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Separation of metronidazole, its major metabolites and their conjugates using dynamically modified silica

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Abstract

Metronidazole has previously been used as a probe when investigating cytochrome P450 isoenzymes and thus the pattern of metabolism of this drug has been extensively studied. However, in previous investigations the conjugates were determined by indirect methods. In this paper we present a high-performance liquid chromatographic (HPLC) system for the simultaneous determination of metronidazole, its major metabolites and their glucuronic acid conjugates in biological fluids. The separation is performed using bare silica dynamically modified with N-cetyl-N,N,N-trimethylammonium bromide contained in the mobile phase. The separation of the acidic metabolites of metronidazole is greatly improved with this system compared to other published reversed-phase HPLC systems intended for the same purpose. Glucuronides of metronidazole and its hydroxy metabolites have been synthesized in vitro using rat liver microsomes and preparative HPLC. The method developed makes it possible to determine the intact glucuronic acid conjugates of metronidazole and the hydroxy metabolite in human urine.

1. Introduction

Metronidazole [1-(hydroxyethyl)-2-methyl-5-nitroimidazole] is a drug used for the treatment of protozoal infections (*Trichomoniasis vaginalis*) and infections caused by anaerobic micro-organisms [1–3]. The therapeutically active plasma concentration is about 5 μg/ml [3]. The drug is further used as a radio sensitiser of hypoxic cells (cancer therapy) [2]. Pharmacokinetic studies (in humans) using radioactive tracers have shown that about 77% of drug is excreted in the urine and about 14% is excreted in faeces (bilary excretion) within 5 days [2]. About 5% of the drug is excreted as CO_2 due to reductive metabolism by the gut flora leading to a breakdown of the imidazole ring [4,5] (Fig. 1); the latter studies were performed in rats.

Oxidative metabolism is the major route for biotransformation of metronidazole in humans and three major oxidative metabolites are found: the hydroxy metabolite, 1-(hydroxyethyl)-2-hydroxymethyl-5-nitroimidazole (HM); the acetic acid metabolite, 2-methyl-5-nitroimidazole-1-acetic acid (MAA) and the 2-carboxy metabolite, 1-(hydroxyethyl)-2-carboxy-5-nitroimidazole (MOOH). In rat the predominant route of biotransformation is conjugation with glucuronic acid and sulphuric acid [2,6,7]. In man the

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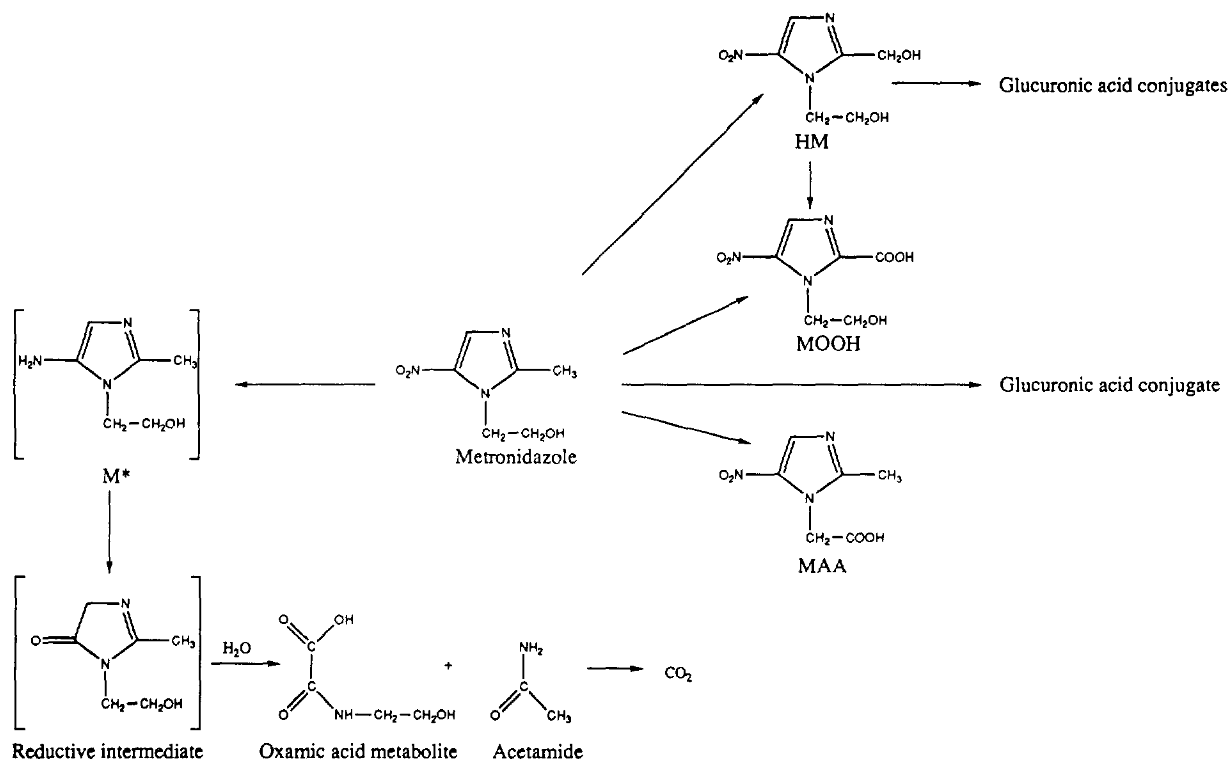


