Eur J Med Chem (1995) 30, 757–762

Identification and full characterization of a new metabolite of metoclopramide

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(Received 25 October 1994; accepted 24 May 1995)

Summary — The metabolism of metoclopramide **1** has been reinvestigated by HPLC coupled with mass spectrometry. The urine of healthy volunteers showed the presence of a new metabolite **2** derived from the enzymatic oxidation of the primary aromatic amino group of **1** to the hydroxylamino moiety. The structure of the new metabolite was ascertained by its fragmentation pattern in the mass spectrometer and unambiguously secured by an independent synthesis. In addition, the tertiary amine group of **1** was selectively oxidized to the corresponding *N*-oxide. This compound and its ring oxidation metabolites were not detected in the urine of the tested volunteers.

metoclopramide metabolism / metabolite / arylhydroxylamino derivative / metoclopramide *N*-oxide

Introduction

Metoclopramide **1** (4-amino-5-chloro-2-methoxy-*N*-[2-diethylaminoethyl]benzamide) (fig 1) is the drug most often used to block nausea and vomiting associated with cancer chemotherapy [1]. Furthermore, metoclopramide is also used to control gastro-intestinal motility disorders.

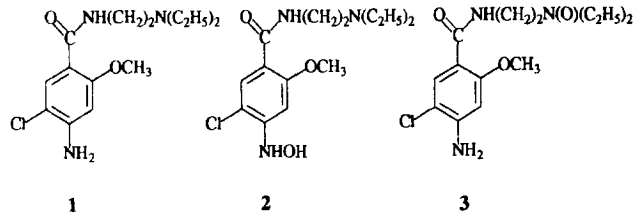
Although the mechanism of action of **1** was initially thought to be mediated *via* dopamine D_2-receptor blockade [2, 3], more recent investigations attribute the gastric prokinetic activity of the drug to an antagonism of the 5-HT_3 receptors located in the area postrema [4, 5], or to the activation of the 5-HT_4 receptors [6]. After oral administration, metoclopramide is rapidly absorbed from the gastro-intestinal tract and is excreted in urine. Its metabolism has been thoroughly investigated in animals and humans. In humans, up to 80% of a dose is excreted in urine within the first 24 h either as unchanged drug (up to 25%) or as glucuronide and sulfate conjugates of non-metabolized drug. The major metabolite is the N-4-sulfate which accounts for 40% of the dose recovered after oral administration [7]. The presence of trace amounts of 2-(4-amino-5-chloro-2-methoxybenzamido)acetic acid was also reported [8]. The results of these studies have recently been reviewed and collected in the analytical profile of metoclopramide [9]. It is worth pointing out that the presence of a metabolite derived from the enzymatic oxidation of the aromatic amino group of **1** was set forth by Arita *et al* [10] by analysing the organic extracts of rabbit urine [10]. Unfortunately, the lability of the compound prevented its full characterization and its presence could not be evidenced in human urine [8].

These outcomes and the evidence that the recovery of the dose was not quantitative prompted us to reinvestigate the metabolism of metoclopramide on healthy volunteers.

This paper deals with the HPLC detection of a new metabolite, the arylhydroxylamino derivative **2** (fig 1), its analysis by mass spectrometry and its full characterization by independent synthesis. Furthermore, the synthesis of metoclopramide *N*-oxide **3** (fig 1) allowed us to exclude its presence in human urine.

Chemistry

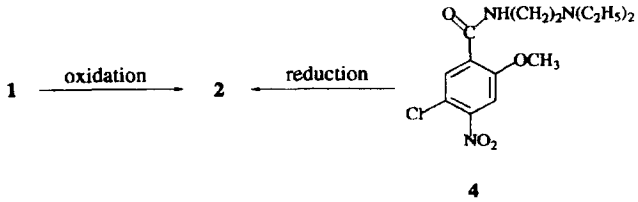
Conceivable strategies for the synthesis of hydroxylamino derivative **2** can be envisaged from both the oxidation of metoclopramide **1** and the reduction of nitro derivative **4** (scheme 1). A survey of the literature evidenced the lack of chemical methods suitable for the selective oxidation of a primary amino group



