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High performance liquid chromatography determination of dextromethorphan and its metabolites in urine using solid-phase extraction[1]

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Abstract

A high performance liquid chromatography assay coupled with fluorescence detection was developed for the determination of dextromethorphan and its metabolites in urine. The products and the internal standard, pholcodine, were separated on an Alltima C18, 5 μm column (250 × 4.6 mm), using a mobile phase containing sodium dodecyl sulphate (1 mM) in a mixture of acetonitrile–sodium dihydrogen phosphate (0.01 M) (40.5:59.5, v/v) (pH* = 2.5). A novel solid-phase extraction procedure with strong cation exchange, non end-capped, Isolute SCX cartridges allows good recovery of the products (mean 85% or more). For all analytes, the assay is sensitive (LOQ 25 ng ml^{-1}, using 200 μl urine), reproducible (RSD < 15%) and accurate (< 15% deviation of the nominal value) over the range evaluated. This method can be used to measure dextromethorphan and its metabolites to phenotype individuals as poor or extensive metabolizers of drugs metabolized by CYP2D6.

Keywords: Dextromethorphan; Fluorescence; Metabolites; RP-HPLC; Solid-phase extraction; Urine

1. Introduction

After oral administration to humans, the antitussive agent dextromethorphan (DM), is rapidly and extensively metabolized by *O*- and *N*-demethylation followed by conjugation (Fig. 1). The *O*-demethylation of DM to dextrophan (DX) is catalysed by the polymorphically expressed CYP2D6 isoenzyme [1]. DX is *N*-demethylated to 3-hydroxymorphinan (3OHM). DM also undergoes *N*-demethylation to form 3-methoxymorphinan (3MM) probably by the CYP3A4 isoenzyme [2] and is further *O*-demethylated to 3-hydroxymorphinan. The main metabolites found in urine as conjugates are DX and 3OHM. DM is now widely used as a suitable probe to phenotype for CYP2D6, based on the determination of the DM/DX metabolic ratio in urine. It can perhaps also be used for the quantitation of CYP3A subfamily activity in vivo (DM/3MM metabolic ratio in urine) [3,4].

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As the concentrations of DM and 3MM in the urine are sometimes very low, sensitive assays for these compounds are necessary. Several high performance liquid chromatography (HPLC) methods have been described for the determination of DM and its metabolites in urine [5–9]. Most HPLC determinations are done with low-UV detection [5,6] or with fluorescence detection [7–9]. UV detection, however, lacks sensitivity [5,9]. Mostly, phenyl [5,6,8,9] or cyano [7] stationary phases are used. For sample preparation from urine, multiple liquid–liquid extraction [5,7,8], precolumn switching [6], and solid-phase extraction with weak ion-exchange columns [9] are used. In most of these methods extraction recoveries are low for the metabolites [7,8] and/or for DM [7–9].

In this work a micellar reversed phase (RP)-HPLC method is developed for the determination of DM and its metabolites in urine, using a pH-stable C18 column. To enhance extraction recovery from urine, extraction of DM and its metabolites is done by solid-phase extraction with strong cation-exchange columns.

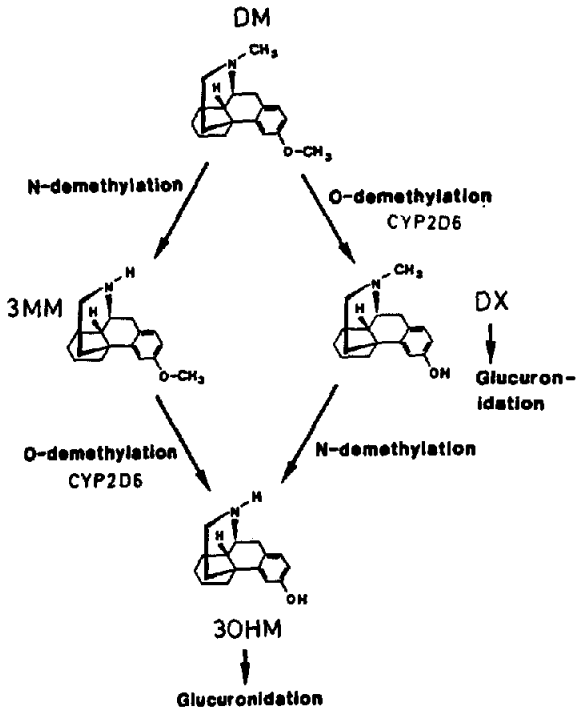

Fig. 1. Chemical structures and metabolic pathways of dextromethorphan.

