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Simultaneous quantification of dextromethorphan and its metabolites dextrorphan, 3-methoxymorphinan and 3-hydroxymorphinan in human plasma by ultra performance liquid chromatography/tandem triple-quadrupole mass spectrometry

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ABSTRACT

A rapid and sensitive ultra performance liquid chromatography/tandem mass spectrometry (UPLC–MS/MS) method has been developed and validated for the simultaneous quantitative determination of dextromethorphan (DM) and its metabolites dextrorphan (DX), 3-methoxymorphinan (3MM) and 3-hydroxymorphinan (3HM), in human lithium heparinized plasma. The extraction involved a simple liquid–liquid extraction with 1 ml *n*-butylchloride from 200 μl aliquots of plasma, after the addition of 20 μl 4% (v/v) ammonium hydroxide and 100 μl stable labeled isotopic internal standards in acetonitrile. Chromatographic separations were achieved on an Aquity UPLC® BEH C_{18} 1.7 μm 2.1 mm × 100 mm column eluted at a flow-rate of 0.250 ml/min on a gradient of acetonitrile. The overall cycle time of the method was 7 min, with elution times of 1.3 min for DX and 3HM, 2.8 min for 3MM and 2.9 min for DM. The multiple reaction monitoring transitions were set at 272 > 215 (*m*/*z*), at 258 > 133 (*m*/*z*), at 258 > 213 (*m*/*z*) and at 244 > 157 (*m*/*z*) for DM, DX, 3MM and 3HM, respectively. The calibration curves were linear ($r^2 \geq 0.995$) over the range of 0.500–100 nM with the lower limit of quantitation validated at 0.500 nM for all compounds, which is equivalent to 136, 129, 129 and 122 pg/ml for DM, DX, 3MM and 3HM, respectively. Extraction recoveries were constant, but ranged from 39% for DM to 83% for DX. The within-run and between-run precisions were within 11.6%, while the accuracy ranged from 92.7 to 110.6%. The applicability of the bioanalytical method was demonstrated and is currently implemented in a clinical trial to study DM as probe-drug for individualized tamoxifen treatment in breast cancer patients.

1. Introduction

Dextromethorphan (DM) is a well known cytochrome P450 2D6 (CYP2D6) phenotyping probe-drug, despite is also being a substrate for CYP3A4 [1]. A probe-drug is a (harmless) drug used to predict the metabolism of a drug of interest in an individual patient at a certain moment in time, by indirectly determining enzyme activity [2]. DM is metabolized by CYP2D6 into dextrorphan (DX) and by CYP3A4 into 3-methoxymorphinan (3MM), while 3-hydroxymorphinan (3HM) is formed through di-demethylation via CYP2D6 and CYP3A4. The combined CYP2D6 and CYP3A4 mediated metabolism makes DM a potential probe to predict tamoxifen pharmacokinetics, as tamoxifen is also metabolized by both CYP2D6 and CYP3A4.

Tamoxifen is an anti-estrogen frequently used in the treatment of breast cancer [3]. The clinical effects of tamoxifen with respect to toxicity and efficacy differ markedly between individuals. This inter-individual variability may lead to diverse clinical responses and drug related toxicities and is influenced by both genetic and environmental factors. For example the role of CYP2D6*4, a single-nucleotide polymorphism in the gene coding for CYP2D6, and leading to a reduced enzymatic activity, is studied intensively [4]. But also non-inherited factors such as co-medication and lifestyle factors contribute to the relatively large inter-individual variability in the metabolism of tamoxifen [5,6]. Although (*in vitro*) literature is consistent with the application of DM as a probe-drug for monitoring both CYP3A4 and CYP2D6 activity, only limited clinical data are available confirming this hypothesis [7].

In view of the use of DM as putative probe-drug for individualized dosing of tamoxifen in breast cancer patients, a bioanalytical

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method of DM and its metabolites DX, 3MM and 3HM in human lithium heparinized plasma needed to be developed and validated.

During the past decade several analytical methodologies have been published describing the development and validation of DM assays, either alone or simultaneously with one or more of its metabolites and/or simultaneously with other CYP probe drugs in biological matrices [8–29]. Only a few of them, however, describe the simultaneous quantification of DM and its metabolites DX, 3MM and 3HM in human urine [9,15,18,19,21], plasma [9,12,23] or saliva [15]. The method published by Spanakis et al. [9] has primarily been developed for the simultaneous analysis of DM and its phase I metabolites for application of *in vitro* CYP2D6 and CYP3A4 inhibitory studies but lacks the sensitivity required for plasma pharmacokinetic analysis of DM and its phase I metabolites, with a lower limit of quantitation (LLQ) of 10 ng/ml for all analytes in 0.5 ml aliquots of plasma. Although the method published by Kristensen [23] has a better sensitivity with an LLQ estimated to be about 0.7 ng/ml for DM and its phase I metabolites, this method is based on capillary electrophoresis and requires 2.5 ml of plasma. The method published recently by Lin et al. [12] is the most sensitive method published so far, with the LLQ validated at 1 nM for DM (271 pg/ml), DX (257 pg/ml), 3MM (257 pg/ml) and 3HM (243 pg/ml), following a double extraction procedure of 1 ml aliquots of plasma. As their method is based on fluorescence detection, adequate base-line separation of the analytes is essential and requires a relative long overall analysis time.

