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Short communication

Simultaneous analysis of dextromethorphan and its three metabolites in human plasma using an improved HPLC method with fluorometric detection

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Abstract

A simple and improved HPLC method with fluorometric detection for simultaneous determination of dextromethorphan (DM) and its three metabolites (dextrorphan (DX), 3-methoxymorphinan (MM), 3-hydroxymorphinan (HM)) in human plasma was developed and validated. The method involved a simple and efficient extraction protocol using an *n*-heptane/ethyl acetate (1:1) solvent mixture that achieved recoveries of 70–90% with an insignificant interference from the plasma matrix. The analysis was performed on a phenyl column with isocratic elution, a mobile phase composed of 20% methanol, 30% acetonitrile, and 50% KH_2PO_4 buffer (10 mM, with adding 0.02% of TEA; adjusted with phosphoric acid to pH 3.5), and a run time of only 15 min. Linear calibration curves were constructed in the concentration range of 1–200 nM for DM and its three metabolites. The lower limit of quantitation (LLOQ) in human plasma was 1 nM for each compound. The coefficient of variation and RSE% of the intraday and interday analyses for DM and its three metabolites all complied with USFDA requirements. This analytical method was preliminarily applied to determine the polymorphic functions of CYP2D6 and CYP3A4 in the metabolic pathway of DM to DX and then to HM.

Keywords: Dextromethorphan; Dextrorphan; Methoxymorphinan; Hydroxymorphinan; Phenyl column

1. Introduction

It was earlier demonstrated by Jones et al. [1,2] that biotransformation of dextromethorphan (DM) can be employed as a simultaneous biomarker of CYP3A4 and CYP2D6 activities. DM is primarily transformed to DX via *O*-demethylation by CYP2D6, which is polymorphically expressed in humans who can be classified as poor, intermediate, and extensive metabolizers [3]. DM is *N*-demethylated via an additional route to MM, which is primarily mediated by CYP3A4 in human liver microsomes and intestinal flora [4,5]. DX and MM are then demethylated to HM via CYP3A4 and CYP2D6, respectively [6,7]. Therefore, the simultaneous analysis of DM and its three metabolites at low levels would be practically useful for employing DM as a marker of CYP3A4 and CYP2D6 activity.

The simultaneous detection of DM and its three metabolites with a variety of techniques has been described. LC–MS/MS assay was developed and validated for the determination of DM and its metabolites in human urine [8], in human saliva and urine [9], and in rat everted gut sacs [10]. Selective LC–MS/MS methods for the simultaneous determination of DM and DX in urine samples were developed with improved selectivity and freedom from complications of the matrix effect [11]. A HPLC method to determine DM and its three metabolites in urine samples was achieved on a phenyl column serially connected to a nitrile column with a mobile phase consisting of a mixture of 1.5% ACH, 0.1% triethylamine and 75% acetonitrile using a fluorescence detector [12]. DM and its metabolites were determined in rat serum by liquid–liquid extraction separated on a phenyl column with a mobile phase consisting of 0.05 M potassium phosphate (pH 3.8), 1 mM heptane sulfonic acid, 20% acetonitrile, 2.5%

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methanol and 1% tetrahydrofuran using fluorescence detection [13]. Simultaneous determination of DM and its metabolites in human plasma with fluorescence detection was performed on a C_{18} column with gradient elution [14].

LC–MS/MS is the preferred technique in terms of sensitivity but not necessarily in terms of costs. However, it was demonstrated by Hendrickson et al. that excellent sensitivity could be obtained with only 100 μL of rat serum using a liquid–liquid extraction with fluorescence detection [13] to characterize the concentration–time profiles of DM and DX. In this study, therefore, an isocratic HPLC method eluted with a simplified mobile phase was developed using liquid–liquid extraction with fluorescence detection and with desirable analytical sensitivity and selectivity for DM and its three metabolites, thus enabling its application to the characterization of plasma concentration–time profiles for DM and its three metabolites for polymorphic profiling following oral administration of 30 mg DM in human subjects.

