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Determination of dextromethorphan and its metabolites in plasma by dual column liquid chromatography and fluorimetric detection*

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Abstract: Dextromethorphan and its metabolites were determined in plasma by a dual column liquid chromatographic switching system, after a prepurification on Sep-Pak C_{18} cartridges. A polar precolumn (CN) and two hydrophobic analytical columns (C_{18}) were used with mobile phases optimised regarding trace enrichment, selectivity and speed by the content of organic modifier. Dextromethorphan and its metabolites could be quantified at 5 ng ml^{-1} with an intra-assay precision better than 6% (RSD).

Keywords: *Dextromethorphan and its metabolites; plasma; liquid solid distribution; liquid chromatography; coupled columns.*

Introduction

Dextromethorphan is a widely used antitussive agent. The drug is rapidly and extensively metabolised by demethylation (Fig. 1) and conjugation [1, 2] with large interindividual variation [1]. Humans can be divided into three groups with different metabolic patterns. In the first, the most common group, conjugated dextrorphan is the main compound. In the second dextromethorphan dominates, while in the third both dextromethorphan and dextrorphan are found.

In previously published methods, dextromethorphan or the metabolite dextrorphan (+17-methylmorphinan-3-ol) have been determined in plasma fluorimetrically [3], by gas chromatography [1, 4–7] by liquid chromatography [8, 9] or by radioimmunoassay [10]. In one paper dextromethorphan and its metabolites in urine were simultaneously determined by liquid chromatography [11]. In a recently published liquid chromatographic method dextromethorphan or dextrorphan and 3-hydroxy-morphinan were determined with different mobile phases [12]. The present method determines dextromethorphan and its metabolites simultaneously in plasma by dual column liquid chromatography with fluorimetric detection. Plasma samples are purified on Sep-Pak

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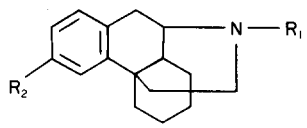

R_1	R_2	
$-CH_3$	$-OCH_3$	(+) -3-Methoxy-17-Methylmorphinan (Dextromethorphan)
$-CH_3$	$-OH$	(+) -17-Methylmorphinan-3-ol (Metabolite I; dextrorphan)
$-H$	$-OCH_3$	(+) -3-Methoxymorphinan (Metabolite II)
$-H$	$-OH$	(+) -Morphinan-3-ol (Metabolite III)

Figure 1
Chemical structures of dextromethorphan and the metabolites.

C_{18} cartridges before chromatography. The technique involves an improvement of detection limits and a reduction of the analysis time compared to previous methods.

Experimental

Chemicals

Dextromethorphan hydrobromide, dextrorphan, 3-methoxymorphinan and 3-hydroxy-morphinan were kindly supplied by Roche (Basel, Switzerland). Heptanesulfonic acid (Eastman–Kodac Rochester, NY, USA), and triethylamine puriss (Fluka, Buchs, Switzerland) were used as received. All other chemicals used were of analytical grade from E. Merck (Darmstad, FRG). The β-glucuronidase (Koch-Light Laboratories Ltd., Berkshire, UK) contained 2000 Fishman units mg^{-1} material and contained also some arylsulphatase activity. Water was purified in a Milli-Q apparatus (Millipore, Bedford, MA, USA). Sep-Pak C_{18} cartridges were obtained from Waters Associates (Milford, MA, USA).

Apparatus

The liquid chromatograph consisted of a Kontron Tracer MCS 670 valve switching unit, with a Tracer timer 210 (Kontron AG. Zürich, Switzerland). The pumps were a double Milton Roy Minipump (Laboratory Data Control, FL, USA) for the analytical columns and a Waters M-6000A for the precolumn. The fluorimetric detector was a Schoeffel FS 970, cell volume 5 μl (Schoeffel Instruments, NJ, USA) with an excitation wavelength of 220 nm. The injector was a Waters WISP 710 B automatic injector. The integrator was an SP 4270 (Spectra Physics, CA, USA). The sample preparation was performed in a Sep-Pak C_{18} cartridge Rack.

