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Identification of the Metabolites of MX/2/120 in the Guinea-pig by High-performance Liquid Chromatography/Thermospray Tandem Mass Spectrometry

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The metabolism of MX/2/120, a new xanthine derivative endowed with potent and long-lasting antibronchospastic activity, was investigated in guinea-pig urine, plasma and bile after intravenous administration of 12.5 mg kg^{-1} by high-performance liquid chromatography/tandem mass spectrometry with thermospray ionization. Following a first series of collisionally activated neutral losses and parent ion scan experiments performed on urine samples, potential quasi-molecular ions of possible metabolites were identified, which were then analysed by collisionally activated fragment ion scans. A side-chain carboxylated, a side-chain hydroxylated and a xanthine-ring demethylated metabolite, along with the unmodified drug, were identified. In urine, MX/2/120 and its hydroxylated metabolite were also present as glucuronic acid conjugates. In plasma and bile, only the unmodified drug was found. The structures of the identified metabolites were then confirmed by comparison with the authentic compounds prepared by synthesis.

INTRODUCTION

We have recently reported on the identification of plasma and urine metabolites of isbufylline, i.e. 1,3-dimethyl-7-(2-methylpropyl)xanthine, a new anti-asthma drug discovered in our laboratories.[1,2] This study showed that in humans, but also in guinea-pigs and rabbits, isbufylline underwent demethylation in position 3 and oxidation of the 7-isobutyl chain; whereas the 3-*N*-demethylated metabolite displayed an antibronchospastic effect comparable to that with the parent compound, the side-chain oxidized metabolites were less or far less active. Thus, with the aim of preventing or at least hindering the metabolic oxidation step by means of a bulkier alkyl group in position 7, we synthesized a series of 7-neopentyl-substituted xanthines bearing various substituents in the remaining positions of the ring.[3]

Among the compounds tested in guinea-pigs, we found that 7-[(2,2-dimethyl)propyl]-1-methylxanthine, laboratory code MX/2/120 (Fig. 1), was endowed with the most potent and long-lasting antibronchospastic activity and negligible side-effects, thus confirming the previous findings that the 3-alkyl substituent was not a prerequisite for activity.[4] The aim of the present work was to identify and characterize plasma, urine and bile metabolites of MX/2/120 in the guinea-pig, after a single i.v. administration of 12.5 mg kg^{-1}.

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EXPERIMENTAL

Animals

Male Dunkin–Hartley guinea-pigs were used for the study (Rodentia, Torre Pallavicina, Bergamo, Italy). Their weight ranged from 400 to 500 g.

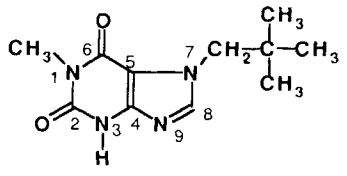

Figure 1. Structure of the anti-bronchospastic drug MX/2/120 (1-methyl-7-neopentylxanthine).

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Drug administration and sample storage

After a fasting period of 16 h, animals were treated i.v. with a solution of MX/2/120 in glycofurol at a concentration of 12.5 mg ml^{-1}. The dose administered corresponded to 12.5 mg kg^{-1}. Urine, plasma and bile samples were collected until 24 h after administration. All the samples were stored at −20 °C before analysis.

Reagents

β-Glucuronidase from *Escherichia coli* was purchased from Sigma (St Louis, MO, USA), glycofurol from Alchymars (Milan, Italy), aluminium chloride, toluene, 3-bromo-2,2-dimethylpropan-1-ol and 3-chloropyvalic acid from Aldrich (Milwaukee, WI, USA), analytical-grade ammonium acetate, acetic acid and trifluoroacetic acid from Merck (Darmstadt, Germany) and HPLC-grade acetonitrile and water from Labscan (Dublin, Ireland).

MX/2/120 was synthesized according to the procedures described in Ref. 3.

