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Determination of dextromethorphan and its metabolite dextrorphan in human urine by capillary gas chromatography without derivatization

Y.J. Wu[a,b], Y.Y. Cheng[b], S. Zeng[a,*], M.M. Ma[a]

[a]*College of Pharmaceutical Sciences, Zhejiang University, Hangzhou, Zhejiang 310031, People's Republic of China*
[b]*Institute of Pharmaceutical Engineering, Zhejiang University, Hangzhou, Zhejiang 310027, People's Republic of China*

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Abstract

A sensitive, simple and accurate method was developed for determination of dextromethorphan (DM) and dextrorphan (DT) in human urine by capillary gas chromatography without derivatization. After an oral dose of 30 mg DM, urine samples were collected and extracted, then analyzed on 0.22 mm×17 m HP-1 capillary column. DM and its metabolite DT were analyzed simultaneously with good separation. Docosane was used as the internal standard (I.S.). The detector used was flame ionization detector (FID). There was a linear relationship between peak area ratios of analytes to I.S. and concentration of analytes over the concentration range 0.37–7.38 μmol/l for DM and 0.39–77.8 μmol/l for DT. The recovery was 88.1~103.9% for DM and 86.7~96.8% for DT. The within-day and between-day coefficients of variation were less than 7.4 and 7.3% (RSD) for the assay of DM and DT in urine, respectively. The limits of detection (LOD) were 0.30 μmol/l for DM and 0.16 μmol/l for DT. The limits of quantitation (LOQ) were 0.37 μmol/l (RSD<6%) for DM and 0.39 μmol/l (RSD<7%) for DT. The method has been applied to determine the oxidative phenotypes of cytochrome P450 2D6 (*CYP2D6*) in a Chinese population with metabolic ratio of DM in human urine.
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1. Introduction

The debrisoquine/sparteine hydroxylase (*CYP2D6*) is responsible for the metabolism of a large group of clinically important drugs including several antiarrhythmic agents, β-adrenergic blockers, antihypertensive drugs, neuroleptics, tricyclic antidepressants, monoamine oxidase inhibitors, analgesic-antitussive opioids and many other drugs [1,2]. Dextromethorphan (DM) is a widely used antitussive ingredient in many over-the-counter cough formulations that is oxidatively metabolized by cytochrome P450 2D6 through *O*-demethylation and *N*-demethylation pathways. The wide interindividual variation in the metabolism of dextromethorphan has been explained by the demonstration of a genetic polymorphism in its hydroxylation. A genetic deficiency in the metabolism of dextromethorphan may be revealed by the excretion pattern of dextromethor-

*Corresponding author. Tel.: +86-57-1721-7203; fax: +86-57-1721-7367.
E-mail address: zengsu@cps.zju.edu.cn (S. Zeng).

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phan and its metabolite dextrorphan (DT). A variety of analytical methods have been developed for the analysis of DM in plasma or urine such as high-performance liquid chromatography (HPLC) [3,4], capillary zone electrophoresis (CZE) [5] and gas chromatography (GC) with nitrogen–phosphorous, electron capture and mass spectrometric detection [6–9]. It is usually necessary to carry out a pre-column derivatization reaction for GC analysis of dextrorphan. In the present paper, we developed a new and direct capillary gas chromatographic method with FID for analysis of DM and DT simultaneously in human urine that pre-column derivatization was not needed.

2. Experimental

2.1. Materials

Dextromethorphan hydrobromide and dextrorphan tartrate were purchased from Hoffmann–La Roche (Switzerland). Docosane was purchased from Shanghai Chemical Reagent Company (Shanghai, PR China). All other chemicals and solvents were chromatographic and analytical reagent and obtained from common commercial sources.

2.2. Apparatus and chromatographic conditions

The Shimadzu GC-15A gas chromatography system (Shimadzu, Japan) was equipped with a cross-linked methyl silicone fused-silica capillary column (17 m long, 0.22 mm I.D., 0.25 μm film thickness), a hydrogen flame ionization detector and a Shimadzu C-R4A chromatopac data system. High purity nitrogen was used as the carrier gas at a head pressure of 1.0 kg/cm^2 and makeup gas was set at a flow-rate of 25 ml/min. The oven temperature was maintained at 190 °C, and the injector temperature and the detector temperature were maintained at 260 °C. Split ratio was set at 50:1, H_2 at 50 ml/min was used as combustion gas and air at 500 ml/min was used as combustion adjuvant. The instrumental sensitivity was set at range 1.