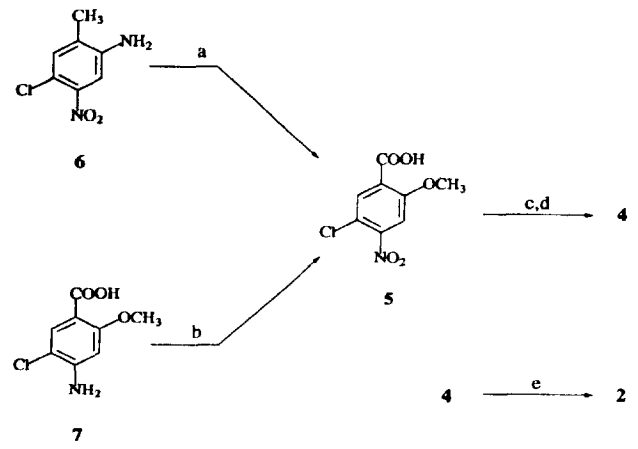
1 **2** **3**

Fig 1.

to the hydroxylamino moiety in the presence of a tertiary amine group. On the other hand, an aromatic nitro group can be cleanly reduced to the hydroxylamino function. For this reason we planned the synthesis of compound **4**, which had already been prepared by condensing 2-methoxy-4-nitro-5-chlorobenzoic acid **5** with diethylaminoethylamine (scheme 2) [11]. In turn, acid **5** was obtained by oxidation of toluene derivative **6**, which is not commercially available but has to be prepared by a tedious procedure [12]. Alternatively, we took into account the possibility of preparing **5** by oxidation of the commercially available amino acid **7** (scheme 2). Reagents such as dimethyldioxirane [13, 14], sodium percarbonate [15] and sodium perborate [16] can reach such a goal. In our hands, the best results were obtained by using the inexpensive sodium perborate. An excess of sodium perborate in acetic acid was reacted with **7** to produce nitro derivative **5** in satisfactory yield (60%). Following the previously reported procedure [11], we prepared key intermediate **4**, which was then reduced with activated zinc and ammonium chloride to give the corresponding hydroxylamino derivative **2** (scheme 2). Compound **2** was characterized by ^{1}H-NMR analysis.



Scheme 1.



Scheme 2. a: reference [11]; b: $NaBO_3/CH_3COOH$; c: $SOCl_2$; d: $H_2N(CH_2)_2NEt_2$; e: Zn/NH_4Cl.

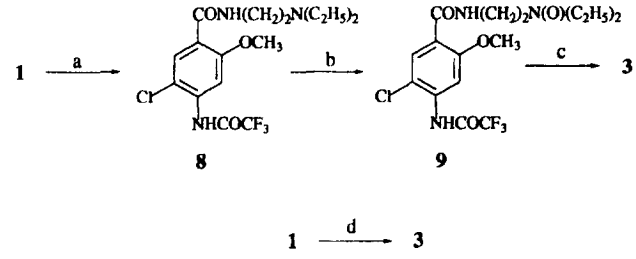
The synthesis of metoclopramide *N*-oxide **3** was accomplished *via* oxidation of metoclopramide with 3-chloroperbenzoic acid (scheme 3). In order to selectively oxidize the tertiary amino group of metoclopramide we chose to protect its primary amino group. For such a purpose we prepared the corresponding trifluoroacetamide **8** by reacting metoclopramide with trifluoroacetic anhydride and triethylamine. Derivative **8** was oxidized to **9** with 3-chloroperbenzoic acid according to a standard protocol. The protecting group at the primary amine was immediately removed by treating **9** with a water/methanol solution of sodium carbonate (scheme 3). We also tested the possibility of preparing **3** by a direct oxidation of metoclopramide. Compound **1** was stirred at room temperature with an excess of 30% hydrogen peroxide then unreacted metoclopramide was removed by extraction with dichloromethane. The aqueous layer was lyophilized to leave pure **3** as a colorless solid. The structure of **3** was ascertained by ^{1}H-NMR analysis.

Experimental protocols

HPLC analysis of the biological samples

HPLC analyses were performed on a Perkin Elmer Series 4 Chromatograph, equipped with a variable UV-visible detector (LC 75), a C_{18} reversed-phase column with 10 μm particle size (25 cm, 4.6 mm id) and a Rheodyne model 7425 injector with a 20 μl loop. Analyses were carried out at room temperature with a flow rate of 1.5 ml/min and at a wavelength of 280 nm.