2. Experimental

2.1. Chemicals

Dextromethorphan hydrobromide, dextrophan tartrate, (+)-3-methoxymorphinan hydrobromide and (+)-3-hydroxymorphinan hydrochloride were kindly supplied by Hoffmann–La Roche (Basle, Switzerland). Pholcodine was from Belgopia NV (Louvain-la-Neuve, Belgium). Acetonitrile (far-UV) HPLC grade was purchased from Labscan (Darmstadt, Germany). The β-glucuronidase/β-arylsulphatase (type H-5, from *Helix pomatia*, 100 000 units ml^{-1}) was from Serva (Heidelberg, Germany). Glucose-6-phosphate dehydrogenase, glucose-6-phosphate and NADP were purchased from Sigma Chemical Company (St. Louis, MO). All other chemicals were reagent grade from Merck (Darmstadt, Germany). Isolute SCX (benzene sulphonic acid, strong cation exchanger, non end-capped, 200 mg per 3 ml) cartridges were purchased from IST (Hengoed, Mid-Glamorgan, UK). Pure water, 18 Ω, was obtained using a Gelman Water-I Laboratory System (Gelman Science, Ann Arbor, MI).

2.2. Preparations of standards

Stock solutions (1 mg ml^{-1}) of the four products and the internal standard were prepared in water and stored at −20°C. Working solutions were prepared from these stock solutions.

2.3. Apparatus

The HPLC system consisted of a SP 8810 solvent pump (Spectra Physics, San Jose, CA), a SP 8780 XR autosampler and a SP 4290 integrator for measuring the peak heights. The detector was a Waters 470 scanning fluorescence detector (Millipore Corporation, Milford, MA), and was used at an excitation wavelength of 200 nm and an emission wavelength of 310 nm. Samples were processed with a Vacmaster™ 20 apparatus (IST).

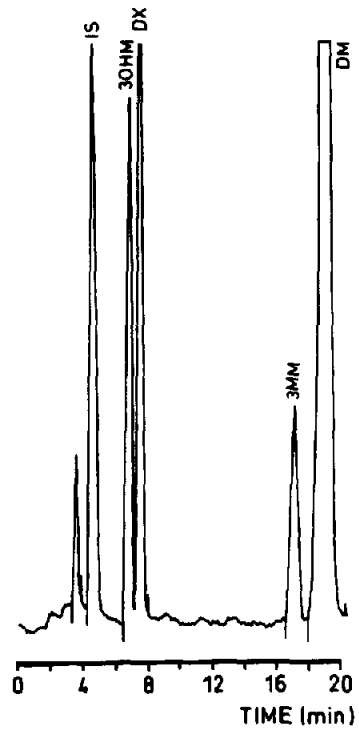

Fig. 2. Representative HPLC chromatogram of a 200 μl extract of drug-free urine spiked with 1 μg DM, 200 ng DX, 50 ng 3MM and 100 ng 3OHM. The internal standard (IS), pholcodine, is also present.

2.4. HPLC conditions

A refillable guard column (50 × 2.0 mm i.d.) was packed with a pellicular C18 material. An Alltima C18 5 μm analytical column (250 mm × 4.6 mm i.d.) (Alltech, Deerfield, IL) was used. A mobile phase containing sodium dodecyl sulphate (1 mM) in a mixture of acetonitrile–sodium dihydrogen phosphate (0.01 M) (40.5:59.5, v/v) (pH* = 2.5) was pumped at a flow rate of 1.0 ml min^{-1}. The volume injected was 40 μl. All operations were carried out at ambient temperature.

Table 1
Absolute recovery of dextromethorphan and its metabolites from urine ($n = 3$)

Compound	Nominal amount (ng[a])	Absolute recovery (%)	RSD (%)
DM	1000	93.3	3.8
DX	1000	84.8	1.0
3MM	100	86.2	2.9
3OHM	1000	84.8	0.6

[a] 200 μl urine was processed.

Table 2
Intra-assay precision and accuracy for quality control samples ($n = 6$)

Compound	Nominal amount (ng[a])	Mean amount found (ng[a])	RSD (%)
DM	10	9.2	7.0
	50	50.8	8.3
	250	269.3	8.7
	1000	1128.2	9.5
DX	10	9.1	9.1
	50	57.4	2.6
	250	264.0	2.7
	1000	923.6	2.7
3MM	10	10.3	14.7
	20	21.1	9.6
	50	52.6	9.9
	100	97.8	2.2
3OHM	10	11.5	8.5
	50	54.7	2.3
	250	240.9	2.5
	1000	963.4	6.0

[a] 200 μl urine was processed.