2.3. Solution preparation

Stock solution of dextromethorphan and dextrorphan were prepared at 400 μg/ml in ethanol, respectively. All stock solutions were stored at 4 °C.

2.4. sample preparation and assay procedure

Fifty milligrams of L-cysteine and 0.3 ml of hydrochloric acid were added to 2.5 ml of urinary solutions containing the drug and its metabolite. The mixture was heated in water bath at 100 °C for 30 min. After cooling to room temperature, 3 ml of diethyl ether was added into the hydrolyte. The sample was mixed thoroughly on a rotary tube mixer and centrifuged at 4000 rpm for 5 min. The upper diethyl ether layer was discarded, the aqueous phase was alkalified with 0.35 ml of 12 mol/L sodium hydroxide and 1 g of solid buffer (sodium bicarbonate–potassium carbonate, 3:2, w/w) and extracted with 2 ml diethyl ether–isopropanol (9:1, v/v) for two times. After centrifugation, the organic layer was transferred to a glass centrifuge tube with a screw cap and evaporated to dryness under a stream of nitrogen in a warm water bath. The drugs were brought into solution with 100 μl of 80.6 μmol/l docosane in ethyl acetate, and 2 μl of the solution was used for injection.

2.5. Calibration curves

The calibration curves for DM and DT were constructed by analyzing a series of blank urine spiked with stock solution in the concentration range of 0.37–7.38 μmol/l for DM and 0.39–77.8 μmol/l for DT. Extractions of the samples were carried out as described under *sample preparation and assay procedure.* Peak-area ratios (DM or DT vs. Docosane) were measured and regressed against the concentration of DM and DT.

2.6. Recovery studies

A series of blank urine samples, spiked with various amounts of DM or DT, were processed as described under *sample preparation and assay procedure.* The peak-area ratios of DM (or DT) and

internal standard docosane were compared with those obtained when equal amounts of DM (or DT) in solvent.

2.7. reproducibility studies

The assay precision was evaluated over the concentration range of 0.37~7.38 μmol/l urine for DM and 0.39~77.8 μmol/l urine for DT by assaying standard urine samples spiked with different concentration of DM and DT on four separate days. The within-day variability and between-day variability were calculated.

2.8. Subjects

All healthy volunteers were between 18 and 38 years of age. Subjects who smoked 10 packs of cigarettes per year, regular alcohol drinkers, subjects with a history of allergy or those currently taking medications were excluded. A 30 mg of dextromethorphan hydrobromide were administered to each volunteer and over-night urine was collected. All volunteers were advised to abstain from any medications 2 weeks before administration.

3. Results and discussion

Typical chromatograms of DM and DT are shown in Fig. 1. In order to confirm the DM and DT in urine, a GC–MSD (5790 gas chromatography/5790 mass spectrometer detector system, Palo Alto, CA, USA) method set at electron impact mode was used to analyze a human urine sample, and mass spectra of DM and DT were shown in Fig. 2. The mass spectra of sample were consistent with the standard spectra of DM and DT. The electron impact mass fragmentation proposed was shown in Fig. 3.

The assay based on the peak-area ratios of the analyte and internal standard (y) versus concentration of the analytes (x) was linear. The linearity of the calibration curves for DM and DT was over the range 0.37–7.38 μmol/l for DM and 0.39–77.8 μmol/l for DT. The regression equations of the calibration curves were $y=0.2245x-0.0912$ for DM and $y=0.209x-0.3695$ for DT. The correlation coefficients were 0.999 and 0.998 for DM and DT, respectively.

The limit of detection (LOD) of the assay were measured as 0.30 μmol/l for DM and 0.16 μmol/l for DT. The limit of quantitation (LOQ) were 0.37 μmol/l (RSD<6%) for DM and 0.39 μmol/l (RSD<7%) for DT.