Here, we report on the development and validation of an ultra performance liquid chromatography/tandem mass spectrometry (UPLC–MS/MS) method for the simultaneous determination of DX, DM, 3MM and 3HM in 200 μl aliquots of human lithium heparinized plasma. The method presented is more sensitive, involves a single liquid–liquid extraction, is more selective and required less plasma than the previously published methods for plasma [9,12,23] and has been thoroughly validated in accordance with the Guidance for Industry, Bioanalytical Method Validation, as specified by the FDA (www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070107.pdf).

2. Experimental

2.1. Chemicals

DM, DX, 3MM and 3HM were obtained from Sigma–Aldrich (Zwijndrecht, The Netherlands). The stable labeled deuterated internal standards dextromethorphan-d3 (DM-d3), dextrorphan-d3 (DX-d3) and 3-hydroxymorphinan-d3 (3HM-d3) were obtained from Toronto Research Chemicals (North York, ON, Canada). All chemicals were of analytical grade or higher. Acetonitrile, methanol and water were purchased from Biosolve BV (Valkenswaard, The Netherlands). Dimethylsulphoxide (DMSO) was from Sigma–Aldrich and *n*-butylchloride from Rathburn (Walkerburn, Scotland). Formic acid and ammonium hydroxide were obtained from J.T. Baker (Deventer, The Netherlands) and 2-propanol from Merck (Darmstadt, Germany). Blank human lithium heparinized plasma was purchased from Biological Specialty Corporation (Colmar, PA).

2.2. Preparation of stock solutions, calibration standards and quality control samples

Individual stock solutions were prepared for DX, DM, 3MM and 3HM at a concentration of 1.00 mM free base in DMSO. Stock solutions were aliquoted and stored at $T < -70\,^{\circ}C$. Separate stock solutions were prepared (i.e., independent weightings) for the construction of the calibration curve standards and the pools of quality control samples. A working stock solution containing 20.0 μM of DX, DM, 3MM and 3HM was prepared in DMSO, aliquoted and stored at $T < -70\,^{\circ}C$, which was used throughout the validation for the construction of the calibration curve standards.

Individual internal standard stock solutions of DX-d3, DM-d3 and 3HM-d3 were prepared at 1 mg/ml free base in DMSO, which subsequently was aliquoted and stored at $T < -70\,^{\circ}C$. The individual stock solutions were simultaneously 80,000-fold diluted in acetonitrile, resulting in an internal standard working solution containing 12.5 ng/ml DX-d3, DM-d3 and 3HM-d3, which was stored in a refrigerator at $T < 8\,^{\circ}C$ for a maximum of 3 months.

Calibration standards were prepared in duplicate on the day of analysis, by addition of 25 μl aliquots of appropriate dilutions of the working stock solution in acetonitrile/DMSO (1:1, v/v) to 475 μl aliquots of human lithium heparinized plasma at the following concentrations: 0.500, 1.00, 5.00, 10.0, 25.0, 50.0, 90.0, and 100 nM DX, DM, 3MM and 3HM as free base.

Five pools of quality control (QC) samples were prepared in human lithium heparinized plasma at concentrations of 0.500 nM (LLQ), 1.50 nM (QC-Low), 40.0 nM (QC-Middle), 80.0 nM (QC-High) and 1600 nM (QC-Diluted). QC-Diluted was processed after a 20-fold dilution in blank human lithium heparinized plasma. Pools of QC samples were aliquoted and stored at $T < -70\,^{\circ}C$ upon processing.

2.3. Plasma sample preparation

Aliquots of 100 μl of internal standard solution and 20 μl of a 4% (v/v) ammonium hydroxide solution were added to 200 μl of plasma samples in 2 ml microcentrifuge tubes. Subsequently 1 ml aliquots of *n*-butylchloride were added. Hereafter, the samples were vigorously mixed for 10 min and then centrifuged at 18,000 × *g* at ambient temperature for 10 min. Aliquots of 950 μl of the organic phase were transferred into 4.5 ml glass tubes and evaporated under nitrogen at $T = 70\,^{\circ}C$. The residues were resuspended in 100 μl aliquots of acetonitrile/water/formic acid (20:80:0.1, v/v/v). After centrifugation of 30 s at 4000 × *g*, the supernatants were transferred into 350 μl 96-well plates, which were placed into the chilled ($T = 10\,^{\circ}C$) autosampler, from which aliquots of 10 μl were injected onto the UPLC column.