Fig. 1. Metabolic pattern of metronidazole.

parent drug as well as the metabolites are conjugated with glucuronic acid [1,2,6,8] (Fig. 1).

Metronidazole has previously been used as a probe when investigating cytochrome P450 isoenzymes. Thus the pattern of metabolism of this drug has been extensively studied [5,6,9] and a number of reversed-phase HPLC systems have been developed [9–16]. However, in these investigations the conjugates were determined by indirect methods (increase in concentration of mother substance before and after treatment with β-glucuronidase) because of lack of reference compounds and lack of a sufficiently selective separation method.

In this paper we present a HPLC system for the simultaneous determination of metronidazole, its major metabolites as well as their glucuronic acid conjugates in biological fluids. The separation is performed using bare silica dynamically modified with N-cetyl-N,N,N-trimethylammonium (CTMA) bromide dissolved in the mobile phase. The retention and separation of the acidic metabolites of metronidazole is greatly improved with this system compared to the earlier published HPLC systems used in studies of the metabolism of metronidazole [9–16].

2. Experimental

2.1. Chemicals

Metronidazole and the metabolites HM, MAA and MOOH were kindly donated by Steffen Loft, Department of Pharmacology, University of Copenhagen, Copenhagen, Denmark and Dumex (Copenhagen, Denmark). Disodium UDP-glucuronate (UDP-GA) and β-glucuronidase (*Escherichia coli*) (200 U/ml) were purchased from Boehringer (Mannheim, Germany). CTMA bromide (analytical grade) was purchased from Merck (Darmstadt, Germany). All other chemicals were of analytical-reagent grade.

2.2. Apparatus

Analytical chromatography

A Waters (Milford, MA, USA) liquid chromatographic system consisting of a Model 6000 A pump, a 715 Ultra WISP autoinjector, a 490E programmable multiwavelength detector was used and data were collected using Maxima 820 software. A Shimadzu (Kyoto, Japan) CTO-6A oven was used for thermostating the columns.

Preparative chromatography

A Merck–Hitachi (Darmstadt, Germany) 655A-12 liquid chromatographic system pump, a 655A variable-wavelength monitor and a D2000 integrator were used. A Rheodyne (Cotati, CA, USA) 7125 injection module with a 1.5-ml loop was used for sample introduction.

NMR spectroscopy

A 400 MHz Bruker (Rheinstetten, Germany) AMX 400 WB instrument was used.

2.3. Enzymatic synthesis of glucuronides

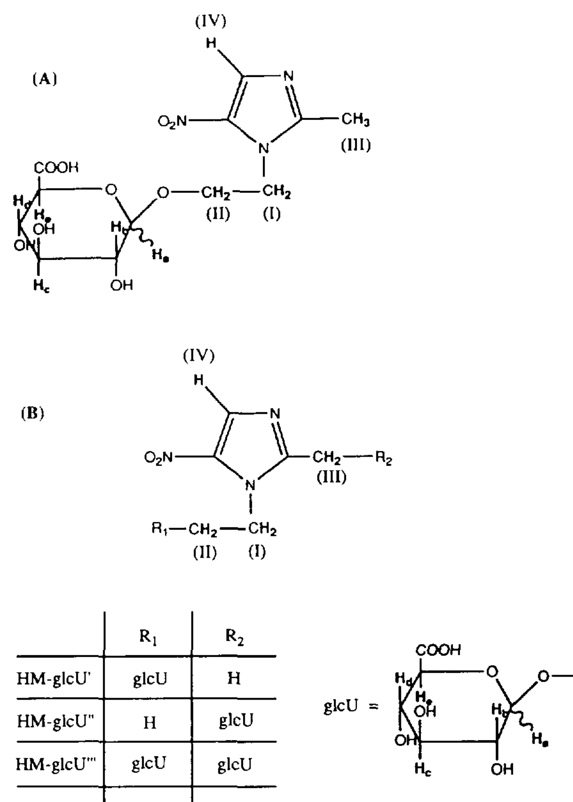
Liver microsomes were prepared as previously described [17]. The liver from 9-weeks-old male Sprague-Dawley rats were used. The protein content of the prepared microsomes was measured by the method of Lowry et al. [18] UDP-glucuronosyl transferase (UDP-GT) activity was measured by the method described earlier [19] except that the change in *p*-nitrophenol concentration was measured by HPLC instead of by spectrophotometry.