2. Experimental procedures

2.1. Reagents

Dextromethorphan (DM) HBr, dextrorphan (DX) tartrate, and 3-methoxymorphinan (MM) HCl were purchased from RBI (USA). (±)-3-Hydroxymorphinan (HM) HBr and levallorphan (LP) (used as the internal standard) were supplied by Sigma–Aldrich Chemie (Germany). Methanol and acetonitrile were LC grade and obtained from Lab-Scan (Ireland). Triethylamine (TEA), acetic acid (ACH), orthophosphoric acid, sodium carbonate anhydrous and heptane were purchased from Merck (Germany). Potassium dihydrogen phosphate and HCl were supplied by Riedel-de Haën (Germany). Ethyl acetate was provided by Mallinckrodt (USA). Medicon® tablet (containing DM salt equivalent to 15 mg DM base) obtained from Shionogi (Japan).

2.2. Liquid chromatography

The HPLC system consisted of a pump (Jasco PU-980, Japan), a fluorescence detector (Thermo Separation Products, FL3000, USA), and an autosampler (Jasco, As-1555-10). DM and its three metabolites were preliminarily tested on a cyano column (Hypersil® BDS CN, 4.6 mm × 50 mm, 5 μm, Thermo Hypersil, UK), three preconditioned silica columns including LiChrospher Si-60 (4 mm × 250 mm, 5 μm, Merck), μ-Porasil (3.9 mm × 300 mm, 10 μm, waters), and Inertsil (4.6 mm × 250 mm, 5 μm, GL Science, Japan), and a phenyl column (4.6 mm × 250 mm, 5 μm, Thermo Hypersil-Keystone, UK). The capacity factor (k') as defined in the following equation was calculated as the basis for comparison

$$k' = \frac{t_R - t_0}{t_0}$$

where t_0 and t_R are respectively the retention times for the solvent peak and the corresponding compound.

2.3. Sample preparation

Standards containing 1, 2, 4, 10, 20, 40, 100 and 200 nM of DM and its three metabolites and quality control (QC) samples containing 3, 80 and 160 nM of the four compounds were prepared in 0.01N HCl with 40 nM LP as an internal standard. Standard curves were constructed by adding 100 μL freshly prepared stock solutions to 1 mL of plasma in a 10-mL screw-top test tube. Plasma samples were then treated with 500 μL saturated sodium carbonate and briefly vortexed. A 3-mL volume of *n*-heptane–ethyl acetate (1:1, v/v) was added, and the sample was vortexed-mixed for 10 min and centrifuged at 3000 rpm for 10 min. The organic layer was transferred to a separate tube containing 0.2 mL 0.01N HCl, and the analytes were back-extracted into the aqueous layer by vortexing for 5 min. After centrifuging at 3000 rpm for 10 min, the aqueous layer was pipetted out, and 100 μL was injected onto a column for LC analysis. The flow rate was set to 1.0 mL/min. The eluent was detected with fluorescence excitation at 230 nm and emission at 330 nm.

2.4. Assay validation

2.4.1. Calibration

The linearity of the response for each analyte was established by plotting the peak area ratio of the compound to that of the internal standard versus the spiked concentration. A plasma concentration range of 1–200 nM was used to generate the calibration curves. Curves were fit to the data using SISC's linear regression analysis function. Slopes, *y*-intercepts, and standard deviations for these curves were determined and reported.

2.4.2. Precision and accuracy

The intra- and inter-assay coefficients of variation and standard deviations of the mean were used to validate the precision and accuracy of the assay by determining standards of DM and its three metabolites in plasma. For interday validation, six sets of control samples at eight different concentrations were evaluated on 6 different days. For intraday validation, six sets of controls at eight different drug concentrations were assayed with one standard curve on the same run. The slope, intercept and correlation coefficient (r^2) were reported for the linear regressions of all standard curves constructed for the interday and intraday assays. Five QC samples (1, 3, 80, 160 and 200 nM) were randomly assayed along with standard samples during the interday and intraday assays to determine the precision and accuracy with respect to the corresponding constructed standard curves.

The assay recoveries for DM and its metabolites were assessed at three standard concentrations (1, 20 and 200 nM) in triplicate by comparing the peak areas obtained after extraction of these three samples from plasma with the peak areas resulting from the same theoretical concentrations of the standard solutions.

2.5. Human study

Healthy volunteers were screened, and one each of three polymorphic differences in CYP2D6 activity designated as poor

(PM), extensive (EM) and ultrarapid (UM) metabolizers were selected to participate in this preliminary study. The volunteers were asked to fast overnight and to continue fasting for 3 h after drug administration. Blood samples were collected into tubes containing EDTA at 0.5 h for predosing and at 0.5, 1.0, 1.5, 2, 3, 4, 5, 6, 8 and 12 h after oral administration of two Medicon® tablets (equivalent to 30 mg DM base). Plasma samples were collected and stored at −80 °C until analysis. The Ethics Committee of Taipei Medical University Hospital approved this study, and written informed consent was obtained from each subject.