7-(2,2-Dimethylpropyl)-1-trideuteromethylxanthine was similarly prepared by methylation of 6-amino-1-benzyluracil (intermediate in MX/2/120 synthesis) with dimethyl-d_6 sulphate. M.p. 209–211 °C; analysis, C calc. 55.21%, found 55.06%, H calc. 6.74%, found 6.76%, N calc. 23.41%, found 23.32%; mass spectrum (*m/z*, electron impact (EI), 70 eV), 239 (100%), 183 (98%), 182 (75%), 169 (50%), 123 (26%), 109 (25%).

1-Methyl-7-(2-methyl-2-hydroxymethyl)propylxanthine was prepared by reaction of the sodium salt of 1-methyl-3-benzylxanthine (synthesis intermediate of MX/2/120) with an equivalent amount of 3-bromo-2,2-dimethylpropan-1-ol, in *N,N*-dimethylformamide for 7 h at 100 °C. The compound obtained (m.p. 121–123 °C) was debenzylated by treatment with anhydrous $AlCl_3$ in toluene at 70 °C for 1 h. M.p. 273–275 °C; analysis, C calc. 52.37%, found 52.44%, H calc. 6.39%, found 6.60%, N calc. 22.21%, found 22.22%; mass spectrum (*m/z*, EI, 70 eV), 252 (20%), 222 (38%), 179 (43%), 166 (100%), 109 (45%).

2,2-Dimethyl,3-(1-methylxanthin-7-yl)propionic acid was prepared by reaction of the sodium salt of 1-methyl-3-benzylxanthine (synthesis intermediate of MX/2/120) with an equivalent amount of 3-chloropyvalic acid in *N,N*-dimethylformamide for 7 h at 100 °C. The compound obtained (m.p. 178–180 °C) was debenzylated by treatment with anhydrous $AlCl_3$ in toluene at 70 °C for 1 h. M.p. 325–327 °C; analysis, C calc. 48.79%, found 48.94%, H calc. 5.58%, found 5.30%, N calc. 20.69%, found 20.52%; mass spectrum (*m/z*, EI, 70 eV), 266 (69%), 221 (22%), 179 (100%), 166 (52%), 109 (40%), 95 (35%), 67 (56%).

7-(2,2-Dimethyl)propylxanthine was synthesized by alkylation of 3-benzylxanthine sodium salt[5] with an equimolar amount of neopentyl bromide in *N,N*-dimethylformamide for 7 h at 100 °C. The product obtained was debenzylated by treatment with anhydrous $AlCl_3$ in toluene at 70 °C for 1 h. M.p. 283–285 °C; analysis for $C_{10}H_{14}N_4O_2 \cdot 0.5\ H_2O$, C calc. 51.89%, found 51.67%, H calc. 6.49%, found 6.39%, N calc. 24.24%, found 24.34%; mass spectrum (*m/z*, EI, 70 eV), 222 (91%), 166 (100%), 152 (22%), 123 (35%), 57 (73%).

Sample preparation

Urine and bile samples were thawed, filtered on a 0.45 μm ACRO LC 25 membrane filter (Gelman Sciences, Ann Arbor, MI, USA) and injected directly into the high-performance liquid chromatographic/mass spectrometric (HPLC/MS) system.

Plasma samples were thawed, an equal volume of acetonitrile was added and the mixtures were filtered and injected.

Hydrolysis with β-glucuronidase

A 1 ml volume of urine was treated with 1000 U of β-glucuronidase in 1 ml of pH 6.8 phosphate buffer at 37 °C for 20 h. The sample was then filtered on a 0.45 μm ACRO LC 25 membrane filter and injected.

HPLC/MS

Thermospray tandem mass spectra were measured on a VG Quattro mass spectrometer with a Q1–X–Q2 configuration (where Q represents a quadrupole analyser and X is an hexapolar collision cell), equipped with Lab-base software and fitted with a VG thermospray ion source operated at 290 °C (Fisons Instruments, Altrincham, UK). The repeller potential was set at 220 V and the vaporizer temperature was set at a constant value of 320 °C.

Collisionally activated dissociation (CAD) MS/MS experiments were performed using argon as the collision gas at an energy of 200 eV and a pressure capable of reducing by about half the intensity of the MX/2/120 quasi-molecular ion (*m/z* 237).

All spectra were the background-subtracted averages of three or four scans centred around the HPLC peak apexes.