The optimal incubation mixture for the two substrates metronidazole and the hydroxy metabolite was found to be the following: rat microsomal protein, 35 mg/ml; substrate, 75 m*M*; potassium phosphate buffer (pH 7.4), 50 m*M*; $MgCl_2$, 5 m*M*; Triton X, 0.01%; UDP-GA, 12 m*M*; water to a total volume of 50 ml. This mixture was incubated in ten vials each containing 5 ml at 37°C for 8 h on a gyratory shaker. If 50 ml were incubated in a single vial the yield of the synthesis was significantly lower due to diffusion problems in the incubation vial.

The synthesis was stopped by adding 10 ml methanol to each vial, the proteins were allowed to precipitate and the mixture was centrifuged 30 min at 5000 *g*. The supernatants were pooled and the methanol removed by rotary evaporation. Finally the water phase was freeze dried. The glucuronides were isolated from this freeze-dried product using preparative HPLC. Identification and purity of the final products were determined by ^1H NMR (400 MHz) in ^2H_2O and by quantitative cleavage with β-glucuronidase followed by HPLC analysis of the parent drug towards a reference standard.

The ether glucuronide of metronidazole

Maximum yield was 0.09 mmol (31 mg) of the metronidazole glucuronide and the purity of the final product was 84%. The ^1H NMR spectrum confirmed the structure of the glucuronic acid conjugate of metronidazole (M-glcU) (Fig. 2A).



	R1	R2
HM-glcU'	glcU	H
HM-glcU''	H	glcU
HM-glcU'''	glcU	glcU

Fig. 2. (A) The glucuronic acid conjugate of metronidazole (M-glcU). (B) The glucuronic acid conjugates of the hydroxy metabolite.

δ = 2.51 ppm [–CH_3(III), s], 3.18 ppm (H_b, t), 3.38–3.45 ppm (H_c, H_d, m), 3.66 ppm (H_e, d), 4.04 ppm [–CH_2(I), m], 4.15 ppm [–CH_2(II), m], 4.31 ppm (H_a, d), and 8.09 ppm [H(IV), s].

The ether glucuronides of the hydroxy metabolite

As shown in Fig. 3. three conjugates of HM were formed in the incubation mixture (t_R = 7.93, 8.99 and 10.22 min). This fact was confirmed by cleavage with β-glucuronidase. The maximum yield of the synthesis was 0.12 mmol

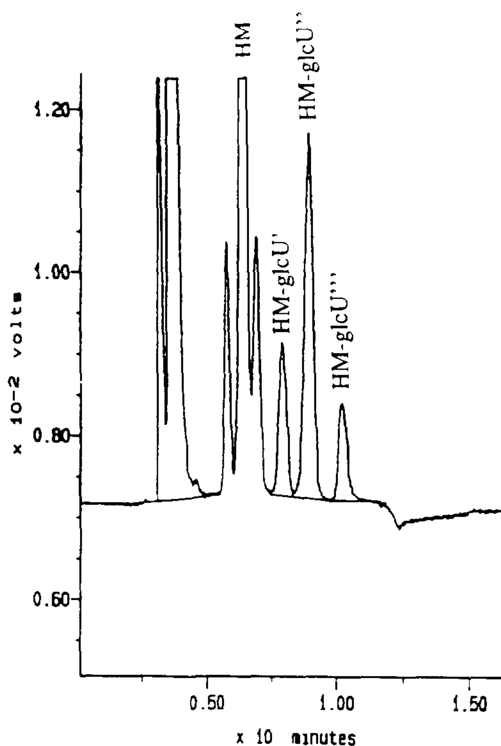


Fig. 3. Chromatogram of a sample from the incubation mixture during in vitro synthesis of conjugates of the hydroxy metabolite of metronidazole (HM). Retention times (min) for the conjugates are: HM-glcU′ (7.93), HM-glcU″ (8.99), HM-glcU‴ (10.22). Chromatographic conditions: saturation column (150 × 4.6 mm I.D.), dry packed with LiChroprep Si 60 (15–25 μm) placed between pump and injector. Analytical column, Knauer column (120 × 4.6 mm I.D.), slurry packed with LiChrosorb Si 60 (5 μm), thermostated at 35°C; eluent, methanol–0.2 *M* potassium phosphate (pH 7.0)–water (25:30:45, v/v/v) with 2.5 m*M* CTMA added. The flow-rate was initially 0.5 ml/min, after 9 min it was increased to 1.0 ml/min. UV detection at 312 nm.

of a mixture of glucuronides. The purity of the final products was poor (about 20%) for all three conjugates but an identification by ^{1}H NMR of the metabolites was performed.