2.4. Equipment

The UPLC–MS/MS system was composed of a Waters Aquity UPLC Sample Manager coupled to a Waters TQ Detector (Waters, Etten-Leur, The Netherlands). The MassLynx V4.1 SCN627 software package was used for the acquisition and processing of data. Quantification was performed using QuanLynx as implemented in the MassLynx software.

2.4.1. Chromatographic conditions

Analytes were separated on an Aquity UPLC® BEH C_{18} column 1.7 μm, 100 mm × 2.1 mm (Waters, Etten-Leur, The Netherlands) thermostatted at $T = 40\,^{\circ}C$. A gradient, at a flow-rate of 0.250 ml/min, was achieved with mobile phase A, composed of water, acidified with 0.1% formic acid and mobile phase B, composed of acetonitrile acidified with 0.1% formic acid. Following a 10 μl injection, analytes were eluted and separated on an isocratic 30% mobile phase B during 4 min, followed by a linear gradient to 100% of mobile phase B from 4 to 5 min to elute matrix components potentially remaining on the column in this relatively hydrophilic mobile phase. Subsequently, a linear gradient was applied from 100% to 30% of mobile phase B over 2 min in order to re-equilibrate the system. The overall run time of the assay was 7 min. The strong needle wash solvent was

Table 1
MS/MS settings.

Analyte	Scan window (min)	Parent (m/z)	Daughter (m/z)	Collision (V)
DM	2.00–7.00	272	215	25
DM-d3	2.00–7.00	275	171	36
DX	0.00–2.00	258	133	35
DX-d3	0.00–2.00	261	157	35
3MM	2.00–7.00	258	213	25
3HM	0.00–2.00	244	157	35
3HM-d3	0.00–2.00	247	160	35

composed of acetonitrile/methanol/water/2-propanol/formic acid (25:25:25:25:0.1, v/v/v/v/v), while the week needle wash solvent was composed of acetonitrile/water (30:70, v/v). The column effluent was passed through the mass spectrometer and monitored.

2.4.2. Mass spectrometry

Tandem mass spectrometry was performed in the positive ion electrospray ionization mode. Mass transitions of m/z were optimized for DX, DM, 3MM and 3HM and the internal standards DX-d3, DM-d3, and 3HM-d3 by infusion of the respective analytes in acetonitrile/water/0.1% formic acid (40:60:0.1, v/v/v) via combined infusion. Optimal MS settings were manually adjusted. The desolvation gas was set at 800 l/h and the cone gas at 25 l/h (nitrogen). The ionspray voltage was kept at 1.50 kV and the cone voltage at 45 V for all compounds, with a source temperature of $T = 150\,^{\circ}C$ and desolvation temperature of $T = 350\,^{\circ}C$. The dwell times were set at 40 ms and the inter-channel delay at 100 ms. Multiple reaction monitoring (MRM) mode was applied as presented in Table 1. The collision cell pirani pressure was set at $\sim 5\,e^{-3}$ mbar (argon).

2.4.3. Quantitation

Calibration curves were generated using peak area ratios of the components to internal standards versus the known concentrations with a linear regression equation of $1/\text{concentration}^2$.

2.5. Method validation

The UPLC–MS/MS method was validated in accordance with the Guidance for Industry, Bioanalytical Method Validation, as specified by the FDA (www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070107.pdf).

The potential presence of endogenous contaminating compounds that may interfere with the analytical assay was determined by analyzing blank human lithium heparinized plasma samples of ten different lots. The following substances were investigated for interference with the analytical method: aprepitant, dexamethasone, domperidon, granisetron, lorazepam, metoclopramide, oxazepam, paracetamol and ranitidine. All drugs have been dissolved and/or diluted in water to a concentration of 1 mg/ml followed by a 500-fold dilution in human lithium heparinized plasma (final concentrations 2 μg/ml). To investigate the potential interference of the validated bioanalytical method in a clinical study in patients on tamoxifen therapy, plasma samples collected from patients prior to the administration of DM (see Section 2.6) were included as well. Aliquots of QC-Diluted (i.e., 1600 nM) have been diluted in the plasma containing the different drugs and the incurred samples of the patients on tamoxifen therapy, to yield final DM, DX, 3MM and 3HM concentrations of 1.50 and 40.0 nM, which have been processed and compared to equal dilutions of QC-Diluted in blank human lithium heparinized plasma.