Liquid chromatographic conditions

The chromatographic system is schematically described in Fig. 2. The precolumn (50 × 4.6 mm i.d.) was packed with Nucleosil CN, 5 μm (Macherey-Nagel and Co., Düren, FRG) and the analytical columns 1 and 2 (150 × 4.6 mm i.d.) were packed with

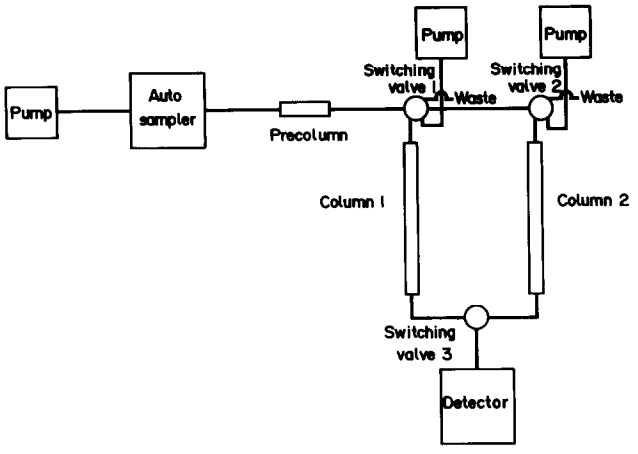

Figure 2
Scheme of the chromatographic system.

Nucleosil C_{18}, 5 μm and Spherisorb ODS, 5 μm (Phase Separations Queensferry, UK) respectively. The precolumn mobile phase was methanol, acetic acid, triethylamine and water (13:1.0:0.1:85.9, v/v/v/v). The flow rate was 0.6 ml min^{-1}. The mobile phase for analytical column 1 was acetonitrile, acetic acid, triethylamine and water (21:1.0:0.1: 77.9, v/v/v/v). The flow rate was 1.2 ml min^{-1}. The mobile phase for analytical column 2 was composed of 9×10^{-5}M heptanesulfonic acid in acetonitrile, acetic acid, triethylamine and water (60:1.0:0.1:38.9, v/v/v/v). The flow rate was 1.4 ml min^{-1}. The mobile phases were filtered and degassed prior to use.

Column switching

Dextromethorphan and the three metabolites were separated into two groups on the precolumn and each group was transferred to an analytical column for further separation.

The detector first monitors the peaks from analytical column 1 and after valve switching the peaks from analytical column 2. The switching events are presented in Table 1. See also Fig. 2.

Sample preparation

Determination of unconjugated concentrations of dextromethorphan and its metabolites.

Sep-Pak C_{18} cartridges were conditioned and purified with successively 5 ml of acetonitrile, 20 ml of 60% (v/v) of acetonitrile in 0.1 M phosphoric acid, 5 ml of acetonitrile and finally 5 ml of phosphate buffer pH 7 $\mu = 0.1$. Plasma (2.00 ml) was loaded and after purification with 10 ml of water and 10 ml of acetonitrile respectively the compounds were eluted with 2.00 ml of 60% (v/v) acetonitrile in 0.1 M phosphoric acid. The eluate was evaporated under a stream of nitrogen in a water bath (35°C). The residue was dissolved in 250 μl of phosphate buffer, pH 4.4 $\mu = 0.01$ and 100–200 μl was injected into the precolumn. A calibration curve was prepared by spiking plasma (2.00 ml) with dextromethorphan and the metabolites in the range of 5–100 ng ml^{-1}.

Table 1
Sequence of column switching procedure

Time after injection	Switch of valve no.	Event
0.00	3	Sample is injected into the precolumn. Analytical column 1 is connected to the detector
3.25	1	The precolumn and analytical column 1 are connected in series and dextrorphan and 3-hydroxy-morphinan are enriched on the analytical column
5.50	1	The precolumn and analytical column 1 are disconnected from each other. Elution of the two metabolites on column 1 starts with its mobile phase
8.00	2	The precolumn and analytical column 2 are connected in series and dextromethorphan and 3-methoxy-morphinan are enriched on column 2
11.00	2	The precolumn and analytical column 2 are disconnected from each other. Elution of dextromethorphan and 3-methoxymorphinan starts with mobile phase for column 2
13.5	3	Analytical column 2 and the detector are connected in series
18	End	The switching cycle is completed

