. Absolute recovery and accuracy

The recovery was calculated by comparing peak heights obtained after extraction of QC samples from plasma with peak heights resulted after injecting standard solutions at the same theoretical concentrations. The accuracy of the measurements were assessed based on four QC samples for each compound in each run.

#### 2.5.3. Precision

The assay procedure described in Section 2.4 was repeated five times for QC samples with in the same day (intra-day precision) and seven times over different days (inter-day precision) both expressed as R.S.D. values.

#### 2.5.4. Human study

Forty healthy volunteers participated in this study. The volunteers were fasted over night and were kept fasted 3 h after drug administration. Blood samples were collected at 0 and 3 h after oral administration of 30 mg of dextromethorphan hydrobromide, into heparinized tubes. Plasma samples were stored at −20 °C until analysis.

This study was approved by the ethics committee of Tehran University of Medical Sciences and written informed consent was obtained from each subject.

## 3. Results and discussion

### 3.1. Mobile phase development

For the starting point, the proportion of acetonitrile and phosphate buffer (pH = 3, 0.01 M) was varied isocratically to obtain the best separation with $0.5 \leq K' \leq 20$. At the best composition of acetonitrile:buffer (26:74), dextrorphan and hydroxymorphinan as well as dextromethorphan and methoxymorphinan were not resolved adequately for quantitative analysis. Lowering the flow rate and changing the pH value or strength of the buffer also did not improve the separation quality. Consequently, a methanol:buffer solvent with the same eluent strength (35:65) was examined and finally a mobile phase containing 33% methanol gave the best separation for dextrorphan and hydroxymorphinan. Since elution times for dextromethorphan and methoxymorphinan were too long, a gradient program was chosen in which the percentage of methanol was 33 at the start time until 13 min, then changed to 45 for the rest of the run.

Unfortunately, there were two endogenous peaks which co-eluted with dextromethorphan In order to separate these peaks, several modifications to the mobile phase were undertaken including changes in the concentration of buffer and pH value, adding low amounts of tetrahydrofuran to mobile phase, changes in percentages of aqueous buffer and organic modifier and the methanol–tetrahydrofuran ratios. The best beneficial variation was the addition of tetrahydrofuran, which reduced the retention times of the analytes. These modifications allowed the complete separation of the analytes from endogenous compounds. Therefore the chromatographic condition described in Section 2.2 was considered as the final mobile phase. Under this chromatographic condition, the analytes had the following retention times; dextrorphan, 9.8 min; 3-hydroxymorphinan, 12.1 min; dextromethorphan, 21.6 min and methoxymorphinan, 23.7 min. Chromatograms from the analysis of enzymatically hydrolyzed blank plasma, hydrolyzed plasma sample of an extensive metabolizer 3 h after administration of dextromethorphan and nonhydrolyzed blank plasma spiked with 75 ng/ml of each compound are presented in (Fig. 2). Precise quantification of concentrations of higher than 20 ng/ml was possible for methoxymorphinan. However, since the quantification of this metabolite was not the purpose of this study, the validation was not performed for that.

### 3.2. Sample preparation

Several methods of protein precipitation such as addition of trichloroacetic acid, perchloric acid, different concentrations of zinc sulfate, different volumes of water-miscible solvents like methanol, acetonitrile, acetone and mixture of

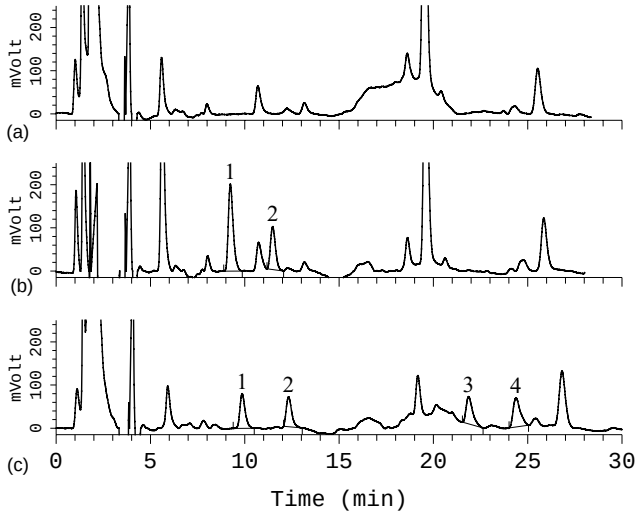


Fig. 2. Chromatograms for dextrorphan (1), hydroxymorphinan (2), dextromethorphan (3), methoxymorphinan (4). (a) Enzymatically hydrolyzed blank plasma, (b) hydrolyzed sample of an extensive metabolizer (the same volunteer) 3 h post administration of 30 mg of dextromethorphan–HBr (197.8 ng/ml dextrorphan, 78.6 ng/ml hydroxymorphinan), (c) nonhydrolyzed blank plasma spiked with 75 ng/ml of each compound.