Five healthy volunteers each received two tablets of commercially available metoclopramide (Plasil 2 × 10.5 mg). Urine samples were collected at intervals and kept frozen until analysis. As a blank we used the 24 h urine of the same volunteers before drug intake. Samples for mass spectrometry analysis were prepared as follows: raw urine was passed through an Amberlite XAD-2 column (bed volume: one half the sample volume) and washed with water (twice the bed volume). The drug-related compounds were then eluted with methanol (three times the bed volume). The methanol solution was



Scheme 3. a: $(CF_3CO)_2O$-NEt_3/CH_2Cl_2; b: *m*-$ClC_6H_4CO_3H/CH_2Cl_2$; c: Na_2CO_3/H_2O-MeOH; d: 30% H_2O_2.

concentrated (10–20 ml for 500 ml urine) and utilized for the mass spectrometry analysis. The original urine or the methanol solution obtained as reported above was filtered through a 0.45 μm Millex-HV filter (Millipore) and 20 μl of the filtrates was directly injected into the HPLC apparatus. The analyses were carried out with the following mobile phases: (a) 240 ml water, 260 ml acetonitrile, 0.8 g sodium laurylsulfate and 5 drops of 85% phosphoric acid; (b) a 65:35 mixture of a 0.05 M aqueous solution of ammonium acetate and acetonitrile. Retention times (t_R, min) with mobile phase (a) were: **1**, 12.7; **2**, 8.5; **3**, 11.5 (see figure 2, which refers to 24 h urine of one subject directly injected in the chromatograph). Retention times (t_R, min) with mobile phase (b) were: **1**, 8.2; **2**, 4.4 (see figure 3, which refers to 24 h urine of one subject after methanol extraction). Due to the lack of an analytical sample of **2** we could not determine the exact amount of the metabolite.

HPLC/MS analyses were performed using a Hewlett Packard HP 1090A solvent delivery system coupled through a thermospray interface to a Hewlett Packard HP 5988A mass spectrometer. Mobile phase (b) was used; the flow rate was 1.5 ml/min. The temperatures were set at 275°C (source), 104°C (stem) and 180°C (tip). The acquisition was made in 'filament off' mode and the positive ions were detected in the range 250–400 Amu.

Fast atom bombardment (FAB) mass spectra were obtained with a VG ZAB 2F instrument on samples of the above reported methanol solutions concentrated to a total volume of 2 ml. Glycerol was used as liquid matrix. Glycerol solutions were bombarded by 8 keV Xe atoms. Collision experiments were performed by colliding 8 keV preselected ions with N_2 in the collision cell placed in second field-free region. The N_2 pressure was adjusted in order to reduce the main beam intensity to 60% of its usual value. The spectra were recorded by collisional activated decomposition (CAD) mass analysed kinetic energy (MIKE) spectroscopy.

Chemistry

Metoclopramide hydrochloride was purchased from Sigma, Amberlite XAD-2 resin from Aldrich. Column chromatographies were carried out on silica gel 63–200 μm and the mobile phases were mixtures of chloroform/methanol in the ratio specified in the appropriate section. TLC analyses were carried out on commercial silica gel GF_{254} plates. Organic solvents were reagent grade. ^{1}H-NMR spectra were recorded at 200 or 400 MHz in $CDCl_3$ solution; chemical shifts (δ) are expressed in ppm and coupling constants (J) in hertz. IR spectra were recorded with a Jasco FT/IR-5000 as a film or as a KBr disc. Mass spectra were obtained with a VG ZAB 2F spectrometer.