Determination of total concentrations of metabolite I (dextrorphan) and metabolite III

Plasma (1.00 ml) was mixed with 2.00 ml phosphate buffer (pH 3.8, $\mu = 1$) containing 0.5 mg ml^{-1} of β-glucuronidase. After incubation overnight at 37°C, the samples were treated as described above. The calibration curve was in the range of 5–700 ng ml^{-1} of the four compounds and 25–100 μl was injected into the precolumn.

Results and Discussion

Sample preparation

In sample preparation from biological material it is important to have a high absolute recovery (>90%) to obtain as low detection limits and as high accuracy and reproducibility as possible. In batch extractions a too high distribution ratio should be avoided since this increases the risk of coextraction of endogenous compounds. A common way to obtain a suitable extraction media is to mix a non-polar organic solvent with a solvent which efficiently solvates the drug and the metabolites. Amines as dextromethorphan and the metabolites are solvated by hydrogen donating agents such as heptafluorobutanol [13, 14]. Extraction of metabolite III (Fig. 1, 3-hydroxymorphinan), the most polar compound, from 0.01 M sodiumhydroxide to cyclohexane + heptafluorobutanol (99.5 + 0.5 v/v) gave an extraction degree of only 7% (equal phase volumes). Increasing the polarity and the H-donating ability by using dichloromethane + heptafluorobutanol (99 + 1) and optimisation of the pH to 11 gave an extraction degree of 92% (equal phase volumes). However, blank plasma gave disturbances in the chromatogram and the recovery was lower compared to buffer solutions. Protein precipitation with trichloroacetic acid (10% w/v) gave a recovery of only about 50%.

Using Sep-Pak C_{18} cartridges the absolute recoveries, with 2.00 ml of eluent, depended on the composition of the eluent as shown in Fig. 3. This retention behaviour indicated a dual retention mechanism which has been described earlier for reversed-phase columns [15–18]. The low recoveries of the compounds in eluents lean in phosphoric acid are probably due to strong adsorption to polar binding sites on the solid phase, which are uncovered in solvents with low polarities. The low recoveries in acetonitrile lean eluents may on the other hand depend on eluents too weak for elution to occur. That the most hydrophilic of the four compounds give the highest recoveries is a further indication for such an explanation. Eluents containing 40–60% (v/v) of acetonitrile in 0.1 M phosphoric acid gave the highest recoveries. The highest possible concentration of acetonitrile gives the fastest evaporation, so 60% was used in the method. Purification of the cartridges with the eluent before sample loading eliminated impurities which interfered with the most polar metabolites.

Hydrolysis with β-glucuronidase was studied with a plasma sample containing 850 ng ml^{-1} of metabolite I and 300 ng ml^{-1} of metabolite III. Solutions (2.00 ml) containing 0.25, 0.5 and 1.0 mg ml^{-1} of β-glucuronidase (in phosphate buffer pH 3.8 μ = 1.0) were incubated with plasma samples (1.00 ml) at 37°C for 17 h. The same concentrations of hydrolysed metabolites were obtained with 0.5 and 1.0 mg ml^{-1} of β-glucuronidase, while 0.25 mg ml^{-1} gave a 10% lower concentration; 0.5 mg ml^{-1} was therefore used in the method.

Chromatographic system

Hydrophobic amines often give peak tailing in reversed-phase liquid chromatography. Addition of an alkylammonium ion in combination with an acidic mobile phase is a common way to improve the symmetry [19].