3. Results and discussion

3.1. Column selection

The influence on the k' value of DM and its metabolites on the cyano column with a variety of mobile phase compositions was examined. The results indicated that poor resolution was found between DM and MM and between DX and HM in all conditions examined. Similarly, DM and its metabolites were found to have no desirable resolution by varying the organic solvent/water or ACH/TEA ratios on all three silica columns (Inertsil, μ-Porasil, and Lichropher). Finally 0.02% (v/v) TEA was added and adjusted with phosphoric acid to pH 3.5 in KH_2PO_4 buffer, and the influence of varying acetonitrile/KH_2PO_4 buffer (10 mM) ratios at a fixed methanol content (20%), varying methanol/KH_2PO_4 buffer (10 mM) ratios at a fixed acetonitrile content (30%), and varying methanol/acetonitrile ratios at a fixed volume of KH_2PO_4 buffer (50%, 10 mM) on the capacity factors for DM and its metabolites on the phenyl column was elucidated. The overall results demonstrated that the resolutions among the four compounds for chromatographic separation were greatly improved using the phenyl column.

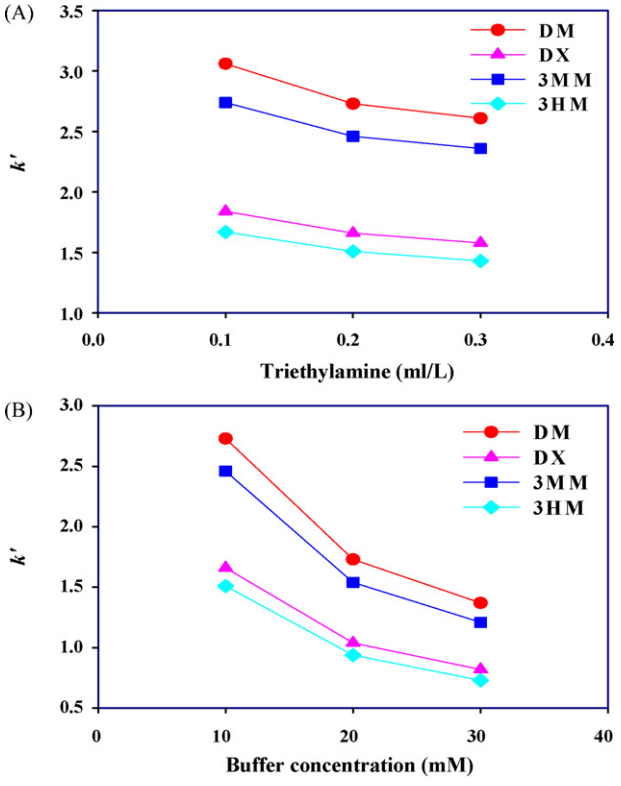

Fig. 1. The influence on the k' value of mobile phases containing different triethylamine concentrations (A) and buffer concentrations (B) in the phenyl column.

3.2. Mobile phase optimization

The phenyl column, therefore, in concert with a mobile phase consisting of 20% (v/v) methanol, 30% (v/v) acetonitrile and 50% (v/v) KH_2PO_4 buffer was further optimized by adjusting with different concentrations of TEA (0.01%, 0.02% and 0.03%) and buffer (10, 20 and 30 mM). The influences of TEA and buffer concentrations on the k' value of DM and its metabolites are shown in Fig. 1A and B, respectively. It obviously indicates that a mobile phase consisting of 20% methanol, 30% acetonitrile and 50% KH_2PO_4 buffer (10 mM, 0.02% of TEA, and pH 3.5) provided the optimal resolution among the four compounds with a 15-min run time. Under these chromatographic conditions, the analytes had the following retention times; HM, 6.1 min; DX, 6.5 min; LP, 7.7 min (internal standard); MM, 9.7 min; and DM, 10.5 min.