The ^{1}H NMR spectrum of the first conjugate (t_R = 7.93 min) confirmed the structure of a mono sugar conjugate (HM-glcU′) (see Fig. 2B). δ = 3.10 ppm (H_b, t), 3.29–3.39 ppm (H_c, H_d, m), 3.62 ppm (H_e, d), 3.95 ppm [–CH_2(I), m], 4.10 ppm [–CH_2(II), m], 4.22 ppm (H_a, d), 4.62 [–CH_2(III), t] and 8.10 ppm [H(IV), s].

The ^{1}H NMR spectrum of the second conjugate (t_R = 8.99 min) confirmed the structure of mono sugar conjugate (HM-glcU″) (see Fig. 2B). δ = 3.09 ppm (H_b, t), 3.25–3.45 ppm (H_c, H_d, m), 3.50 ppm (H_e, d), 3.95 ppm [–CH_2(I), m], 4.02 ppm (H_a, d), 4.50 ppm [–CH_2(II), m], 4.59 [–CH_2(III), t] and 8.00 ppm [H(IV), s].

The ^{1}H NMR spectrum of the last conjugate (t_R = 10.22 min) confirmed that the substance contains two glucuronic acid moieties (HM-glcU‴) (Fig. 2B). The glucuronic acid protons show a complex pattern δ = 3.41–4.39 ppm. δ = 4.20 ppm [–CH_2(I), m], 4.30 ppm [–CH_2(II), m], 4.60 [–CH_2(III), t] and 8.05 ppm [H(IV), s].

2.4. Preparative HPLC

The isolation procedure for the glucuronide of metronidazole (M-glcU) was performed in two steps. A Knauer column (Berlin, Germany) packed with LiChrosorb NH_2 (Merck), 5 μm, was used (250 mm × 16 mm I.D.), with an eluent consisting of methanol–0.2 *M* ammonium carbonate (pH 7.8)–water (90:2:8, v/v/v). The flow-rate was 5 ml/min. Further clean up was performed on a polystyrene–divinylbenzene polymer column (PRPL-S 100A 8 μm; Polymer Labs., Shropshire, UK; 300 mm × 7.5 mm I.D.), with a mobile phase consisting of acetonitrile–water (5:95, v/v) containing 0.1% trifluoracetic acid and a flow-rate of 6 ml/min.

The glucuronides of the hydroxy metabolite were separated on a bare silica column, a Knauer column (120 × 4.6 mm I.D.) packed with LiChrosorb Si 60 (Merck) (5 μm), with a saturation column (150 × 4.6 mm I.D.) dry packed

with LiChroprep Si 60 (Merck) (15–25 μm) installed between the pump and the autoinjector. The mobile phase was methanol–0.2 *M* potassium phosphate (pH 7.0)–water (25:30:45, v/v/v) with 2.5 m*M* CTMA added. The flow-rate was 0.5 ml/min. The isolated glucuronides were finally purified on a PRPL-S 100A column, 8 μm, 300 mm × 7.5 mm I.D., using a mobile phase of acetonitrile–water (5:95, v/v) containing 0.1% trifluoracetic acid with a flow-rate of 5 ml/min.

2.5. Sample preparation

The urine samples were diluted 1:2 with methanol and after 1 h centrifuged for 12 min (5000 *g*). The supernatant was diluted 1:1 with water and 20 μl were injected into the liquid chromatograph.

Samples from the incubation mixtures containing liver microsomes were diluted 1:2 with methanol to stop the reactions and to precipitate the proteins. After centrifugation for 12 min (5000 *g*) 20 μl of the supernatant were injected into the HPLC system.

Cleavage of glucuronides with β-glucuronidase was performed as follows: 5 μl β-glucuronidase were added to 200 μl of the sample (pH 7.4) and the mixture was incubated at 37°C. After 18 h 400 μl methanol were added and the sample was centrifuged for 12 min (5000 *g*). If a urine sample was tested the supernatant was further diluted 1:1 with water before analysis.