Mobile phases containing triethylamine (0.1%, v/v), acetic acid (1.0%, v/v) and varying percentages of acetonitrile were investigated on both hydrophilic and hydrophobic reversed-phase columns. The two polar *O*-demethylated metabolites have

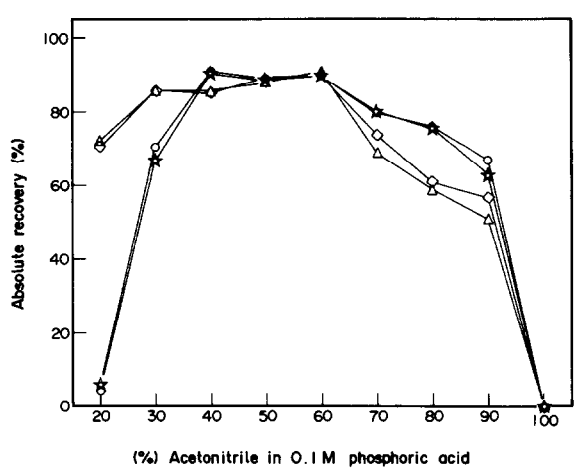

Figure 3
Influence of the composition of the eluent on the absolute recovery from Sep-Pak C_{18} cartridges. Applied amount was 50 ng of each in 2.00 ml plasma. Compounds ○, dextromethorphan; ◇, metabolite I ☆, metabolite II; △, metabolite III.

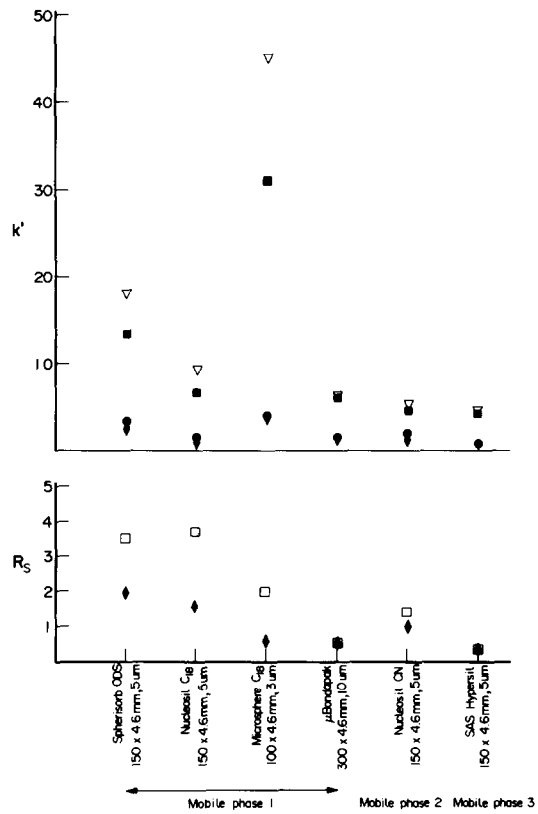

Figure 4
Retention (k') of metabolites I–III and of dextromethorphan and resolution (R_s) between metabolite I and III and between metabolite II and dextromethorphan, respectively, on different reversed-phase columns. Mobile phase 1 consisted of acetonitrile:acetic acid:triethylamine:water (25:1.0:0.1:73.9, v/v/v/v). Mobile phase 2 consisted of acetonitrile:acetic acid:triethylamine:water (10:1.0:0.1:88.9, v/v/v/v). Mobile phase 3 consisted of acetonitrile:acetic acid:triethylamine:water (30:1.0:0.1:68.9, v/v/v/v). ●, Metabolite I; ■, Metabolite II; ◆, Metabolite III; ▽, Dextromethorphan; ◆, R_s Metabolite I/Metabolite III; □, R_s Metabolite II/ Dextromethorphan.

considerably shorter retention compared to the *N*-demethylated metabolite and dextromethorphan on all the columns studied (Fig. 4). None of the columns was suitable for separating all the four compounds since when the resolution was adequate (e.g. on the Spherisorb ODS column and on the Nucleosil C_{18} column), the difference in retention was too large. However, the differences in resolution and retention between the columns were utilised to an advantage in the dual column-system. On the CN-column the four compounds were separated into two groups, which were further separated either on the Spherisorb ODS column or on the Nucleosil C_{18}-column.