Table 1
LOQ values for dextromethorphan, dextrorphan, and hydroxymorphinan

| | Concentration (ng/ml) | Inter-assay (R.S.D., %) | Accuracy (%) |
|---|---|---|---|
| Dextromethorphan | 10 | 16.7 | 104.2 |
| Dextrorphan | 10 | 12.9 | 87.0 |
| Hydroxymorphinan | 10 | 13.9 | 88.4 |

$n = 6$.

them followed by vortexing and centrifugation were examined. In all of the above methods the recoveries were poor except for a mixture of 100 μl methanol and 20 μl of a zinc sulfate solution (35%) which was applied to prepare the samples.

### 3.3. Selectivity and specificity

Over 40 hydrolyzed blank plasma obtained from different individuals were injected to check endogenous interferences. No endogenous substance was found to co-elute with analytes.

Several structurally similar compounds such as codeine and dihydrocodeine as well as some inhibitors of CYP2D6 like fluoxetine, propranolol and imipramine also were assessed to check for possible interferences. None of these drugs interfered with the determination of the analytes.

No change in column efficiency and back pressure was observed after 400 injections of samples processed by this method.

### 3.4. Limit of quantitation (LOQ)

LOQ was determined as the lowest concentration which produced a signal-to-noise ratio of at least 5:1 and could be quantified with an inter-assay R.S.D. of <20% and accuracy of 80–120% [23]. LOQ values for four analytes are reported in Table 1.

### 3.5. Linearity

Assay linearity was evaluated over the concentration range of 10–500 ng/ml. The equations for means ($n = 6$) of six standard curves are: for dextromethorphan, $y = 0.79x+0.20$ ($r^2 = 0.999$); for dextrorphan, $y = 1.10x + 2.26$ ($r^2 = 0.998$); and for hydroxymorphinan, $y = 0.79x + 1.50$ ($r^2 = 0.998$). R.S.D. (%) values (slopes, intercepts) were (9.5, 8.9), (5.8, 12.2) and (8.7, 4.6) for dextromethorphan, dextrorphan and hydroxymorphinan, respectively.

### 3.6. Intra- and inter-day precision

The method was found to be reproducible, as indicated by values obtained for R.S.D. (<11.5%) for three analytes. For this reason internal standard was not used. Values are shown in Table 2.

### 3.7. Accuracy and recovery

Recoveries and accuracies ranged from 88.1 to 108.6% and 88.2 to 111.5%, respectively and are reported in Table 2.

### 3.8. Retention time reproducibility

Twenty successive spiked plasma sample were injected into HPLC device to determine the retention time reproducibility. The R.S.D. (%) assessed were 2.5, 2.8, 2.0 and 1.9 for dextrorphan, hydroxymorphinan, dextromethorphan and methoxymorphinan, respectively.

### 3.9. Stability

Stability of QC samples was evaluated at −20 °C for 4 months, at room temperature for 24 h, and after three freeze-thaw cycles. Stability of treated samples was also assessed after 24 h [23]. In all of the conditions, samples preserved their potency (>90%) during the mentioned period.

Table 2
Intra- and inter-assay variability, accuracy and recovery from quantitation of dextromethorphan, dextrorphan, and hydroxymorphinan

| | Concentration (ng/ml) | Intra-assay variability ($n = 5$) | | Intra-assay variability ($n = 5$) | | Recovery ($n = 10$) | |
|---|---|---|---|---|---|---|---|
| | | R.S.D. (%) | Accuracy (%) | R.S.D. (%) | Accuracy (%) | (%) | R.S.D. (%) |
| Dextromethorphan | 20 | 5.8 | 102.9 | 7.7 | 98.8 | 108.6 | 5.4 |
| | 50 | 7.4 | 88.2 | 7.8 | 97.6 | 100.7 | 6.7 |
| | 100 | 5.6 | 95.1 | 6.1 | 107.6 | 98.2 | 8.1 |
| | 200 | 5.8 | 91.7 | 6.6 | 91.0 | 94.2 | 6.3 |
| Dextrorphan | 20 | 4.5 | 100.5 | 3.6 | 95.1 | 97.6 | 4.1 |
| | 50 | 5.4 | 109.3 | 6.4 | 103.7 | 88.5 | 6.3 |
| | 100 | 3.8 | 111.5 | 4.9 | 110.5 | 94.4 | 6.3 |
| | 200 | 6.5 | 103.7 | 4.5 | 104.3 | 88.0 | 5.3 |
| Hydroxymorphinan | 20 | 7.3 | 93.7 | 5.9 | 96.5 | 107.5 | 3.7 |
| | 50 | 6.1 | 101.3 | 3.6 | 102.9 | 93.2 | 4.8 |
| | 100 | 4.6 | 105.9 | 5.6 | 107.7 | 101.1 | 4.5 |
| | 200 | 6.2 | 96.3 | 5.2 | 102.8 | 91.2 | 7.1 |

### 3.10. Application of the method to dextromethorphan phenotyping

The proposed method was applied to determine plasma concentrations of 40 healthy subjects at 0 and 3 h after an oral dose of 30 mg of dextromethorphan–HBr.