2.6. Analytical chromatography

A saturation column (150 × 4.6 mm I.D.) drypacked with LiChroprep Si 60 (15–25 μm) was installed between the pump and the auto-injector. The analytical column was a Knauer column (120 × 4.6 mm I.D.) packed with LiChrosorb Si 60 (5 μm). Both columns were thermostated at 35°C. The final mobile phase developed for the assay of metronidazole, its metabolites and their conjugates was methanol–0.2 *M* potassium phosphate (pH 7.0)–water (25:30:45, v/v/v) with 2.5 m*M* CTMA added. The flow-rate was initially 0.5 ml/min and after 9 min increased to 1.0 ml/min. The UV detector was operated at 312 nm.

For the assay of *p*-nitrophenol the mobile phase was methanol–0.2 *M* potassium phosphate (pH 6.0)–water (60:5:35, v/v/v) containing 1.25 m*M* CTMA, with a flow-rate of 1.0 ml/min, the UV detector was operated at 405 nm.

3. Results and discussion

3.1. Enzymatic synthesis of glucuronides

The enzyme activities for the formation of glucuronic acid conjugates of *p*-nitrophenol in freeze-dried rat liver microsomes compared to non-freeze-dried liver microsomes were 25.9 ± 5 nmol/min · mg protein and 31.5 ± 5 nmol/min · mg protein, respectively. An activity in fresh rat liver microsomes of 28 nmol/min · mg protein was reported earlier [19]. It is favourable to use the freeze-dried product when synthesizing glucuronic acid conjugates because of the possibility for long-term storage (− 18°C for up to 6 months) [20]. Furthermore, higher protein concentrations may be used.

The development of optimal conditions for the synthesis of ether glucuronides of metronidazole and its hydroxy metabolite (HM) was based on the method previously described [21].

Metronidazole was found to be a poor substrate in vitro for the glucuronosyl transferase enzymes as the yield of glucuronic acid conjugates using standard conditions [21] for enzymatic synthesis was low. One of the reasons may be the high hydrophilicity of the drug itself decreasing the need for glucuronic acid conjugation. Even though the substrate specificity of the UDP-GT enzymes is broad, higher affinity of the enzymes towards the more lipophilic compounds is seen [22]. However, it was possible to increase the yield of the synthesis by increasing the substrate and protein concentration. The yield of the incubation was further optimized changing pH of the incubation mixture as well as the temperature during incubation. A maximum yield (evaluated as the area of the chromatographic peak from the glucuronic acid conjugate

formed) was observed after 8 h using an incubation mixture with pH 7.4 and a temperature of 37°C. The same yield could be observed after 24 h incubating at 25°C (pH 7.4). The incubation conditions that were used for synthesis M-glcU were found to be optimal for the synthesis of glucuronides of the hydroxy metabolite as well. In this study only the ether glucuronides of metronidazole and HM were synthesized for the identification of unknown peaks in the chromatograms. These were the only glucuronides observed in urine obtained from humans. No detectable amounts of acyl glucuronides of the acidic metabolites could be observed. This was determined using β-glucuronidase tests of urine samples, examining the concentration of the phase I metabolites before and after incubation with β-glucuronidase.

3.2. Chromatography

The separation methods for metronidazole and its major oxidative metabolites described in the literature are all based on reversed-phase chromatography performed on chemically bonded phases using mobile phases with a very low content of organic modifier (5–10%) [6,9,11,12,15,23]. However, these systems were not well suited for separation of the very polar acidic metabolites of metronidazole (MOOH, MAA) or the glucuronides. Instead we used bare silica dynamically modified with a long-chain quaternary ammonium compound (CTMA bromide). This results in a reversed-phase HPLC system where metronidazole and HM are retained by a reversed-phase mechanism. Anions such as the acidic metabolites (MAA, MOOH) and glucuronides (M-glcU, HM-glcU′, HM-glcU″, HM-glcU‴) form hydrophobic ion pairs with the CTMA ions which are separated by reversed-phase chromatography.

The chromatographic system was evaluated with respect to pH, amount of methanol, ionic strength and concentration of CTMA in the eluent to obtain satisfactory separation of metronidazole, its metabolites and the conjugates.

Organic modifier

When the amount of organic modifier was increased in the eluent the separation of metronidazole and HM was lost (Fig. 4A). Substituting acetonitrile for methanol or using combinations of the two modifiers was not successful as metronidazole and HM could not be separated. The final eluent was chosen to contain 25% (v/v) of methanol. Lower amounts were not used as the retention of the acidic metabolites would then be unnecessarily long.

pH

The effect of changing pH in the mobile phase can be rather complex in a HPLC system based on dynamically modified silica. The silanol groups have a pK_a value of 6.5–7.0 and when the pH of the mobile phase is increased the adsorption of CTMA to the silica surface increases. Thus, the increase in pH should lead to an increase in retention.