The most polar column (CN-column) was ideal as a precolumn since it is important to have the lowest possible content of organic modifier in the precolumn mobile phase in order to obtain on column concentration of the eluates to the analytical columns. Furthermore, impurities in the precolumn mobile phase were enriched on the analytical column, and therefore the volume of the fraction from the precolumn should be low. This was obtained with a precolumn giving symmetrical peaks and high efficiency. The separation on the Nucleosil-CN precolumn is shown in Fig. 5. In order to increase the

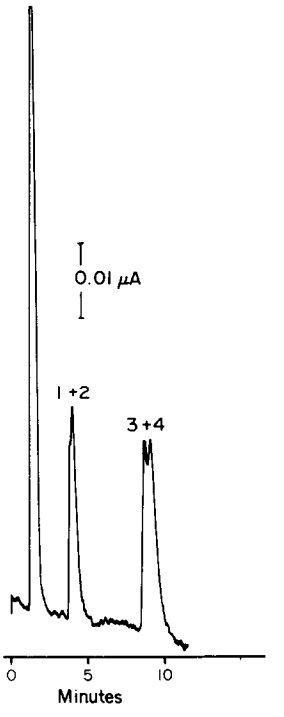

Figure 5
Separation of an aqueous solution on the precolumn (Nucleosil-CN). Injected amount was 20 ng of each compound. The mobile phase consisted of methanol: acetic acid:triethylamine:water (13.0:1.0:0.1:85.9, v/v/v/v). Fluorimetric detection; excitation wavelength: 220 nm. Compounds: 1, metabolite III; 2, metabolite I; 3, metabolite II; 4, dextromethorphan.

ruggedness of the methodology the transferred volumes were approximately 0.5 ml higher than the peak volumes. With the CN-precolumn the fractions transferred to analytical columns 1 and 2 were 1.4 and 1.8 ml, respectively. The dual-cut principle utilised on the precolumn gave plasma chromatograms without any disturbance from plasma components, Fig. 6.

The differences in acetonitrile content between the mobile phases of the two analytical columns (e.g. 21%, v/v and 60%, v/v, respectively) gave a negative baseline shift when column 2 was connected to the detector after column 1. Addition of a low content of heptanesulfonic acid (9×10^{-5}M) to mobile phase 2 changed the negative baseline shift to a small positive shift. This indicated a lower content of fluorescent impurities in acetonitrile compared with the aqueous part of the mobile phase. Furthermore, heptanesulfonic acid either increased the fluorescence of the impurities already present or introduced new ones, which compensated for the negative baseline shift.

Column lifetimes

The precolumn was used both for guard purposes and as a preseparation column. After about 100 injections it was discarded, due to loss in resolution. The switching events were adjusted every day and every 10th injection a calibration solution was injected to check the stability of the retention times on the precolumn. When hydrolysed plasma samples were used the calibration solution was injected every 5th sample and the switching times had to be changed more frequently. The reproducibility was 9% (RSD, $N = 15$) between different slurry packed columns. Two different batches of particles were used and the analytical columns have been used for more than 1000 injections without any signs of deterioration.