The mass spectrometer was interfaced with a model 600 MS liquid chromatograph (Waters, Milford, MA, USA), equipped with a U6K injection valve and a 20 μl loop.

The separation column was a LiChrospher 100 RP-18 cartridge (25 cm × 4.0 mm i.d., particle size 5 μm) (Merck) with a LiChrospher 100 RP-18 pre-column (4.0 mm × 4.0 mm i.d., particle size 5 μm). The mobile phase was a mixture of 0.1 M ammonium acetate in water containing acetic acid (2.5%, v/v), adjusted to pH 4.0 with trifluoroacetic acid, and acetonitrile (95:5, v/v) at a flow rate of 1.0 ml min^{-1}. In order to effect separation a gradient was used, running linearly from 5% to 40% acetonitrile in 40 min, with a 5 min hold at the final value. The injection volume was 20 μl.

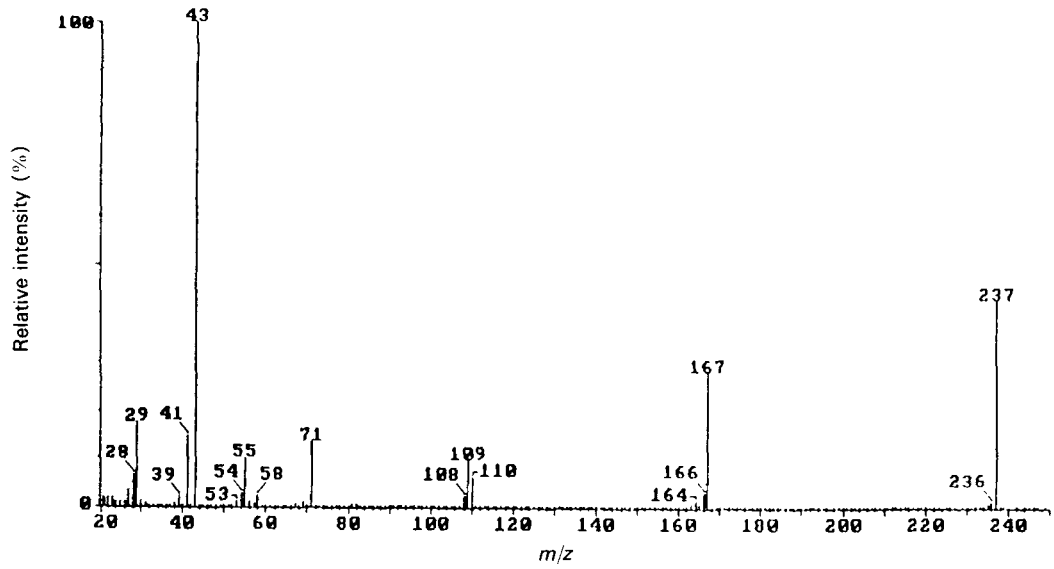

Figure 2. CAD fragment ion spectrum of MX/2/120.

RESULTS

Urine analysis

A first HPLC/thermospray MS analysis of a 24-h urine sample gave only limited information, owing to the presence of a high background and a large number of co-eluting peaks in the total ion chromatogram. Some differences with respect to a blank urine sample allowed three small peaks to be located, at about 20.8, 24.2 and 25.3 min, which were tentatively assigned to metabolites; their putative quasi-molecular ions were at *m/z* 253, 267 and 253, respectively, but no structural information was obtained owing to lack of fragmentation.

In order to confirm the above preliminary data and to detect other possible metabolites, an HPLC/MS/MS screening was then performed, using the parent–neutral loss scan technique.[6–13] According to this method, first described by Perchalski *et al.*,[6] drug metabolites can be identified on the basis of the fact that they normally still maintain some structural characteristics of the original drug. This yields common characteristic fragment ions, and common neutral losses, with respect to the mass spectrum of the drug. The first step consists in measuring a fragment ion tandem mass spectrum of the pure drug, preferably using a soft ionization technique in which the total current is carried mainly by the quasi-molecular ion. Then neutral loss or precursor ion tandem mass spectra are obtained from a biological sample, selecting appropriate neutral losses and fragment ions occurring in the drug spectrum; the ions obtained by this search are candidate metabolite quasi-molecular ions, which are then selected for fragment ion experiments to identify their full fragmentation pattern. The combined neutral loss (or precursor ion)–fragment ion scan experiments may then be repeated selecting new losses or fragment ions arising from the fragment ion spectra of the first identified metabolites.