For the determination of the LLQ, blank human lithium heparinized plasma of 10 different donors was spiked at a concentration of 0.500 nM for all compounds at 2 different days. Accuracy (ACC), within-run precision (WRP) and the between-run precision (BRP) were determined by analyzing 5 replicates of pools of LLQ and QC samples independently over a 4-run period (3-run period at the LLQ), with the calibration curve standards processed in duplicate. The ACC, WRP and BRP at the level of the LLQ and QC samples were calculated by one-way analysis of variance, using the run as the variable as described previously [30,31].

The evaluation of the matrix effect for both compounds was tested by comparing the MS/MS response of DX, DM, 3MM and 3HM at a concentration of 25.0 nM in triplicate in six lots of blank lithium heparinized plasma to the MS/MS responses of the analytes spiked in triplicate into blank plasma extracts as described recently [30].

Extraction recovery (RE) was determined by comparing the MS/MS response of DX, DM, 3MM and 3HM at 25.0 nM spiked in triplicate into six lots of lithium heparinized plasma before extraction, to the MS/MS response of DX, DM, 3MM and 3HM spiked in triplicate into extracts of blank processed human lithium heparinized plasma [30], corrected for the evaporated volume of organic phase.

The stability of the compounds in human lithium heparinized plasma was tested with QC-Low and QC-High following an overnight (i.e., ~18 h) storage at ambient temperature, following 3 freeze–thaw cycles, in which the samples were thawed for at least 15 min before refreezing them for at least 18 h and during long-term storage stability at $T < -20\,^{\circ}C$ and $T < -70\,^{\circ}C$. The storage stability of processed samples in the autosampler was tested in triplicate at the same concentrations. QC samples were processed in triplicate and repeatedly injected at different time points.

2.6. Application of method to clinical samples

To demonstrate the applicability of the validated bioanalytical method, blood samples were collected from breast cancer patients on steady state tamoxifen therapy receiving a single oral dose of DM (see www.trialregister.nl; NTR study number 1751). Blood samples (4 ml) were collected in the presence of lithium heparin as anticoagulant at pre-dose and 30 min, 1, 1.5, 2, 4, 6, 10 and 22 h after the administration of 30 mg DM–hydrobromide monohydrate (i.e., 22 mg DM as free base). Blood samples were centrifuged within 15 min after collection for 10 min at 2800–3000 × g at $4\,^{\circ}C$. The plasma was stored at $T < -70\,^{\circ}C$, until analysis. All patients gave written informed consent and the local institutional review boards approved the clinical protocol (METC 09-157), which was written in accordance with the declaration of Helsinki (see: http://www.wma.net/e/policy/b3.htm).

2.6.1. Pharmacogenetic analysis

An additional blood sample was collected for pharmacogenetic analysis. Genetic polymorphisms encoding differences in CYP2D6 activity (http://www.cypalleles.ki.se/cyp2d6.htm) were determined by TaqMan 5-nuclease and XL-PCR assays, analyzing for the null variants *3, *4, *5 and *6, the decreased activity alleles *10, *17 and *41 alleles, and for gene duplications. Patients were designated as poor (PM), intermediate (IM), extensive (EM) or ultrarapid (UM) metabolizers based on detection for PM of two null alleles, for IM a null allele in combination with a wild type allele or detection of two decreased activity alleles, for EM absence of variant alleles and for UM presence of a gene duplication in absence of variant alleles.

3. Results and discussion

3.1. LC–MS/MS conditions and method development

The product ion spectra (Fig. 1) yield abundant product ions suitable for use in multiple reactions monitoring of DM, DX, 3MM