3.3. Plasma sample preparation

The addition of several organic solvents like *n*-heptane, *n*-hexane, ethyl acetate, dichloromethane, and their 1:1 mixtures as liquid–liquid extracts of DM and its metabolites from the plasma sample was examined. Overall, the recovery from a mixture of *n*-heptane/ethyl acetate (1:1, v/v) was among the best with the least interference as shown by Fig. 2. Extraction recov-

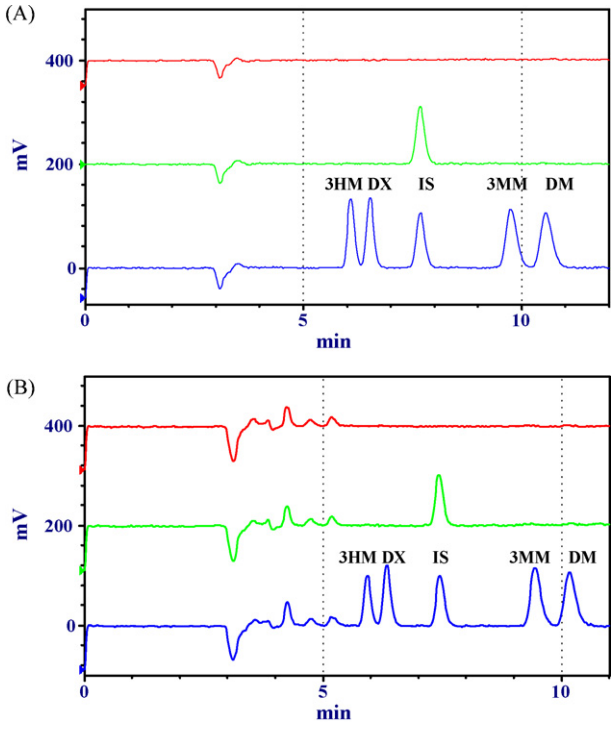

Fig. 2. Chromatograms of an aqueous sample (A) and a plasma sample (B). A blank solution and samples containing known concentrations of DM and its three metabolites (DX, MM), and HM) were separated on the phenyl column.

eries from plasma at the highest (200 nM), intermediate (20 nM), and lowest concentrations (1 nM) for DM, its three metabolites, and IS are determined to be 91.1 ± 0.5, 87.3 ± 2.3, 89.6 ± 2.1, 69.3 ± 3.0 and 91.1 ± 2.2, respectively. Excellent recoveries and precision values were obtained as indicated by the absolute percentages and standard deviations found for them.

3.4. Calibration

The correlation coefficient and variability in the slopes and intercepts of the linear least square equation of the calibration curves for DM and its metabolites were compared from both the interday and intraday assays to examine the precision and accuracy of the quantitative determination. The linearity of the calibration curves for the interday and intraday assays is demonstrated. Regression parameters are listed in Table 1. Results indicate that the correlation coefficients were all >0.999, and the variabilities of the slopes and intercepts were all <5% for all calibration curves constructed from the interday and intraday assays.

3.5. Precision and accuracy

Table 2 shows the accuracy and the precision for the intraday and interday assays at the lower limit of quantitation (LLOQ) and the highest limit of quantitation, and the three QC concentrations for all four compounds. The RSE% and CV% were better than 20% at the LLOQ and better than 15% at the remaining concentrations for all four analytes in both the intraday and interday analyses. This demonstrates the compliance with FDA regulations (both the RSE% and CV% at the LLOQ and at the remaining concentrations must be better than ±20% and ±15%, respectively), so that the accuracies of the pharmacokinetic parameters so derived are not compromised. It was concluded that this analytical method for determining DM and its metabolites in the concentration range of 1–200 nM with an LLOQ of 1 nM is validated to be applicable for analyzing DM and its three metabolites in biological samples.

3.6. Preliminary human study

Utilization of this analytical method for phenotyping CYP2D6 and CYP3A4 in a preliminary human study by assaying DM and its three metabolites was then demonstrated. One subject each of three phenotypes (CYP2D6) designated ultrarapid (UM, subject C), extensive (EM, subject B), and poor metabolizers (PM, subject L) were selected, and the concentration–time profiles of DM and its three metabolites were measured following the oral administration of 30 mg DM with water. The plasma concentration–time profiles are illustrated in Fig. 3, which demonstrates that the validated concentration range of 1–200 nM was sufficiently high to quantify DM concentrations that might be found in a poor metabolizer (subject L) and low enough to quantify that for the ultrarapid metabolizer (subject C). It was also true that concentrations of DX, MM and HM for the three metabolizers with different metabolic rates fell within the validated concentration range of