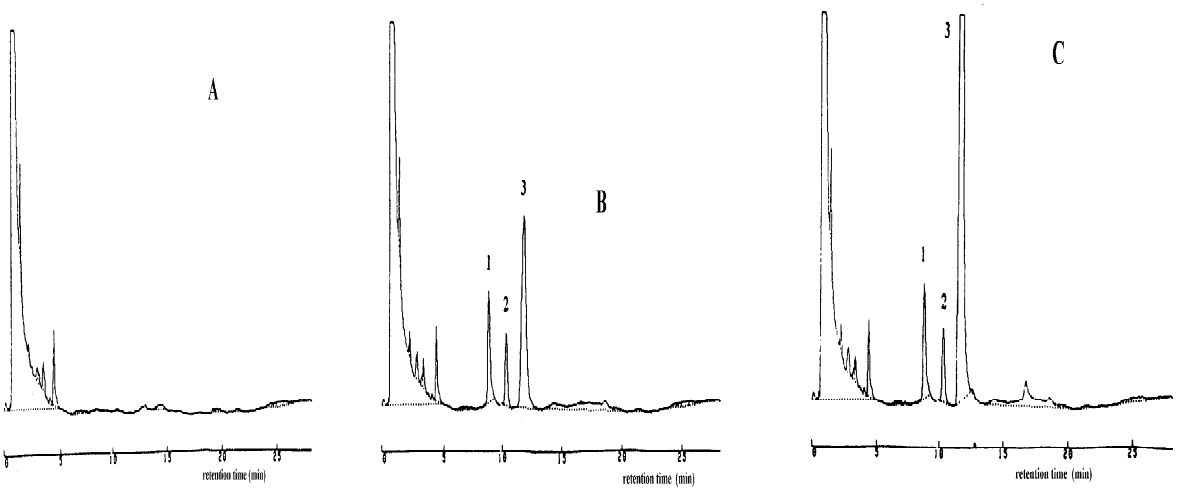

Fig. 1. Chromatograms of DM and DT. (A) Blank human urine; (B) urine spiked with DM (2.21 μmol/l) and DT (3.11 μmol/l); (C) human urine obtained after an oral dose of 30 mg dextromethorphan hydrobromide (DM 2.25 μmol/l, DT 10.44 μmol/l) 1. DM, 2. I.S., 3. DT.