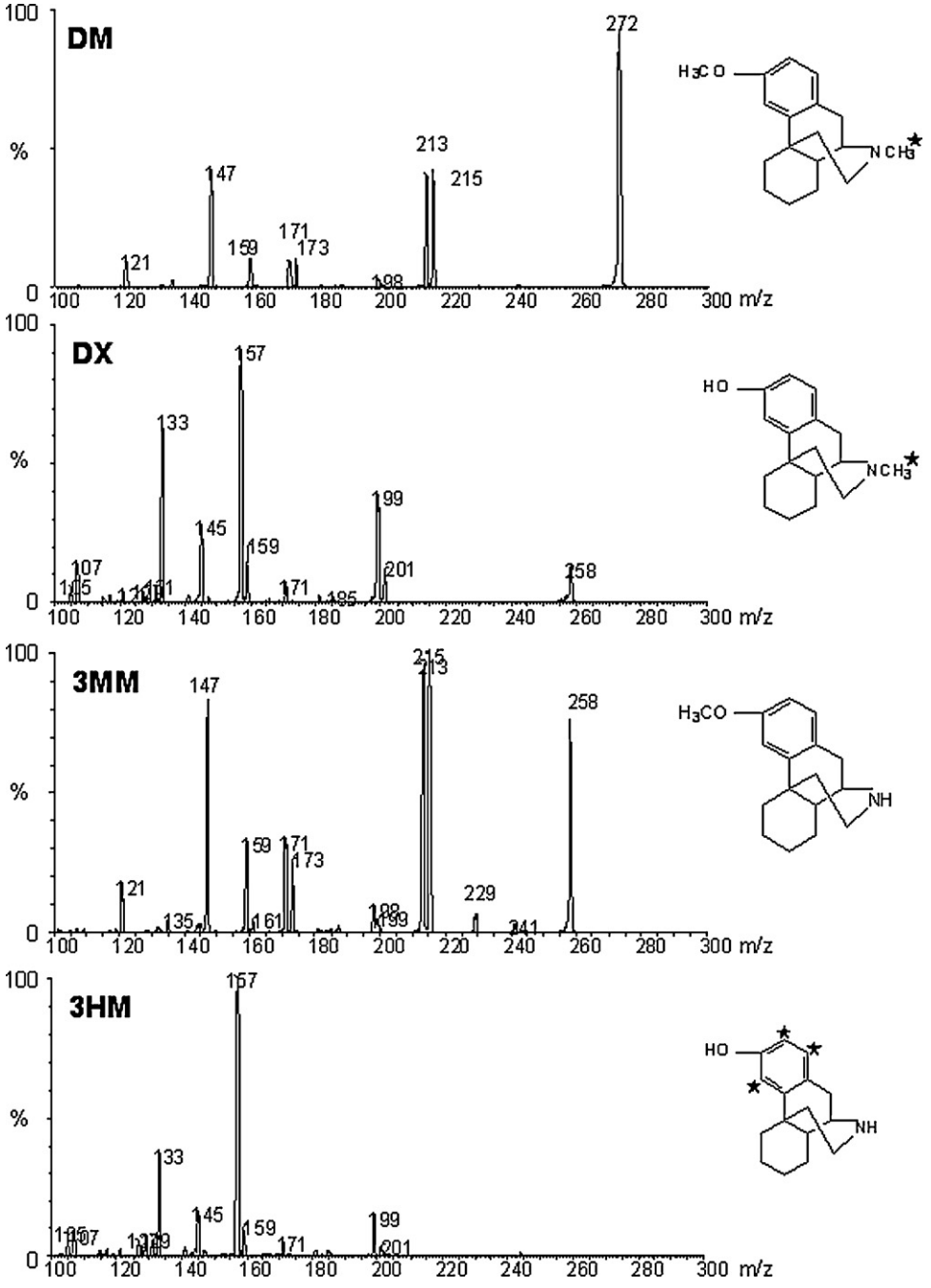

Fig. 1. Mass spectrum and chemical structures of dextromethorphan (DM), dextrorphan (DX), 3-methoxymorphinan (3MM) and 3-hydroxymorphinan (3HM). The stars represent the deuterium atoms in the isotopic internal standards DM-d3, DX-d3 and 3HM-d3.

and 3HM. See Table 1 for selected product ions and collision energies of the analytes and their respective isotopic internal standards. The collision of DM and DM-d3 and of DX and DX-d3 results in equal daughter ions. Primarily during the method development, the same daughter ions for DM and DM-d3 and for DX and DX-d3 were used for the quantitation. However, small peaks of DM and DX were observed in blank processed plasma samples (i.e., blank samples spiked with the internal standard solution). Although the exact underlying explanation is not clear (most likely cross-talk between the different MRM transitions) we resolved this by selecting other daughter ions for the internal standards.

By applying a simple isocratic elution, the metabolites DX and 3MM with equal parent masses and overlapping daughter ions, could be adequately base-line separated, while maintaining a relative short injection to injection time of 7 min with elution times of 1.3 min for DX and 3HM, 2.8 min for 3MM and 2.9 min for DM (Fig. 2). No equilibration time at the end of the gradient of 7 min was required, as the system is fully equilibrated during the time required for the sample injection, which takes approximately 0.5 min.

For 3MM no stable labeled internal standard was available and it was anticipated that DM-d3 would be the most suitable internal standard for the quantitation of 3MM as they co-eluted from the analytical column. During the validation, however, DM-d3 turned out to be a poor internal standard for 3MM, better results were instead obtained with DX-d3.