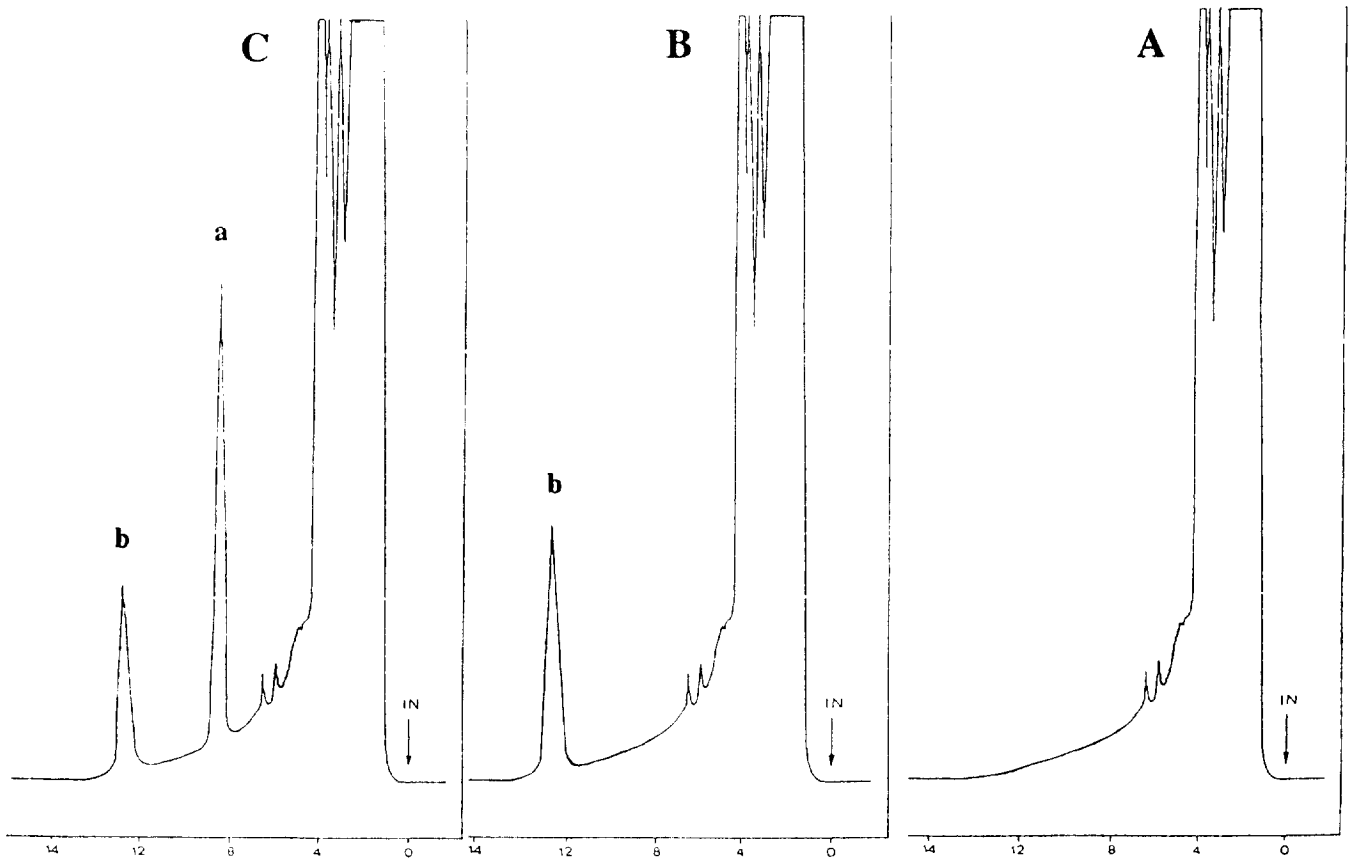


Fig 2. Typical chromatograms obtained after injection of 20 μl of blank (**A**), spiked with metoclopramide (**B**) and sample (**C**) 24 h filtered urine. Mobile phase (a) (see *Experimental protocols*). Sensitivity: 0.01 AUFS; **a**: metabolite; **b**: metoclopramide.