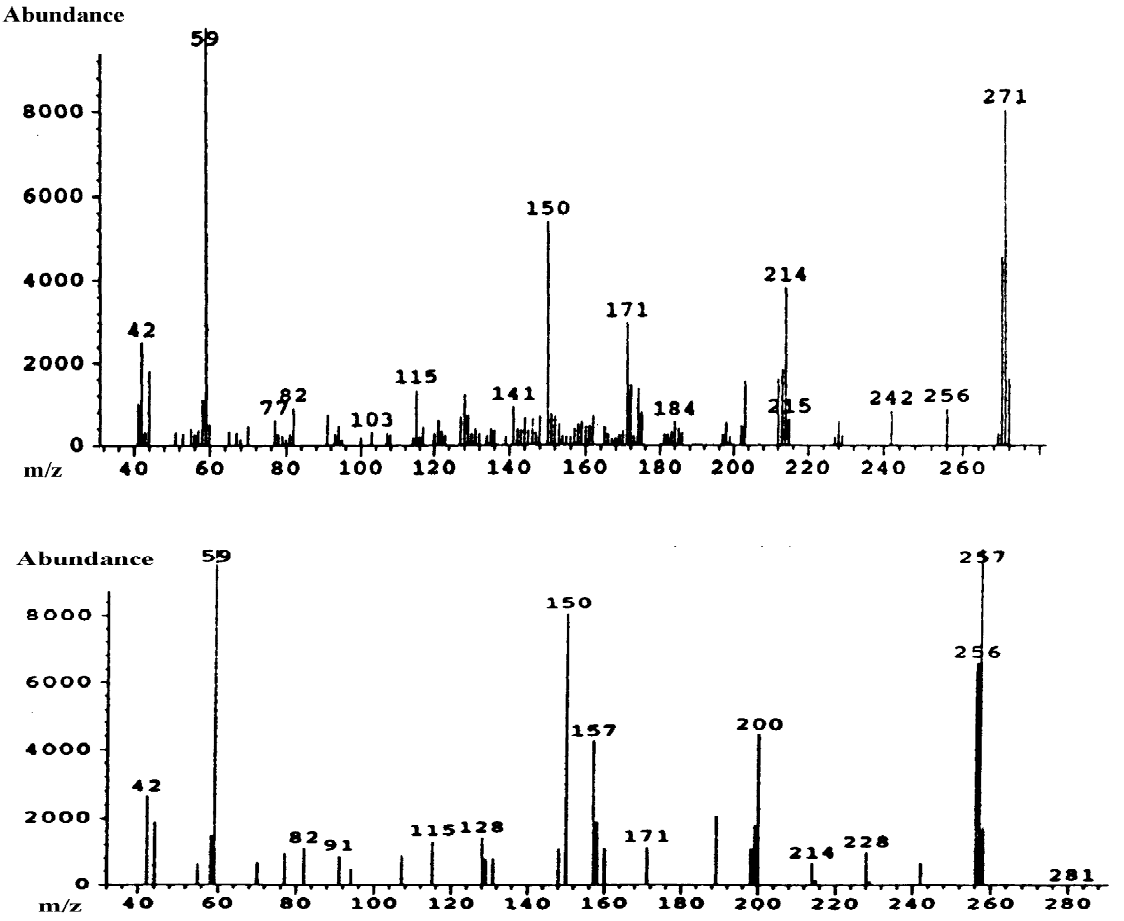

Fig. 2. Mass spectra of DM and DT in urine.

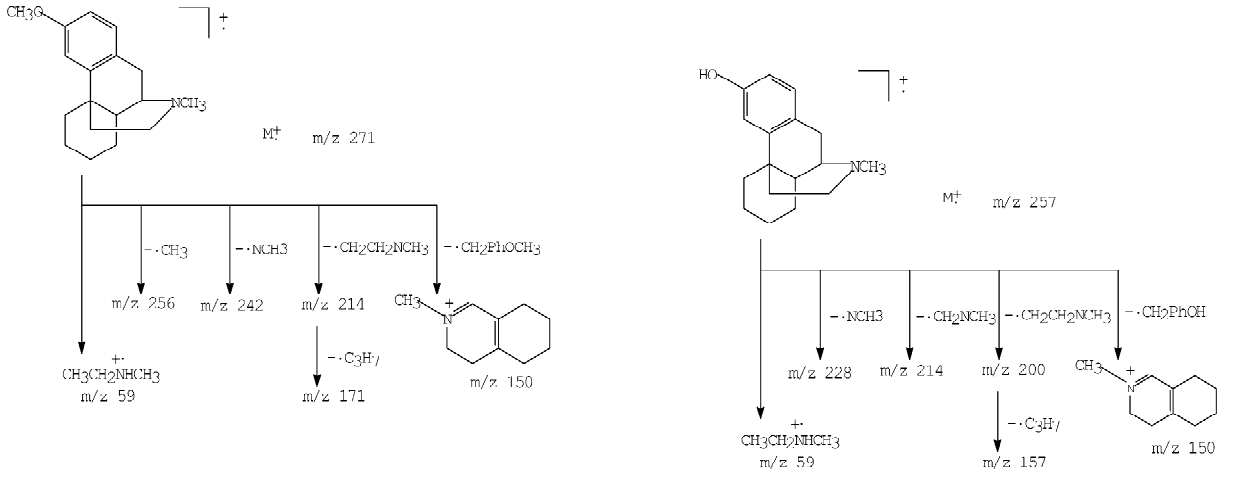

Fig. 3. Proposed electron impact mass fragmentation of DM and DT.

Table 1
The recoveries of DM and DT measured at three different concentrations ($n=6$)

Concentration added (μmol/l)	Concentration measured (μmol/l)	Recovery (%, mean±s)
Dextromethorphan		
0.37	0.326±0.013	88.1±3.4
2.95	2.81±0.08	95.2±2.8
7.38	7.66±0.12	103.9±1.7
Dextrorphan		
0.39	0.338±0.018	86.7±4.7
31.1	30.1±0.70	96.8±2.3
77.8	73.7±2.20	94.7±2.9

The recoveries of analytes were summarized in Table 1. The average recoveries of this analytical method were 88.1~103.9% and 86.7~96.8% for DM and DT, respectively.

The precision of this analytical method was shown in Tables 2 and 3. The within-day and between-day coefficients of variation were less than 7.4 and 7.3% (RSD) for the assay of DM and DT in urine. Therefore, we concluded that the reproducibility and precision of this GC method for determination of DM and DT in urine was satisfactory.

Table 2
Within-day reproducibility of the assay of DM and DT in urine ($n=5$)

Concentration added (μmol/l)	Concentration measured (mean±s) (μmol/l)	RSD (%)
Dextromethorphan		
0.37	0.314±0.018	5.7
2.95	2.84±0.15	5.2
7.38	7.45±0.41	5.5
Dextrorphan		
0.39	0.342±0.023	6.9
31.1	30.6±0.89	3.0
77.8	76.6±2.26	3.0

Table 3
Between-day reproducibility of the assay of DM and DT in urine

Concentration added (μmol/l)	Concentration measured (mean±s) (μmol/l)	RSD (%)
Dextromethorphan		
0.37	0.328±0.022	6.8
2.95	2.69±0.18	6.7
7.38	7.53±0.55	7.4
Dextrorphan		
0.39	0.323±0.023	7.3
31.1	31.0±1.21	3.9
77.8	75.8±2.65	3.5

The GC method developed in the present article has been applied to analyze the human phenotypes of *CYP2D6* in a Chinese population with metabolic ratio of DM in human urine. Table 4 showed the oxidative phenotypes results of 13 Chinese.