Table 1
Validation of the calibration equation for dextromethorphan and its metabolites in plasma

	DM	DX	3-MM	3-HM
Interday				
Curve 1	$y = 0.03450x - 0.01051$ ($r^2 = 0.99993$)	$y = 0.02382x - 0.00469$ ($r^2 = 0.99999$)	$y = 0.03593x - 0.02339$ ($r^2 = 0.99993$)	$y = 0.01891x + 0.09025$ ($r^2 = 0.99999$)
Curve 2	$y = 0.03456x - 0.02025$ ($r^2 = 0.99999$)	$y = 0.02381x - 0.00584$ ($r^2 = 0.99998$)	$y = 0.03589x - 0.03248$ ($r^2 = 0.99998$)	$y = 0.01951x - 0.00259$ ($r^2 = 0.99998$)
Curve 3	$y = 0.03469x + 0.00543$ ($r^2 = 0.99995$)	$y = 0.02407x - 0.00300$ ($r^2 = 0.99999$)	$y = 0.03582x - 0.00242$ ($r^2 = 0.99999$)	$y = 0.01951x + 0.00562$ ($r^2 = 0.99999$)
Curve 4	$y = 0.03439x + 0.01835$ ($r^2 = 0.99980$)	$y = 0.02386x + 0.00398$ ($r^2 = 0.99991$)	$y = 0.03592x + 0.00978$ ($r^2 = 0.99981$)	$y = 0.01920x + 0.00598$ ($r^2 = 0.99996$)
Curve 5	$y = 0.03377x + 0.01637$ ($r^2 = 0.99996$)	$y = 0.02367x + 0.00425$ ($r^2 = 0.99995$)	$y = 0.03509x + 0.01143$ ($r^2 = 0.99996$)	$y = 0.01910x + 0.00821$ ($r^2 = 0.99999$)
Curve 6	$y = 0.03453x - 0.00001$ ($r^2 = 0.99998$)	$y = 0.02416x - 0.00427$ ($r^2 = 0.99999$)	$y = 0.03627x - 0.00993$ ($r^2 = 0.99998$)	$y = 0.01949x + 0.00534$ ($r^2 = 0.99996$)
Intraday				
	$y = 0.03423x + 0.00700$ ($r^2 = 0.99999$)	$y = 0.02379x + 0.00318$ ($r^2 = 0.99998$)	$y = 0.03566x - 0.00394$ ($r^2 = 0.99998$)	$y = 0.01919x + 0.00728$ ($r^2 = 0.99996$)

Table 2
Intraday and interday precision and accuracy for quality control in plasma ($n = 6$)

Spiked (nM)	Intraday		Interday	
	Calculated (nM, mean ± S.D.[a])	CV[b], % (RSE[c], %)	Calculated (nM, mean ± S.D.[a])	CV[b], % (RSE[c], %)
DM				
1	1.08 ± 0.14	12.79 (7.60)	1.17 ± 0.07	6.38 (16.69)
3	3.32 ± 0.14	4.13 (10.53)	2.94 ± 0.35	11.81 (−2.01)
80	80.27 ± 1.27	1.58 (0.33)	80.10 ± 0.90	1.12 (0.12)
160	160.23 ± 2.02	1.26 (0.15)	160.48 ± 1.52	0.94 (0.30)
200	199.20 ± 1.45	0.73 (−0.40)	200.53 ± 3.28	1.64 (0.26)
DX				
1	1.03 ± 0.12	11.48 (2.76)	1.08 ± 0.08	7.67 (8.36)
3	3.16 ± 0.08	2.66 (5.33)	2.76 ± 0.36	13.06 (−7.96)
80	80.82 ± 0.89	1.10 (1.03)	80.13 ± 0.75	0.93 (0.17)
160	160.57 ± 2.27	1.42 (0.36)	160.83 ± 1.22	0.76 (0.52)
200	199.36 ± 1.65	0.83 (−0.32)	199.13 ± 2.27	1.14 (−0.43)
3MM				
1	1.14 ± 0.10	8.58 (14.33)	1.14 ± 0.12	10.32 (13.66)
3	3.30 ± 0.19	5.88 (9.98)	2.98 ± 0.24	7.97 (−0.54)
80	79.86 ± 2.19	2.75 (−0.17)	78.85 ± 2.59	3.28 (−1.43)
160	159.17 ± 2.26	1.42 (−0.52)	157.96 ± 4.48	2.84 (−1.28)
200	198.34 ± 3.37	1.70 (−0.83)	199.56 ± 2.35	1.18 (−0.22)
3HM				
1	1.15 ± 0.17	14.90 (14.63)	1.17 ± 0.10	8.88 (17.33)
3	3.28 ± 0.19	5.75 (9.49)	3.00 ± 0.40	13.35 (−0.07)
80	80.79 ± 1.74	2.15 (0.99)	78.37 ± 4.28	5.46 (−2.04)
160	158.93 ± 3.85	2.42 (−0.67)	155.48 ± 9.16	5.89 (−2.83)
200	201.09 ± 2.07	1.03 (0.55)	200.00 ± 4.41	2.21 (0.00)