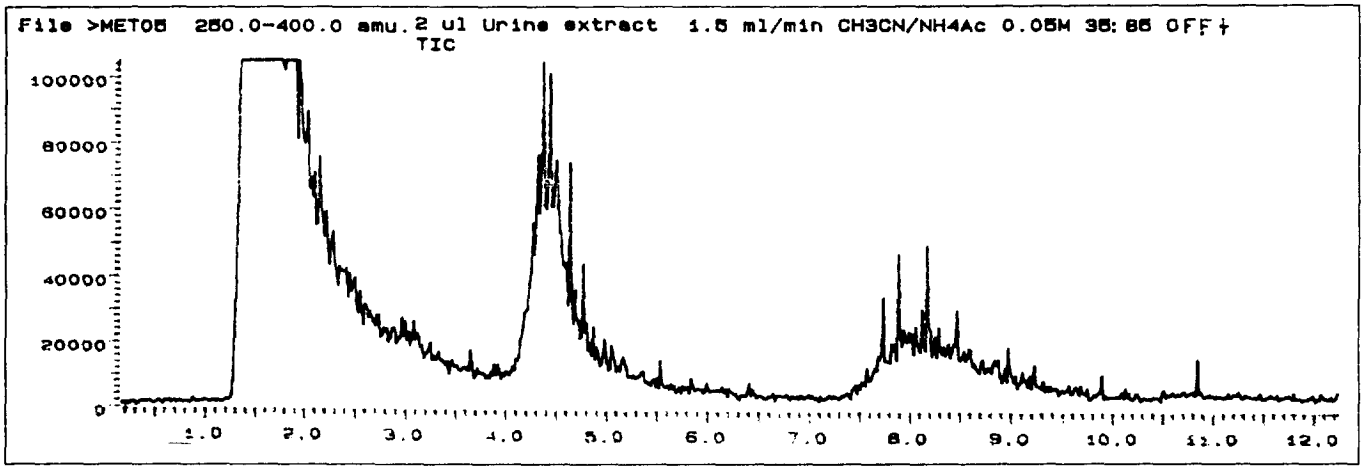


Fig 3. A typical HPLC-thermospray chromatogram of a treated volunteer's urine. Mobile phase (b) (see *Experimental protocols*). Scanning range: 250 to 400 *m/z*.

Synthesis of 2-methoxy-4-nitro-5-chlorobenzoic acid **5**

A 500 ml Erlenmeyer flask charged with 5.5 g (27.3 mmol) of **7**, 24 g (0.156 mol) of sodium perborate tetrahydrate and glacial acetic acid (350 ml) was heated at 50–55°C and magnetically stirred overnight. The precipitate was filtered off and the clarified solution was then diluted with water (500 ml) and extracted with chloroform (3 x 100 ml). The organic extracts were dried over anhydrous sodium sulfate. After evaporation of the solvent under vacuum, acid **5** was collected as a yellow solid (3.8 g; 60% yield). Compound **5** crystallized from EtOH/water as yellow needles, mp 168–169°C (lit [11] 176–177°C). IR (nujol): ν(cm^{-1}) 1710, 1690, 1540, 1460, 1375. ^{1}H-NMR ($CDCl_3$): 4.13 (s, 3, OMe); 7.54 (s, 1, H-3); 8.29 (s, 1, H-6).

Synthesis of 2-methoxy-4-nitro-5-chloro-N-[2-diethylaminoethyl]benzamide **4**

Compound **4** was prepared according to the procedure reported in reference [11]. Mp 58–59°C. ^{1}H-NMR ($CDCl_3$): 1.01 (t, 6, $2CH_2CH_3$; J = 6.8); 2.58 (bq, 6, $CH_2N(CH_2)_2$); 3.53 (q, 2, $NHCH_2$); 4.03 (s, 3, OCH_3); 7.50 (s, 1, H-3); 8.35 (s, 1, H-6); 8.42 (bs, 1, $CONHCH_2$). IR ν(cm^{-1}): 3350, 1660, 1530, 1465, 1380.

Reduction of **4** *to* **2**

A 25 ml Erlenmeyer flask was charged with 0.6 g (1.82 mmol) of **4**, 0.1 g (1.87 mmol) of ammonium chloride and a 1:1 mixture of ethanol/water (12 ml). To the mixture, cooled at 0°C and magnetically stirred, was added 1.0 g of activated zinc. The reaction went to completion in 5 min (HPLC analysis). The excess zinc was removed by filtration and the solution was extracted with chloroform (3 x 10 ml). The organic extracts were dried over sodium sulfate. After evaporation of the solvent, compound **2** (0.43 g, 75% yield) was collected as a yellow solid which partly decomposed on attempted crystallization. IR (KBr): ν(cm^{-1}) 3245, 1600, 1520, 1495, 1470, 1420. ^{1}H-NMR ($CDCl_3$): 1.15 (t, 6, $2CH_2CH_3$; J = 7.2); 2.79 (q, 4, $N(CH_2CH_3)_2$; J = 7.2); 2.87 (t, 2, NCH_2CH_2; J = 5.9); 3.60 (t, 2, $CONHCH_2$; J = 5.9); 3.50–3.70 (bs, 2, NHOH; disappears by treatment with D_2O); 3.86 (s, 3, OCH_3); 6.78 (s, 1, H-3); 7.95 (s, 1, H-6); 8.31 (bs, 1, $CONHCH_2$).