2.5. Assay application

Volunteers took orally 30 mg of DM (TOUXIUM®). Urine was collected over a 0–8 h period. The urine samples were analysed after enzymatic hydrolysis of the conjugated metabolites with ß-glucuronidase. Urine (100 μl or 200 μl for phenotyping for CYP2D6 and 350 μl for the determination of the DM/3MM ratio in urine), was incubated with 10 000 units ml^{-1} ß-glucuronidase at pH 5.0 [acetate buffer (pH 5.0; 0.5 M) until 0.5 ml final volume], for 24 h at 37°C.

2.6. Extraction procedure

Urine samples were processed as follows: 40 μl (4 μg) internal standard pholcodine and 0.3 ml of

Table 3
Inter-assay precision and accuracy for quality control samples ($n = 3$)

Compound	Nominal amount (ng[a])	Mean amount found (ng[a])	RSD (%)
DM	10	11.1	9.3
	50	54.2	5.6
	1000	1016.3	7.5
DX	10	9.6	11.2
	50	53.3	5.3
	1000	970.3	5.7
3MM	10	10.6	12.8
	20	20.4	6.6
	100	99.5	4.5
3OHM	10	11.1	9.3
	50	56.2	4.6
	1000	980.3	6.2

[a] 200 μl urine was processed.

sodium carbonate buffer (pH 9; 1M) were added to the samples. The SCX cartridges were pre-wetted with 4 ml of methanol followed by 4 ml of water. The samples were applied to the cartridges and washed with 3 ml of water, followed by 1.5 ml acetate buffer (pH 4.0; 0.1 M) and then 3 ml of methanol. The columns were dried under full vacuum for 3 min. The analytes were then eluted with 4.5 ml of a mixture of dichloromethane–isopropanol (70:30, v/v) containing 3% (v/v) ammonium hydroxide. After evaporation of the organic phase under nitrogen at 40°C, the residue was dissolved in 50 μl of the mobile phase.

2.7. Validation

Peak-height ratios of the four compounds (DM, DX, 3MM, 3OHM) to the internal standard were calculated, and used to generate standard calibrations lines of the peak-height ratios versus the drug concentrations. Linear regression analysis using the method of least-squares and weighting factors of 1/concentration squared was performed to determine the slope, the intercept, and the correlation coefficient of the calibration lines.

Intra- and inter-assay precision and accuracy were evaluated by spiking 200 μl urine samples with known amounts of the compounds and by

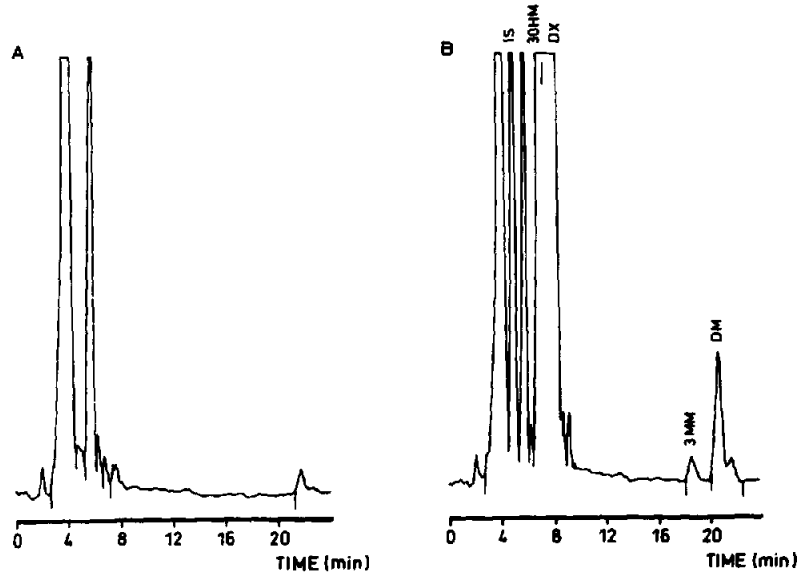

Fig. 3. Representative HPLC chromatograms of 350 μl extracts of (a) drug-free enzymatically hydrolysed urine of an extensive metabolizer and (b) a (0–8 h) hydrolysed urine sample of the same volunteer after oral intake of 30 mg DM (amounts: DM, 98.0 ng; DX, 5.10 μg; 3OHM, 3.81 μg; 3MM, 7.1 ng). Pholcodine, the internal standard (IS), is also present.