This approach, which permits rapid drug metabolite identification with very little sample preparation, is generally used by directly introducing the sample after an extraction step in a chemical ionization[6–9] or fast atom bombardment[10–13] ion source without on-line chromatographic separation. In the present case, we coupled this technique with HPLC in order to add retention time information and to minimize matrix interferences.

The CAD fragment ion spectrum of MX/2/120 is shown in Fig. 2 and is in agreement with both the fragmentation scheme described for the CAD tandem mass spectrum of the quasi-molecular ion of isbufylline[1] and the general decomposition pathway of the xanthine nucleus observed for EI[14] and EI/mass-analysed ion kinetic energy (MIKE)[15] spectra of methylxanthines. This scheme is shown in Fig. 3; accordingly, the main ions in the drug spectrum are at *m/z* 237 (MH^+), 167 (ion *a*), 110 (ion *b*), 109 (ion *b* − 1), 71 ($C_5H_{11}^+$, side-chain), 43 ($C_3H_7^+$) and 41 ($C_3H_5^+$). The last two ions

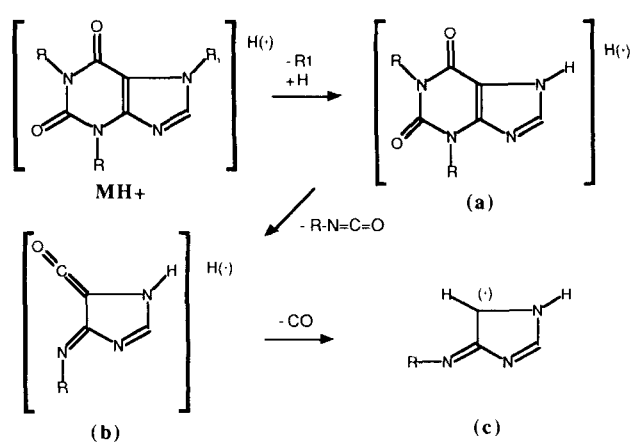

Figure 3. Fragmentation scheme for CAD fragment ion mass spectra of MX/2/120 and related compounds. As a rule, ions *a* − 1 and *b* − 1 are also observed, whereas ion *c* has low abundance or is absent.

Table 1. CAD MS/MS experiments performed in the parent–neutral loss screening for the identification of MX/2/120 metabolites

Metabolite substructure	MS/MS experiment[a]
Unchanged xanthine ring	P 167
N^1-Demethylated xanthine ring	P 153
Hydroxylated xanthine ring	P 181
Unchanged side-chain	NL 70
Hydroxylated side-chain	NL 86
Carboxylated side-chain	NL 100

[a] NL = neutral loss; P = parent.

Table 2. HPLC retention times and putative quasi-molecular ion masses of the possible metabolite peaks found through the parent–neutral loss scan technique

HPLC retention time (min)	Candidate MH^+ (m/z)	MS/MS experiment[a]
20.72	253	P 167, NL 86
24.34	267	P 167, NL 100
25.21	237	P 167
25.36	253	P 167, NL 86
28.86	223	P 153
35.22[b]	237	P 167

[a] NL = neutral loss; P = parent.
[b] Unchanged drug.

arise from the rearrangement of the side-chain neopentyl group.

From the results obtained in previous studies with structurally related compounds,[1,2,16] three possible phase 1 metabolite species were expected for MX/2/120, that is, oxidation products of the side-chain in position 7, with formation of hydroxylated and carboxylated compounds, products with a modified xanthine moiety (e.g. N^1-demethylated or ring-oxidized metabolites) and products arising from combination of the two above processes.

On the assumption that the possible metabolites had the same fragmentation pattern as the drug, MS/MS parameters were set in order to reveal the expected structural characteristics as shown in Table 1.