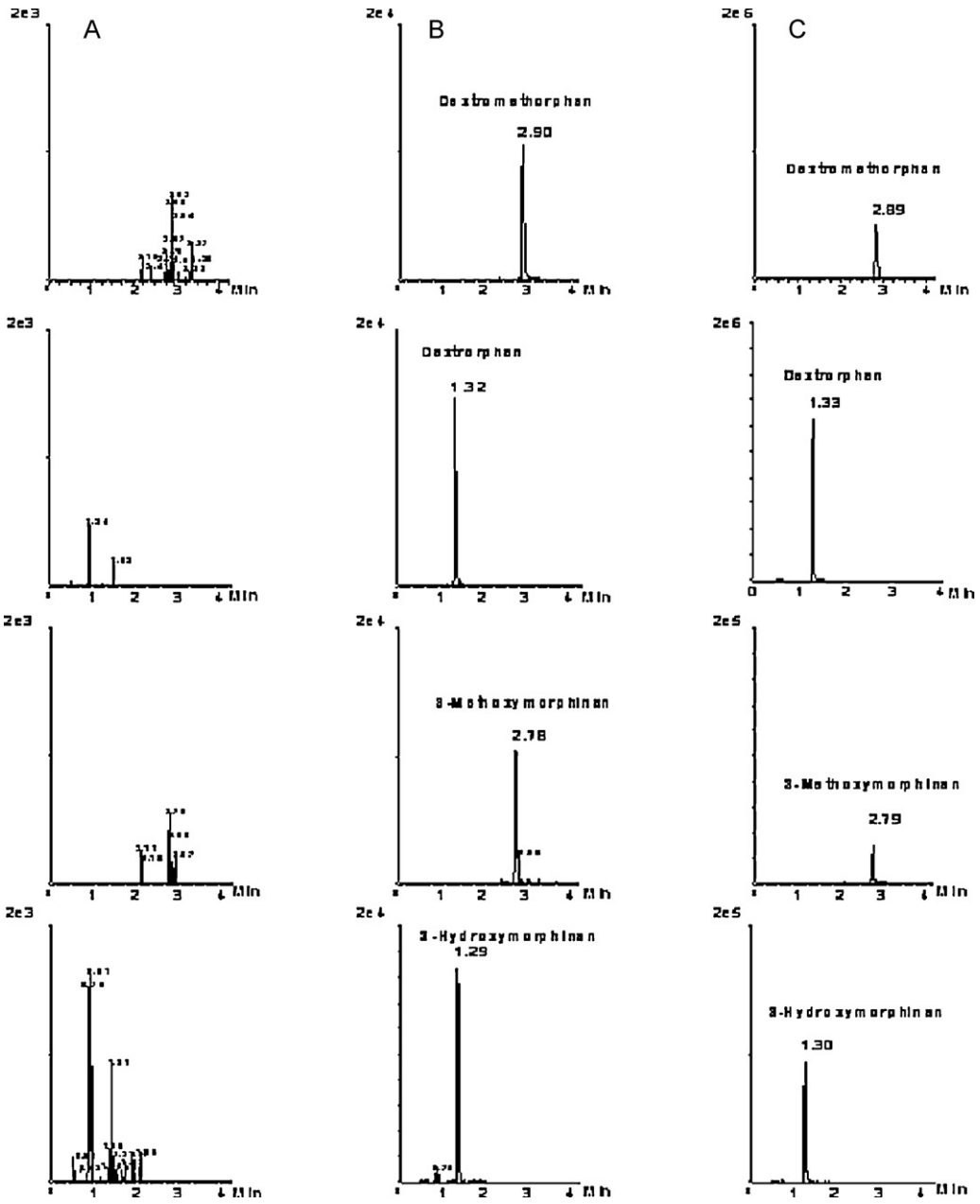

Fig. 2. Representative chromatograms of (A) a double blank processed plasma sample collected pre-dose prior to the first administration of 22 mg DM, (B) a plasma sample spiked at a concentration of 0.500 nM (i.e., LLQ) and (C) a plasma samples collected 1 h after the administration of 22 mg DM containing 16.2 nM of DM, 47.5 nM of DX, 1.43 nM of 3MM and 3.62 nM of 3HM.

3.2. Assay performance

The method results were linear for all compounds ($r^2 \geq 0.995$) in the concentration range of 0.500–100 nM in human lithium heparinized plasma and none of the blank plasma samples showed potential interference for DM, DX, 3HM, MM or any of the labeled internal standards.

None of the tested, potentially co-administered drugs interferes with the quantitation of DM, DX or 3HM. A minor impact of metoclopramide (19% at the concentration of 40 nM and 29% at the concentration of 1.50 nM) on the quantitation of 3MM was observed. This effect is related to the absence of a stable labeled isotope for 3MM. As a result of a minor ionization effect on DX-d3, the concentrations of 3MM were slightly over-estimated. As the validated method will be used, amongst others, for the quantitation of DM and its metabolite concentrations in patient samples enrolled in a clinical study in patients on oral tamoxifen therapy, samples collected from those patients were spiked with DM, DX, 3MM and 3HM. No interference was observed for DM, DX, 3MM and 3HM.