DM is metabolized in vivo to DT via *O*-demethylation that is primarily mediated by *CYP2D6*, DT is further glucuronidated. So it is necessary that DT-glucuronide should be hydrolyzed completely to quantify the total amount of DT by β-D-glucuronidase pretreatment or acid deconjugation. In the present paper, DT-glucuronide was completely hydrolyzed with hydrochloric acid at 100 °C water bath for 30 min. The solvent extraction procedures after hydrolysis was used to eliminate interference and clean up sample.

Because DT is a polar compound, a derivatization reaction was ordinarily employed before GC assay. At the beginning of the experiment, we also tried to derivatize DT in urine with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) according to the reported method [4], but the experimental results showed that the chromatogram of the sample was

Table 4
Human CYP2D6 phenotypes of a Chinese population

Number	Concentration of DM (μmol/l)	Concentration of DT (μmol/l)	MR
1	2.25	10.44	0.22
2	0.41	8.41	0.049
3	1.31	4.92	0.27
4	0.35	11.13	0.031
5	0.46	4.69	0.098
6	0.68	7.20	0.094
7	0.94	12.76	0.074
8	0.75	4.14	0.18
9	1.63	6.62	0.25
10	0.75	7.64	0.098
11	0.58	4.10	0.14
12	0.69	1.26	0.55
13	0.45	1.29	0.35

Notes: $\mathrm{MR}=\frac{\mathrm{DM}\ (\mu\mathrm{mol/l})}{\mathrm{DT}\ (\mu\mathrm{mol/l})}$.

deteriorating in comparison with that of non-derivatization. When DT and its derivative in the derivatized sample was identified with GC–MS, both of DT and its derivative were found. This result indicated that DT could be directly injected onto GC column without pre-column derivatization. It made the analysis simple and easily operated.

When OV-101WCOT capillary column was employed to analyze DM and DT at 180 °C of oven temperature, the peaks of DM and DT was not symmetrical with a tailing factor more than 2.

In order to optimize the separation of DM and DT, the chromatographic conditions, such as column temperature, flow-rate, split ratio, were modified to improve the peak shape and retention characteristics of DM and DT. When HP-1 capillary column was used with 160 to 260 °C of oven temperature at 5 °C/min program rate, there was partial overlap between the peaks of DM, I.S. and DT, and the baseline drifted seriously. Changing the column temperature to 120, 150, 170, 190 and 210 °C, respectively, we found that the peaks shape were symmetrical and retention time of DM and DT were suitable at 190 °C. No obvious effect on chromatographic behavior was observed as changing the flow-rate of carrier gas. We changed the split ratio from 10:1 to 100:1 and found that it had a remarkable influence on sensitivity and precision of analysis. The sensitivity, precision and peak shape were satisfactory when split ratio was set at 25:1–75:1. The chromatographic response was reduced as split ratio was increased. The precision of peak area was unsatisfactory (RSD>15%) when split ratio was larger than 75:1 and concentration of DM was less than 0.5 μmol/l. As concentration of DT was larger than 50 μmol/l and split ratio was less than 25:1, the peak shape of DT became asymmetrical with tailing factor more than 2 because of loading limit of column.

The concentration of DM and DT in human urine for 13 subjects were at least 0.35 μmol/l, indicating that the sensitivity of the present technique is adequate for the purpose of phenotypes analysis for human urine samples. If concentration of DM in human urine is lower than LOD, the larger volumes of urine sample could be extracted to increase the chromatographic response.

Compared with published HPLC methods [10,11], the present GC method is satisfactory in precision and recovery. The sensitivity of GC method is similar with HPLC methods. HPLC methods are slightly rapid, its typically analysis time is less than 20 min but analysis time (<30 min) of GC method is also acceptable for usual analysis.

In summary, a sensitive, simple and accurate non-derivatization capillary gas chromatographic method for determination of dextromethorphan and dextrorphan was developed. This improved method has been be used to *CYP2D6* phenotypes analysis of oxidative metabolism in a Chinese population.

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