Synthesis of derivative **8**

A 50 ml Erlenmeyer flask equipped with a dropping funnel and a magnetic stirrer was charged with metoclopramide (0.6 g, 2 mmol), triethylamine (0.9 ml, 8 mmol) and chloroform (20 ml). To the ice-cooled mixture, a solution of trifluoroacetic anhydride (560 µl, 4 mmol) in chloroform (10 ml) was added dropwise under stirring. The progress of the reaction was monitored by TLC (eluent: chloform/methanol 2:1). The mixture was washed with a solution of 10% sodium carbonate and dried over sodium sulfate. After the usual workup, the residue (0.68 g, 86% yield) crystallized from isopropanol as colorless prisms, mp 126–128°C. ^{1}H-NMR ($CDCl_3$): 1.04 (t, 6, $2CH_2CH_3$; J = 7.1); 2.59 (m, 6, $2NCH_2CH_3$ and NCH_2CH_2); 3.50 (m, 2, $CONHCH_2$); 3.99 (s, 3, OCH_3); 8.16 (s, 1, H-3); 8.29 (s, 1, H-6); 8.40 (bs, 2, NHCO).

Synthesis of metoclopramide N-oxide **3**

Method A. A mixture of **8** (1.19 g, 3.0 mmol), *m*-chloroperbenzoic acid (0.9 g, 5.2 mmol) and chloroform was stirred at room temperature until disappearance of the starting material. The solvent was removed under vacuum and the residue was column chromatographed on neutral alumina (eluent: chloroform/methanol 2:1). Derivative **9** was collected in quantitative form as yellowish oil. ^{1}H-NMR ($CDCl_3$): 1.34 (t, 6, $2CH_2CHz$; J = 7.2); 3.42 (q, 4, $2NCH_2CH_3$; J = 7.2); 3.56 (t, 2, NCH_2CH_2); 3.94 (m, 2, $CONHCH_2$); 4.00 (s, 3, OCH_3); 4.49 (bs, 1, $NHCOCF_3$); 8.12 (s, 1, H-3); 8.19 (s, 1, H-6); 9.15 (bt, 1, CONH).

A 20% aqueous solution of sodium carbonate (15 ml) was added to a solution of **9** (1.0 g, 2.43 mmol) in methanol (15 ml) and stirred at room temperature until disappearance of the starting material. Methanol was removed from the mixture by evaporation under vacuum and the resulting solution was lyophilized at room temperature. The residue was taken up with ethyl acetate to give **3** in quantitative yield as a colorless solid. Derivative **3** crystallized from absolute EtOH/acetone as colorless prisms, mp 152–153°C, dec. R_F (chloroform–methanol 2:1) 0.16; ^{1}H-NMR ($CDCl_3$): 1.32 (t, 6, $2CH_2CH_3$; J = 7.2); 3.33 (q, 4, $2NCH_2CH_3$; J = 7.2); 3.42 (dd, 2, NCH_2CH_2; J = 6.2 and 6.2); 3.85 (s, 3, OCH_3); 3.93 (dd, 2, $CONHCH_2$; J = 6.2 and 6.2); 4.42 (bs, 2, NH_2; disappears by treatment with

D_2O); 6.24 (s, 1, H-3); 8.03 (s, 1, H-6); 8.79 (bt, 1, CON*H*; disappears by treatment with D_2O).

Method B. Metoclopramide (0.6 g, 2 mmol) was added to 30 ml of 30% hydrogen peroxide. The suspension was magnetically stirred at room temperature overnight and became homogeneous. The solution was extracted with dichloromethane (2 x 5 ml), the aqueous layer was frozen and lyophilized at room temperature to leave **3** (0.487 g, 77%) as a colorless solid.