The LLQ was validated at 0.500 nM for all analytes which is equivalent to 136, 129, 129 and 122 pg/ml for DM, DX, 3MM and 3HM, respectively. The LLQ has been validated in two separate runs in which the analytes were spiked to ten different lots of human lithium heparinized plasma. The measured concentrations of DM for all 20 independently spiked plasma samples fell within the acceptable range of accuracy of 80–120%, with an average measured concentration of 0.506 ± 0.040 nM. For DX, also the back-calculated concentration of all 20 processed samples fell within the acceptable range of accuracy, with an average observed concentration of 0.506 ± 0.030 nM. For 3MM, measured concentrations in 17 of 20 independent samples fall within the acceptable range of accuracy, with an average concentration, using all 20 observed values,

Table 2
Calculations of the between-run and within-run precisions and the average accuracy of the LLQ and QC samples.[a]

Sample	Spiked (nM)	GM (nM)	ACC (%)	WRP (%)	BRP (%)	n[b]
Dextromethorphan (DM)						
LLQ	0.500	0.540	108.0	6.58	0.21	15 of 15
Low	1.50	1.39	92.7	6.37	1.50	18 of 20
Middle	40.0	39.0	97.5	2.80	#[c]	20 of 20
High	80.0	79.0	98.8	2.34	2.16	20 of 20
Diluted	1600	1585	99.1	2.08	0.97	20 of 20
Dextrorphan (DX)						
LLQ	0.500	0.474	94.8	6.09	2.71	15 of 15
Low	1.50	1.40	93.3	3.87	3.46	18 of 20
Middle	40.0	39.3	98.3	2.75	3.00	20 of 20
High	80.0	79.0	98.8	1.92	2.89	20 of 20
Diluted	1600	1597	99.8	1.39	#[c]	20 of 20
3-Methoxymorphinan (3MM)						
LLQ	0.500	0.553	110.6	7.71	#[c]	13 of 15
Low	1.50	1.44	96.0	3.91	8.76	19 of 20
Middle	40.0	37.9	94.8	6.25	9.49	17 of 20
High	80.0	79.2	99.0	4.08	11.64	17 of 20
Diluted	1600	1738	108.6	4.09	2.78	18 of 20
3-Hydroxymorphinan (3HM)						
LLQ	0.500	0.502	100.4	10.16	#[c]	15 of 15
Low	1.50	1.55	103.3	3.95	3.56	20 of 20
Middle	40.0	41.0	102.5	3.51	#[c]	20 of 20
High	80.0	81.1	101.4	3.11	1.73	20 of 20
Diluted	1600	1626	101.6	2.21	2.84	20 of 20

Abbreviations: GM, grand mean; WRP, within-run precision; BRP, between-run precision; ACC, average accuracy.
[a] $n = 5$ in 4 separate runs (3 runs at the LLQ).
[b] Number of individual samples falling within acceptable range of accuracy of 85–115% (80–120% at LLQ).
[c] No additional variation observed by performing the assay in different runs.

of 0.453 ± 0.044 nM. The average concentration for 3HM in the 20 samples (19 in acceptable range) was 0.518 ± 0.048 nM. A representative chromatogram at the level of the LLQ is presented in Fig. 2B.

The within-run and between-run precisions and the accuracies at five tested concentrations, including at the level of the LLQ, are summarized in Table 2 and all fell within the accepted ranges as specified by the FDA (www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070107.pdf). As shown, the between-run precisions for 3MM are higher than those observed for the other analytes, most likely due to the absence of a commercially available stable labeled isotope of 3MM that can be used as internal standard.

The extraction recovery (RE) and matrix effect (ME) were determined in lithium heparinized plasma from six different lots spiked with DM, DX, 3MM and 3HM at a concentration of 25.0 nM. The mean measured extraction efficiencies and matrix effect are shown in Table 3. As shown, enhanced signals were observed for DM, 3MM and 3HM, while no matrix effect was observed for DX. The recoveries ranged from 39% for DM to 83% for DX.

Table 3
Extraction recovery (RE) and matrix effect (ME) in lithium heparinized plasma from six different lots spiked with all analytes at a concentration of 25.0 nM.