The observed decrease in retention times of the acidic metabolites results (Fig. 4B) from a combination of equilibrium states. As the pH increases the concentration of potassium ions increases and thereby the ionic strength in the system. The potassium ions will compete with CTMA for the solute as well as for the silanol groups of the column (resulting in lower retention as the pH increases). The negative charge of the phosphate ions increase as the pH increases and the phosphate ions might compete with the acids for CTMA interaction. The pH value of the system did not significantly influence the retention of metronidazole and HM. To keep retention of the acids as short as possible a pH of 7.0 was used in the eluent.

Ionic strength

The acidic metabolites have fairly long retentions while metronidazole and HM elute early in the chromatogram. A high ionic strength in the eluent has previously been proven to be able to selectively reduce the retention of anionic solutes [24]. Concomitantly, the increase in ionic strength will not change the retention of non-

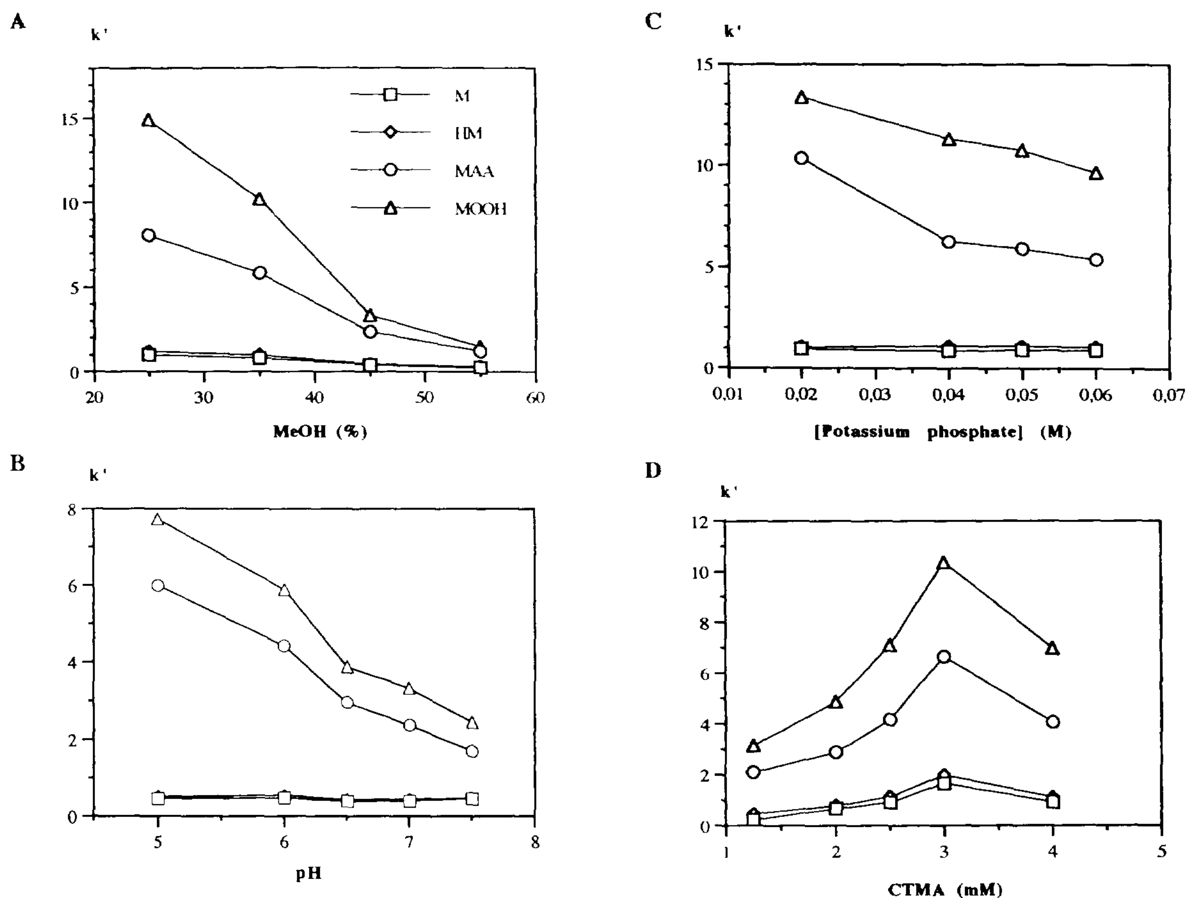


Fig. 4. (A) Relationship between the concentration of methanol and the capacity factor (k') for metronidazole (□) and its three major metabolites (◇ = HM; ○ = MAA; △ = MOOH). Columns as in Fig. 3; eluent, methanol–0.2 *M* potassium phosphate (pH 7.0)–water [(25–55):30:(15–45), v/v/v], with 2.5 m*M* CTMA added. (B) Relationship between pH of the buffer and k' for metronidazole and its three major metabolites. Columns as in Fig. 3; eluent, methanol–0.2 *M* potassium phosphate (pH 5.0–7.5)–water (25:30:45, v/v/v), with 2.5 m*M* CTMA added, symbols as in (A). (C) Relationship between potassium phosphate concentration in the buffer added and k' for metronidazole and its three major metabolites. Columns as in Fig. 3; eluent, methanol–0.2 *M* potassium phosphate (pH 7.0)–water [25:(10–30):(45–65), v/v/v], with 2.5 m*M* CTMA added, symbols as in (A). (D) Relationship between the concentration of CTMA and k' for metronidazole and its three major metabolites. Columns as in Fig. 3; eluent, methanol–0.2 *M* potassium phosphate (pH 7.0)–water (25:30:45, v/v/v), with 1.25–4.0 m*M* CTMA added, symbols as in (A). The hold-up time t_0 was determined as the retention time of deuterium oxide.

ionic solutes. Keeping the methanol concentration low favours the separation of metronidazole and HM. A combination of the parameters low methanol concentration and high ionic strength would thus be favourable. The result of increasing the ionic strength in the eluent with a methanol concentration of 25% is seen in Fig. 4C.

CTMA