Results and discussion

The 24 h urine of five healthy volunteers, before and after oral administration of a dose of metoclopramide (Plasil® 2 x 10.5 mg), was analyzed by HPLC according to the procedure reported in the *Experimental protocols*. Figure 2a–c reports typical chromatograms of the blank, sample and spiked urine. In addition to common large peaks due to less retained endogenous substances, the chromatogram of the sample urine shows two additional peaks. One of them, which is characterized by a retention time of 12.7 min, can easily be attributed to metoclopramide. This finding is not surprising since it is well known that up to 25% of metoclopramide is excreted as such [7]. The second peak, characterized by a retention time of 8.5 min, must be attributed to a metabolite of metoclopramide. In order to gain insight into its structure we carried out an HPLC-MS analysis equipped with a thermospray source. A typical HPLC-MS run of treated volunteers' urine is shown in figure 3. We chose to scan the range between *m/z* 250 and 400 in order to avoid any interference of endogenous substances. The peak characterized by a retention time centered at 8.2 min was attributed to metoclopramide by comparing its mass spectrum with that of an authentic sample. The other peak with a t_R of 4.4 min showed a protonated molecular ion $(M + H)^+$ at *m/z* 316. A protonated M + 2 isotopic species and its relative ratio indicates the presence of a chlorine atom. The simultaneous presence of a protonated molecular ion at *m/z* 300 and the related peak at *m/z* 302 was attributed to a different molecular species originating from a thermal decomposition of the compound with the protonated molecular ion at *m/z* 316 during the desolvation step. The mass spectrum of such a metabolite is reported in figure 4. It is quite clear that this compound is not 2-(4-amino-5-chloro-2-methoxybenzamido)acetic acid, a metabolite previously found in human urine [8], since its molecular weight should be 258. The new metabolite possesses a molecular weight 16 units higher than metoclopramide; it is straightforward to deduce that such a metabolite is derived from the addition of an oxygen atom to the structure of metoclopramide. In order to confirm the above findings and to elucidate the structure of the metabolite, we undertook a series of experiments based on CAD-MIKE spectroscopy. The FAB analysis of the treated urine gives rise to a very complex spectrum which is almost useless. We selected the ion at *m/z* 316 and, by means of CAD-MIKE spectroscopy, we obtained a fragmentation pattern reported in figure 5. In contrast with what was observed for metoclopramide, a primary loss of 17 Amu was observed to give the ion at *m/z* 299, which suggests that the oxygen atom is present in the molecule as a hydroxyl group. This result is confirmed by the presence of the ion at *m/z* 284, corresponding to the loss of the NHOH moiety. Loss of two ethyl radicals or the diethylamino group from the ion at *m/z* 316, the parent ion, gave rise to the ions at *m/z* 258 or 244 respectively. Since the collision spectrum indicates the presence of the NHOH group, which is totally absent in metoclopramide, we tenta-