Figure 4 shows some HPLC thermospray CAD MS/MS traces obtained for the parents of the m/z 167 ion: besides the peak at 35.22 min, assignable by retention time and precursor ion mass of 237 to the original drug, there are four other peaks at 20.72, 24.34, 25.21 and 25.36 min, corresponding to precursor ion masses of 253, 267, 237 and 253, respectively, related to candidate metabolites with the unmodified xanthine ring.

The noisy baseline did not permit to reveal the peak of the unchanged drug from the reconstructed chromatogram of the ions in the m/z range 200–300; the chromatogram of the m/z 237 ion, however, clearly showed the presence of MX/2/120 and a small additional peak at 25.21 min.

Analogously, the HPLC/thermospray CAD MS/MS trace obtained for the parents of the m/z 153 ion showed a peak at 28.86 min, displaying a precursor ion mass of 223, as a possible metabolite in which, according to Table 1, the xanthine ring should be N^1-demethylated.

Among the other MS/MS experiments reported in Table 1, the chromatograms of the precursors of m/z 181 and the neutral losses of 70 u did not show significant peaks, whereas the chromatograms of the neutral losses of 86 and 100 u confirmed the presence of the known peaks having precursor ion masses of 253 and 267, without giving additional information.

Table 2 summarizes HPLC retention times and putative quasi-molecular ion masses of the possible metabolite peaks found through the parent–neutral loss scan technique.

In order to allow identification of the candidate metabolites detected above, a series of CAD fragment

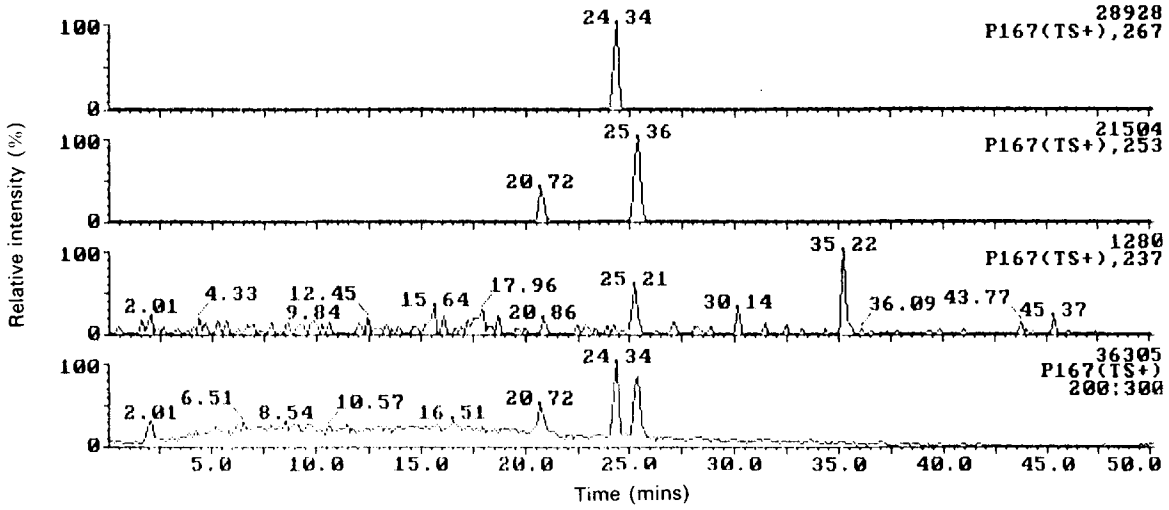

Figure 4. HPLC thermospray CAD MS/MS traces obtained for the parents of the m/z 167 ion: the peaks at 20.72, 24.34, 25.21 and 25.36 min are candidate metabolites in which the xanthine ring is unchanged. The peak at 35.22 min is the original drug.