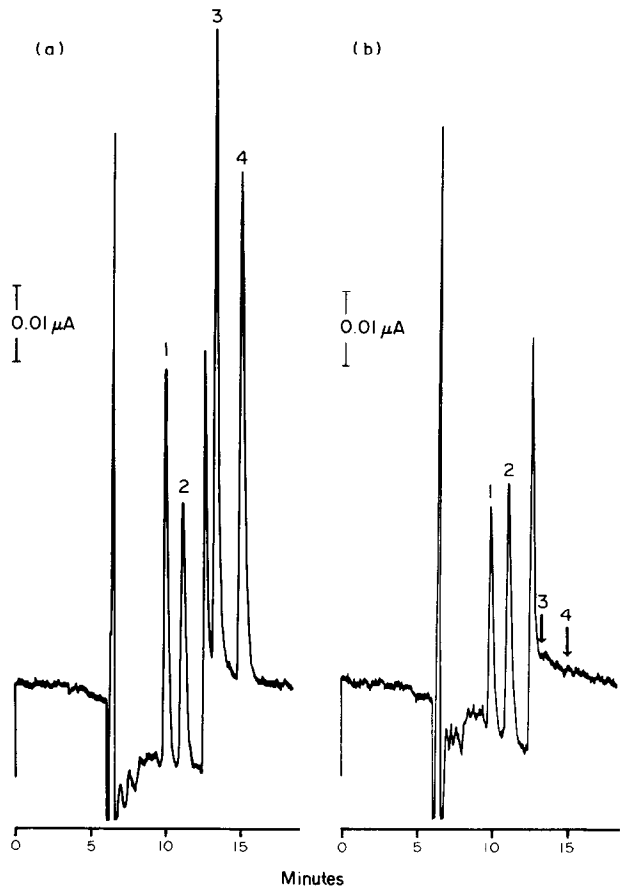

Figure 6
Comparison between chromatograms obtained by injection of an aqueous sample (A) containing all the four compounds, and a hydrolysed plasma sample (B) containing 200 ng ml^{-1} of metabolite III and 280 ng ml^{-1} of metabolite I. Symbols as in Fig. 5. Injected amount of the aqueous solution was 40 ng of each compound. The precolumn mobile phase consisted of methanol:acetic acid:triethylamine:water (13.0:1.0:0.1:85.0, v/v/v/v). The mobile phase for analytical column 1 was acetonitrile:acetic acid:triethylamine:water (21:1.0:0.1:77.9, v/v/v/v). The mobile phase for analytical column 2 was acetonitrile:acetic acid:triethylamine:water (60:1.0:0.1:38.9, v/v/v/v), containing 9×10^{-5}M heptanesulfonic acid. Fluorimetric detection; excitation wavelength: 220 nm.

Recovery, precision and sensitivity

Results were obtained from a calibration curve of peak area plotted against known concentration. The absolute recoveries and the intra-assay precisions are shown in Table 2. The absolute recoveries were obtained by comparison with direct injection of the compounds dissolved in phosphate buffer of pH 4.0. Plasma levels down to 5 ng ml^{-1} could be determined for all the four compounds using 2.00 ml of plasma.

Application

Plasma levels from a human volunteer receiving 60 mg of dextromethorphan as a single oral dose is shown in Fig. 7. Conjugated dextrorphan is the prevailing compound

Table 2
Intra-assay precision (%) and absolute recovery (%) of the four compounds at different levels of concentration

	5 ng ml^{-1}			100 ng ml^{-1}			250 ng ml^{-1}		
	RSD (%)	Absolute recovery (%)	*N*	RSD (%)	Absolute recovery (%)	*N*	RSD (%)	Absolute recovery (%)	*N*
Dextromethorphan	1.8	99.5	7	2.0	97.5	7	2.2	94.7	7
Metabolite I	5.9	87.2	7	1.8	93.9	7	3.9	90.6	7
Metabolite II	1.5	97.8	7	1.4	94.2	7	3.7	92.8	7
Metabolite III	6.0	100.4	7	2.0	95.0	7	1.9	96.8	7

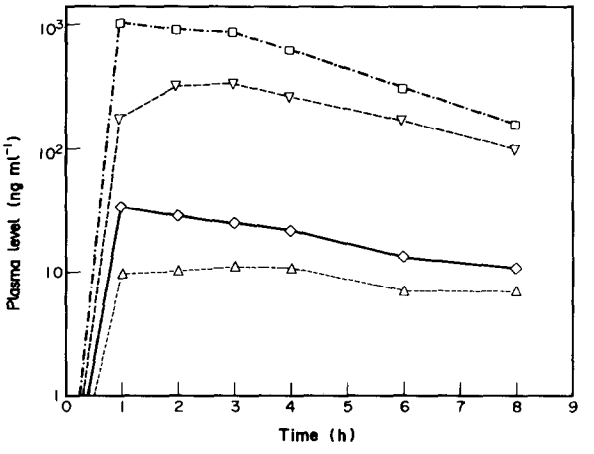

Figure 7
Plasma levels of metabolite I and metabolite III after an oral dose of 60 mg of dextromethorphan. ◇, Metabolite I, unconjugated △, metabolite III, unconjugated □, metabolite I, total ▽, metabolite III, total.

in plasma and the concentration of dextromethorphan is below the limit of quantitation which indicate that this subject belongs to the most common metabolic group.

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