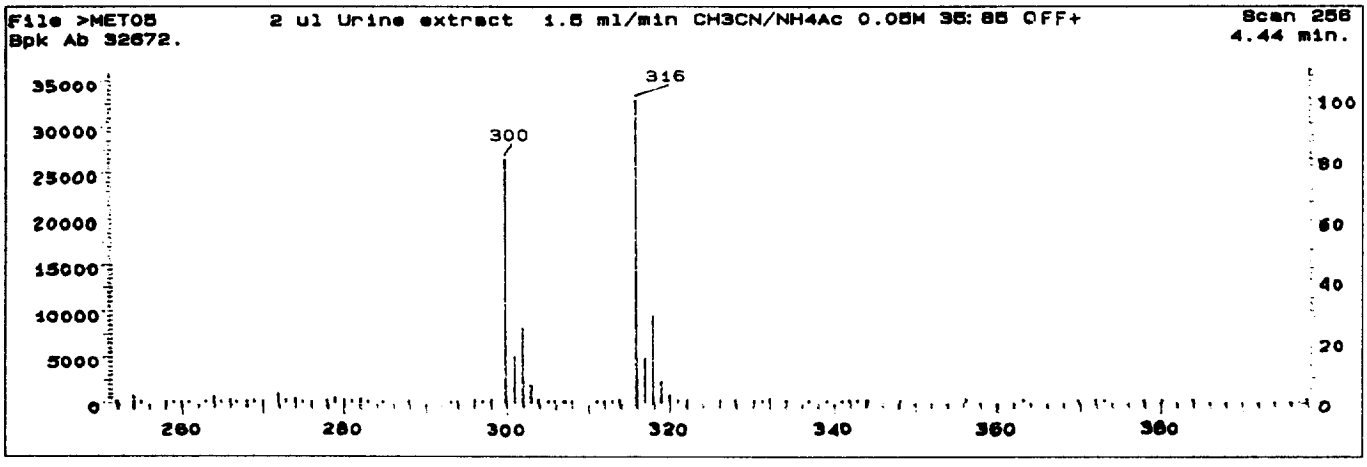


Fig 4. Thermospray mass spectrum of the compound at t_R 4.4 min (see fig 3).

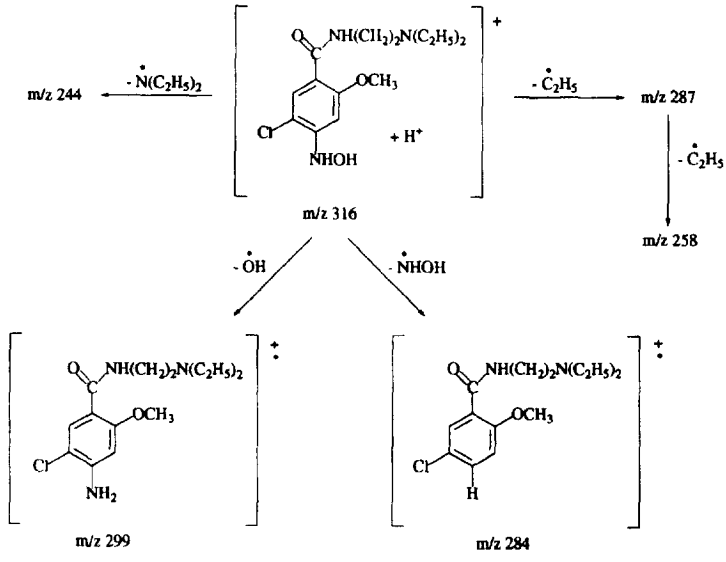


Fig 5. MS fragmentation pattern for metabolite **2**.

tively assigned the structure of the hydroxylamino derivative **2** to the new metabolite.

Since the presence of an arylhydroxylamino derivative among the metabolites of metoclopramide is of utmost importance, we planned the synthesis of **2** according to the strategy reported above. In such a way we attributed the structure of the new metabolite with certainty. The addition of a small amount of pure **2** to the sample urine enhanced the peak at 8.5 min in the HPLC chromatogram. Furthermore, the fragmentation pattern of pure **2** was identical to that discussed above for the unknown metabolite.

Unfortunately, we could not determine the exact amount of the metabolite in the sample urine. A rough evaluation of the amount of **2** could be deduced from the ratio between the areas of **2** and metoclopramide which spanned the range 1.8–2.2.

An alternative metabolic path of metoclopramide could involve the oxidation of the tertiary amine group to give metoclopramide *N*-oxide **3**, whose molecular weight is identical to that of derivative **2**. In order to exclude such a possibility we prepared **3** which was submitted to HPLC analysis. Under the same experimental conditions compound **3** displayed a t_R of 11.5 min, which is quite different from that of **2** (t_R 8.5 min). In such a way, we excluded the presence of **3** among the metabolites of metoclopramide.

Moreover, we were unable to detect ring oxidation compounds, which represent a further metabolic path of primary aromatic amines [17].

In summary, the results of the present research provide evidence for the presence of a new metabolite of metoclopramide in the urine of healthy volunteers. Its structure, inferred by HPLC-MS and FAB MS-MS analyses and fully confirmed by an independent synthesis, is that of **2** and is characterized by the presence of the hydroxylamino moiety. The biological relevance of such a new metabolite will be studied in the near future and will be published in due course.

Acknowledgments

For FAB and CAD-MIKE spectroscopy measurements we thank P Traldi from Servizio di Spettrometria di Massa, CNR, corso Stati Uniti, 4, Camin 35100 Padova (Italy). This work was supported by a grant from Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST, Rome).

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