The CTMA concentration was varied in the final system with 25% (v/v) methanol and 0.06 *M* potassium phosphate pH 7.0. The results are seen in Fig. 4D. In the evaluation of the optimal amount of CTMA separation of metronidazole and its metabolites as well as their conjugates were investigated. The retention of met-

ronidazole and its metabolites increases until a concentration of 3 mM is reached as more CTMA adsorbs to the silica surface. At 4 mM CTMA the critical micellar concentration (CMC) was most likely exceeded [25] and the capacity factors decreased. When CMC is exceeded a "secondary lipophilic phase" which migrate with the speed of the eluent is formed. Some of the solute molecules migrate into the micelles instead of interacting with the stationary phase and thus the retention decreases.

3.3. Assay validation

The detection limits for metronidazole, its metabolites and the conjugates in urine were determined to be the following: metronidazole 2 ng/ml, HM 9 ng/ml, M-glcU 8 ng/ml, MAA 20 ng/ml and MOOH 37 ng/ml. This was estimated as three times the standard deviation (σ) of the peak-to-peak noise ($N_{p\text{-}p}$), where $\sigma = N_{p\text{-}p}/5$ which is a good estimate when the noise is assumed to be normal distributed. The limit of quantitation was 5 ng/ml for metronidazole, 28 ng/ml for HM, 25 ng/ml for M-glcU, 65 ng/ml for MAA and 120 ng/ml for MOOH. The limit of quantitation was estimated as 10σ of the peak-to-peak noise. The calibration curves in urine of metronidazole (0.01–50 μg/ml), its phase I metabolites (0.1–50 μg/ml) and the conjugates (0.05–50 μg/ml) were linear within the concentration ranges specified ($r^2 > 0.99$).

Recovery studies of metronidazole and its metabolites in human urine were done by adding known amounts of the reference compounds to drug-free urine ($n = 6$). The recoveries for all solutes were found to be 87–103% and are seen in Table 1. The intra-assay relative standard deviations (R.S.D.s) are sufficiently low for the method to be used for routine analysis.

3.4. Applications

The metabolism of metronidazole in vivo was investigated in a single dose experiment. A 500-mg amount of metronidazole was given orally to two healthy volunteers (female, 23 years old,

Table 1
Analysis of samples of urine spiked with metronidazole and metabolites of metronidazole

Sample	Added (μg/ml)	Found (μg/ml)[a]	Recovery (%)	R.S.D. (%)
Metronidazole	2.76	2.57	93.5	3.4
	5.88	6.03	102.6	1.5
	62.66	57.96	92.5	3.0
HM	2.43	2.47	101.6	6.6
	5.50	5.30	96.7	2.2
	30.67	29.75	97.0	2.7
MAA	2.91	2.50	86.2	4.2
	7.14	6.78	95.8	1.4
	30.67	30.03	97.9	1.7
MOOH	2.67	2.72	102.4	7.7
	7.00	6.20	88.9	5.2
	28.00	27.65	98.8	0.8
M-glcU	1.85	1.85	100.0	1.6
	4.00	3.67	91.8	5.3
	10.08	9.77	87.1	1.8

[a] Mean, $n = 6$.

mass 55 kg and female, 25 years old, mass 62 kg) and urine was properly collected for 72 h.