Molar metabolic ratios were calculated according to the previously reported procedures [1,18] and antimode of 0.3 was used to differentiate poor and extensive metabolizers of CYP2D6.

A clearly determinable concentration of dextromethorphan was found in the plasma of a poor metabolizer based on the above criterion (35.2 and 100.4 ng/ml, 0.33 for dextromethorphan, dextrorphan and molar metabolic ratio respectively). On contrary to this, in 35 out of 39 extensive metabolizers, dextromethorphan was not detected.

Using more sensitive assay procedures in three different studies [6,18,24], dextromethorphan was not detectable in 35% [6] or all of the extensive metabolizers [18,24] participated in the studies. Eichhold et al. [25] stablished a highly sensitive method to determine the pharmacokinetic profile of dextromethorphan following peroral administration, in which the reported mean $C_{max}$ (0.772 ng/ml) for three extensive metabolizers was below the LOQs achieved by most procedures designed to screen dextromethorphan polymorphism [6,7,18–20,24]. The fact is that plasma concentrations of higher than 10 ng/ml have been detected in poor metabolizers [8,20,26], which is significantly different from those of extensive metabolizers.

In the quick test suggested by Hartter et al. [18], high concentrations of dextromethorphan, which is not conjugated to glucuronic or sulfuric acid in nonhydrolyzed samples, could be used to distinguish poor and extensive metabolizers.

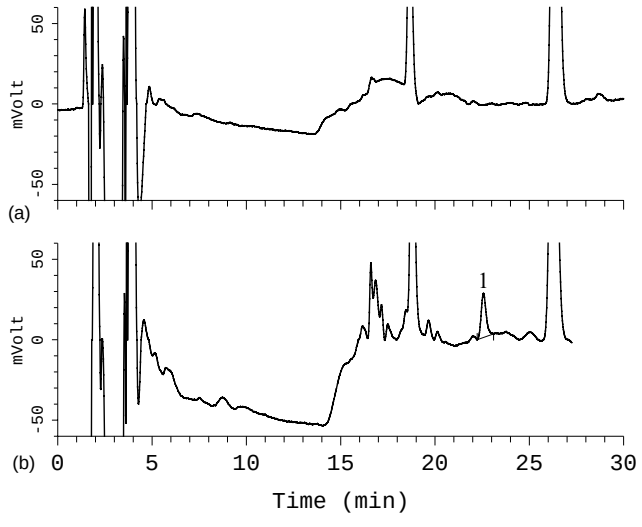


Fig. 3. Chromatogram for dextromethorphan (35.2 ng/ml) (1) (a) nonhydrolyzed blank plasma, (b) nonhydrolyzed sample of the same volunteer (a poor metabolizer) 3 h post-administration of 30 mg of dextromethorphan–HBr. One hundred microliters of acetate buffer was added instead of enzyme solution to prepare the samples.

In a poor metabolizer identified in our study, a measurable concentration of dexteomethorphan was determined without hydrolysis (Fig. 3). Accordingly, although higher than those of previously reported methods, the LOQ achieved in this procedure seems sufficient to determine poor and extensive metabolizers, which is the purpose of this work.

To discriminate extensive metabolism from low absorption, total dextrorphan and hydroxymorphinan were also determined, which ranged between 112.0 and 261.8 ng/ml and 22.7 to 94.8 ng/ml in extensive metabolizers.

## 4. Conclusion

The aim of this study was to develop a selective method for determination of dextromethorphan and its metabolites in plasma samples to assess CYP2D6 activity. The method was designed to be rapid, selective, reproducible, inexpensive and easy to perform. The principal advantage of the method, is the use of a simple protein precipitation sample preparation as part of the chromatographic procedure, yielding recoveries of higher than 88.0%, better than those reported in other papers which use liquid–liquid extraction techniques [7,20]. In addition it is less cumbersome and polluting and a small volume of plasma (100 μl) is needed to quantify three analytes in a single run.

In conclusion the newly developed method allowed the simultaneous analysis of dextromethorphan and its main metabolites with LOQ values that were comparable to those of some previously reported procedures [18,21]. The method is suitable to separate poor metabolizers of CYP2D6.

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