extracting them as described earlier. For the intra-day validation of each compound, six samples were determined at four different concentration levels. For the inter-day validation for each compound three different concentration levels were analysed six times each in three different assays. Absolute recovery was assessed by comparison of the peak heights of the extracted products to those obtained with direct injection of pure standards dissolved in the mobile phase. The lower limit of quantition (LOQ) was determined for each analyte by analysing six times the lowest concentration of the standard curve, possessing 200 μl urine and 350 μl urine.

3. Results and discussion

3.1. Chromatography

Micellar liquid chromatography (MLC) with sodium dodecyl sulfate and a pH-stable column was used to separate DM and its metabolites. MLC enhances selectivity and fluorescence [10]. A good resolution of all four compounds and the internal standard with good peak shape and with a reasonable run time (≈ 20 min) was obtained as shown by the chromatogram of an extract of 200 μl drug-free urine spiked with the four products and the internal standard (Fig. 2).

3.2. Extraction

The extraction method gives an excellent recovery from urine for the four compounds (Table 1). Similar recoveries were obtained from calculations based on the slope of the calibration curves with and without extraction. These high recoveries are mainly important for DM and 3MM as low concentrations can be present in the urine of, e.g., very extensive metabolizers (EM). Absolute extraction recoveries for DM and 3MM reported in the literature are between 57% and 90% for DM and between 60% and 80% for 3MM [5,7–9]. The extraction efficiency of the solid-phase extraction columns, regenerated twice with successively 3 ml of dichloromethane and 3 ml of methanol, third use, was not significantly different from that of new columns.

3.3. Internal standard

Nalorphine, quinine, quinidine, codeine and pholcodine were tested for use as internal standard. Although the fluorescence properties of pholcodine are lower than for DM and its metabolites, it is a suitable internal standard with a reproducible recovery (74.6 ± 4.9%) ($n = 3$), when extracted with the SCX solid-phase cartridges. The other products have low recoveries, inconvenient retention times or are not fluorescent.

3.4. Validation

The seven-point calibration curves showed a good linearity between peak-height ratios (product/internal standard) and the tested concentrations (5–2000 ng for DM and DX, 5–1500 ng for 3OHM and 5–150 ng for 3MM, processing 200 μl urine). Typical standard curves are: for DM, $y = 0.0040x + 0.0116$ ($r = 0.9943$); for DX, $y = 0.0065x + 0.1753$ ($r = 0.9933$); for 3OHM, $y = 0.0087x + 0.1390$ ($r = 0.9962$); and for 3MM, $y = 0.0072x - 0.0107$ ($r = 0.9921$). The LOQ for the four compounds is 25 ng ml^{-1}, using 200 μl urine and about 15 ng ml^{-1}, using 350 μl urine. About 350 μl can be used without increasing background interference on the chromatogram. At the LOQ the precision and deviation of the nominal value are below 20%. Reported LOQs (lowest point of the calibration curve) for DM are 50 ng ml^{-1} (using 0.5 ml urine [7] or 2 ml urine [5]) and between 20 ng ml^{-1} and 0.5 μg ml^{-1} (using 1 ml urine) [6,8,9]. The intra- and inter-assay precision were consistent over a wide concentration range, using 200 μl urine, with relative standard deviation (RSD) values of less than 15%. The intra- and inter-assay accuracy are also acceptable, with no more than 15% deviation of the nominal value (Tables 2 and 3).

Fig. 3 shows an example of a chromatogram of 350 μl of a drug-free enzymatically hydrolysed urine sample of an extensive metabolizer and the chromatogram of a 350 μl hydrolysed (0–8 h) urine sample of the same volunteer after oral intake of 30 mg of DM. Large amounts of the metabolites DX (4.4 mg) and 3OHM (1.6 mg) and only a small amount of DM (85.3 μg) and 3MM

(6.2 μg) are present in the (0–8 h) urine of this volunteer.

In summary, the procedure described, allowing a rapid sample preparation and use of a small sample volume, offers significant advantages in sensitivity over existing HPLC methods for the determination of DM and its metabolites in human urine.

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