[a] Standard deviation.
[b] Coefficients of variation.
[c] Relative standard error.

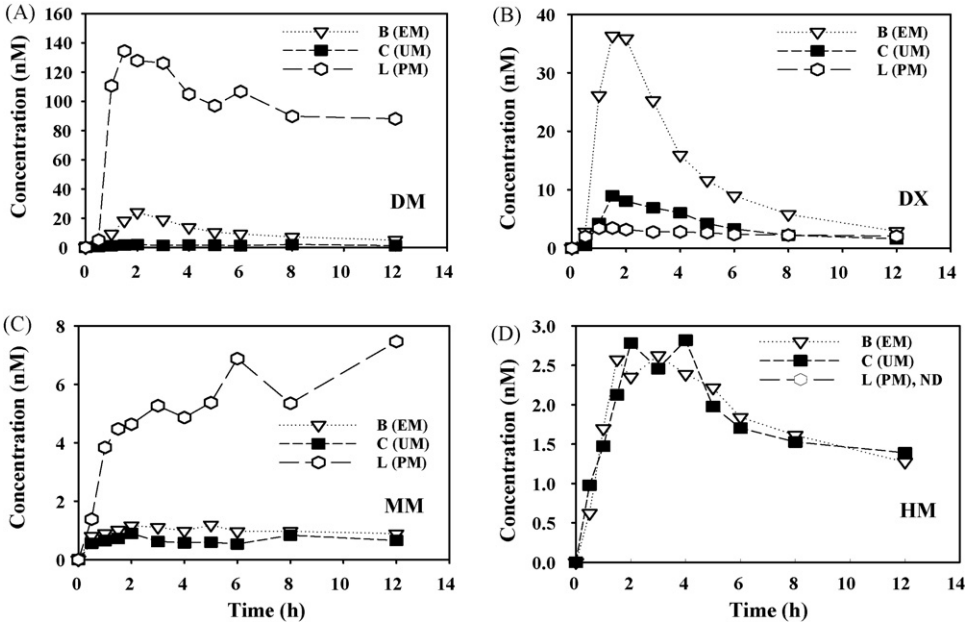

Fig. 3. Individual plasma concentration time profiles of DM (A), DX (B), MM (C) and HM (D) in three volunteers (an extensive metabolizer (EM), subject B; an ultrarapid metabolizer (UM), subject C: and a poor metabolizer (PM), subject L) after oral administration of 30 mg DM base with water (ND: not detectable).

1–200 nM, except that plasma concentrations of MM for EM and UM were so low as to fall below the LLOQ of 1 nM. The metabolic pathway from DM to MM and then to HM is mediated by CYP3A4 and CYP2D6, respectively, whereas that from DM to DX and then to HM is mediated by CYP2D6 and CYP3A4, respectively. Since *N*-demethylation of DM and MM is mainly mediated by CYP2D6, the concentration of MM for those EM and UM subjects for whom the metabolic activity of CYP2D6 is higher than that of CYP3A4 is expected to be at a very low level as a result of the lower extent of biotransformation from DM to MM and the faster metabolism of MM to HM.

4. Conclusions

An inexpensive and reproducible HPLC method using a phenyl column for simultaneous determination of DM and its metabolites in the concentration range of 1–200 nM in human plasma was developed. The recovery of the liquid–liquid extraction was improved and the interference was significantly reduced with a 1:1 solvent mixture of *n*-heptane/ethyl acetate. Under these chromatographic conditions, the run time was shortened to within 15 min, and the LLOQs for DM and its three metabolites in human plasma were determined to be 1 nM. The improved method was validated to be robust and enabled the determination of phenotypes of human subjects following the metabolic pathway from DM to DX and then to HM.

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