Analyte	ME (%)	RE (%)
DM	178 ± 6.6	39 ± 7.5
DM-d3	175 ± 5.8	38 ± 7.1
DX	107 ± 11.7	83 ± 8.3
DX-d3	102 ± 5.1	82 ± 4.3
3MM	143 ± 3.9	58 ± 4.9
3HM	135 ± 6.1	58 ± 4.2
3HM-d3	136 ± 5.6	53 ± 3.8

Data presented as mean ± SD ($n = 6$).

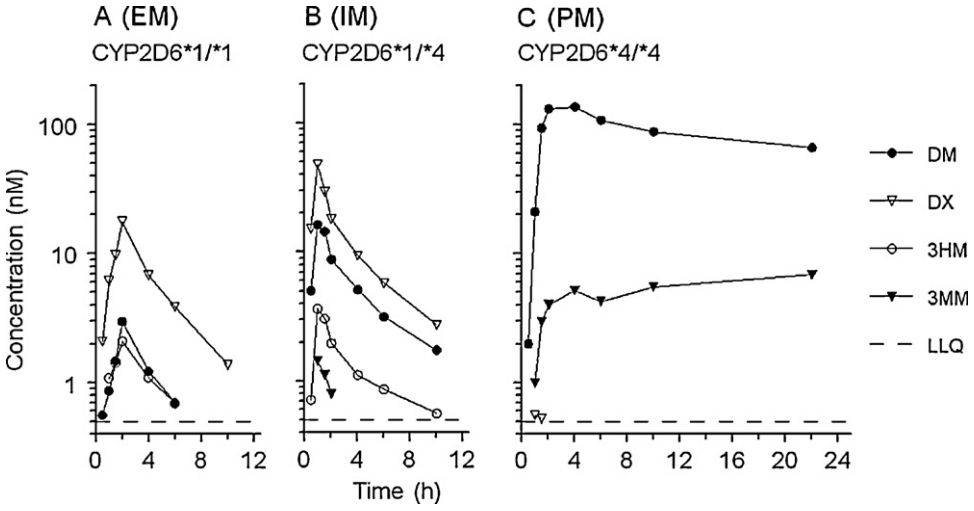

Fig. 3. Plasma concentration–time profiles of DM (filled circles), DX (open triangles), 3MM (filled triangles) and 3HM (open circles) after an oral dose of 22 mg DM to an extensive (CYP2D6*1/*1 (A)), an intermediate (CYP2D6*1/*4 (B)) and a poor (CYP2D6*4/*4 (C)) CYP2D6 metabolizer. The dotted line represents the level of the LLQ of 0.500 nM.

DM, DX, 3MM and 3HM were stable in lithium heparinized plasma at ambient temperature during overnight incubation, following 3 consecutive freeze–thaw cycles, as processed samples for at least 16 h in the chilled ($T = 10\,^{\circ}C$) autosampler and for at least 7 months when stored at $T < -20$ and $T < -70\,^{\circ}C$. Mean values of triplicate back calculated concentrations at the level of QC-Low (1.50 mM) and QC-High (80.0 nM) fall within 90–103% of the reference values for DM, within 90–104% for DX, within 83–113% for 3MM and within 91–108% for 3HM.

3.3. Clinical application

The described analytical method was applied in our institute to a pharmacokinetic study following a single oral administration of 22 mg DM to breast cancer patients on steady state tamoxifen therapy. Representative plasma concentration–time profiles of an extensive, an intermediate and a poor CYP2D6 metabolizer are shown in Fig. 3. As shown, in the EM, 3MM concentrations were all below the LLQ, while 3MM was the most abundant metabolite in the PM, in which no 3HM could be quantitated and DX only in 2 samples. Representative chromatograms of the intermediate metabolizer are shown in Fig. 2A and C.

4. Conclusion

A selective, sensitive and accurate method has been developed and validated for the simultaneous analysis of DM and its three phase I metabolites, DX, 3MM and 3HM. The validation method meets the current requirements of bioanalytical method validation and will be implemented in current and future clinical pharmacokinetic studies with DM as probe-drug for individualized tamoxifen treatment.

Although the method has been validated in lithium heparinized plasma, plasma derived from blood collected in the presence of other anticoagulants (i.e., potassium EDTA), would in all probability be applicable as well. Note that similar to other changes in the procedure, e.g., change of analytical column or extraction solvent, a partial validation of the method using other anti-coagulants will be required.

In addition, DM and DX are known to be glucuronidated and as described by Kristensen [23], the sum of the unconjugated and conjugated metabolites might be quantitated following a deglucuronidation step prior to the sample extraction procedure by the addition of a β-glucuronidase solution. In our clinical study, in which we intent to correlate the phase I metabolic profiles of tamoxifen and dextromethorphan, we do not require the metabolic profile of the conjugated metabolites. Nevertheless, the method can in all likelihood be adapted to quantitate the sum of unconjugated and conjugated metabolites as well.

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