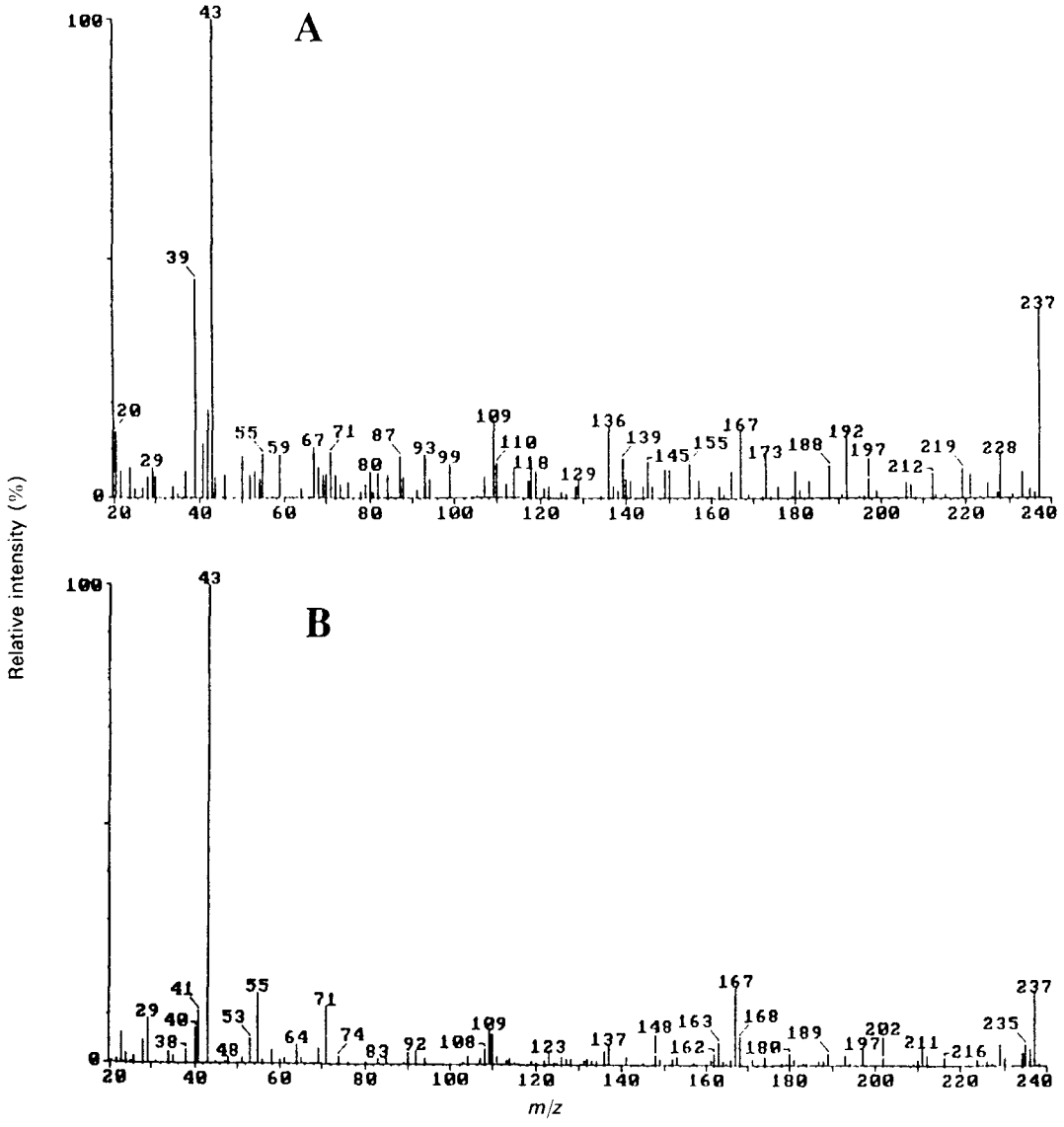

Figure 5. CAD MS/MS fragment ion spectra of the two chromatographic peaks having *m/z* 237 as the possible quasi-molecular ion. (A) Peak at 25.21 min; (B) peak at 35.3 min, assignable to the unchanged drug.