Three metabolites and two conjugates were identified in the urine: HM, MAA, MOOH and the glucuronic acid conjugates of metronidazole (M-glcU) and one of the conjugates of the hydroxy metabolites (HM-glcU"). The two other conjugates of HM (HM-glcU' and HM-glcU‴) could not be detected. The conjugate of HM has not been assayed directly in earlier published work regarding metabolism of metronidazole [9–16]. An example of a chromatogram of an authentic urine sample from one of the volunteers is shown in Fig. 5A along with a blank urine sample Fig. 5B. None of the acyl glucuronides of the acidic metabolites could be detected, not even indirectly using β-glucuronidase tests.

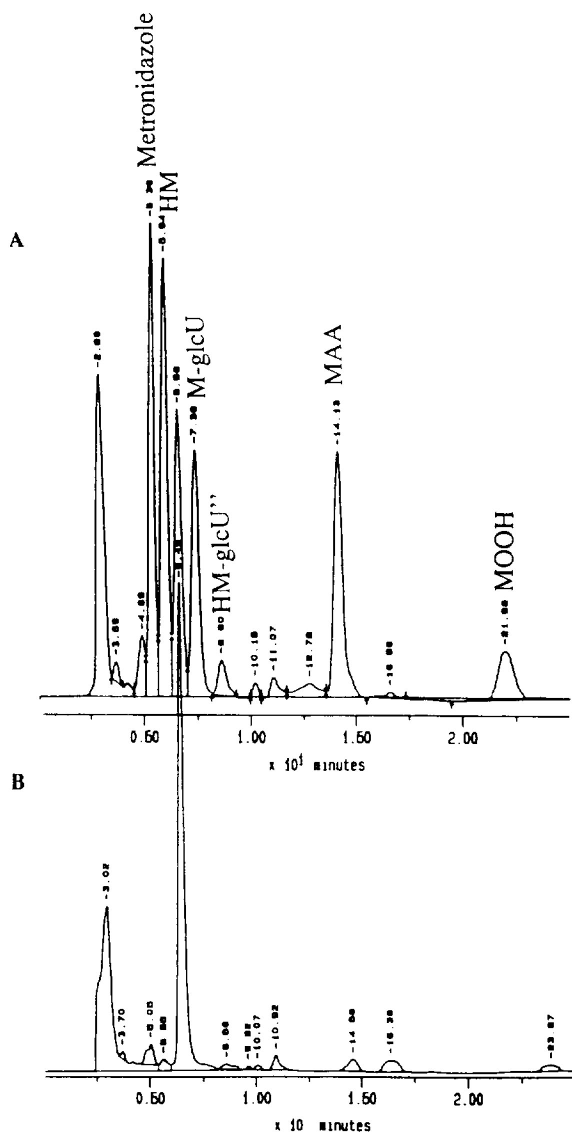


Fig. 5. Chromatograms of urine samples from (A) a volunteer 4 h after oral intake of 500 mg metronidazole and (B) the same volunteer before intake of 500 mg metronidazole. Chromatographic conditions were as described in Fig. 3.

The totally excreted amount of the given dose was 82% in the urine from the first volunteer and 74% from the second, which corresponds to the 77% earlier found [7]. The cumulated amounts of metronidazole, the three major metabolites and the glucuronic acid conjugates excreted over 72 h from the two volunteers are compared in Table 2. There are some differences between the metabolic profiles of the two volunteers especially with respect to excretion of metronidazole, its hydroxy metabolite and the acetic acid metabolite. One could speculate in polymorphism of the P450 isoenzymes but this would require extended investigations.

The single-dose study of metronidazole in human volunteers emphasizes the applicability of the chromatographic method in biological fluids. The HPLC system based on dynamically modified silica is very suitable for the separation and quantitation of compounds which differ in

Table 2
Cumulated urinary excretion of metronidazole and its metabolites and conjugates after oral administration of 500 mg metronidazole to two volunteers (1 and 2)

Compound	Excretion (% of dose) within 72 h	
	1	2
Metronidazole	9.6	16.4
HM	28.7	22.0
MAA	10.1	19.5
MOOH	17.9	15.6
M-glcU	5.0	6.3
HM-glcU"	2.4	2.0
Total% of dose excreted	73.7	81.8

chemical properties such as drugs and their phase I and phase II metabolites.

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