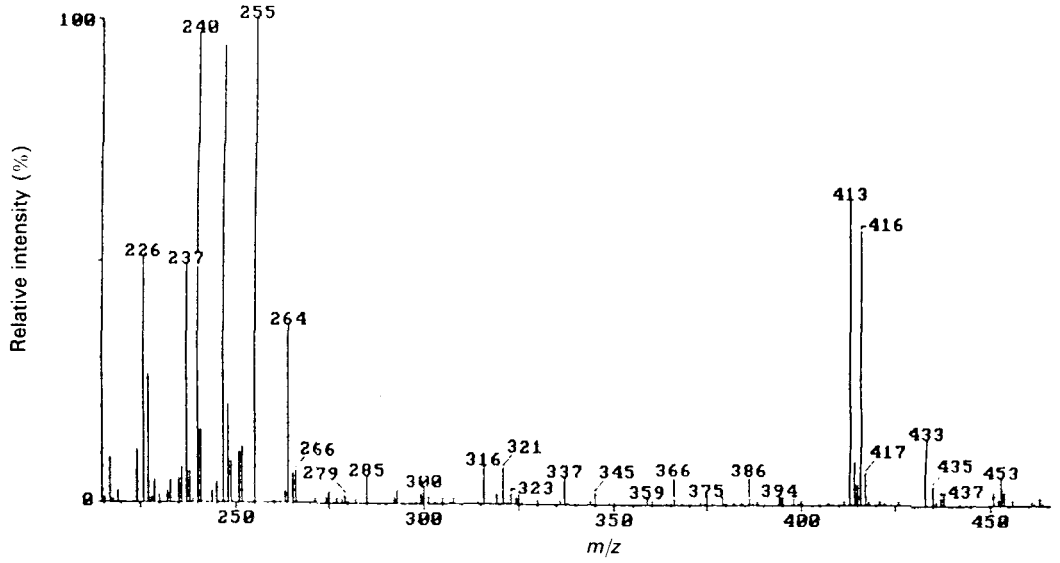

Figure 6. Background-subtracted thermospray mass spectrum of the peak at 25.21 min, obtained from urine of a guinea-pig treated with an equimolar mixture of MX/2/120 and its N^1-deuterated analogue: the two pairs of peaks at *m/z* 237 and 240 and *m/z* 413 and 416 suggest a glucuronide of the unchanged drug.

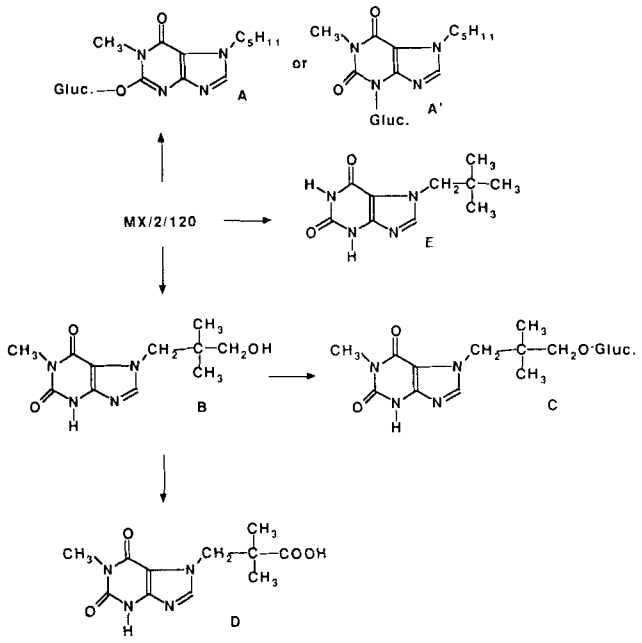

Figure 7. Scheme of the metabolic transformations for MX/2/120 as indicated by guinea-pig urine analysis.

ion experiments were performed, selecting the found putative quasi-molecular ions as precursors. The results were as follows.

Fragments of *m/z* 237. The CAD fragment ion spectra of the two chromatographic peaks having *m/z* 237 as the possible quasi-molecular ion are shown in Fig. 5. The spectrum in Fig. 5(B), corresponding to the peak at 35.3 min, is as expected in agreement with the spectrum of the authentic drug in Fig. 2, although the background peaks interfere with the lower abundance peaks of the spectrum and the relative abundances of the ions are slightly different.

The spectrum of Fig. 5(A), corresponding to the peak at 25.4 min, shows almost the same peaks with the exception of *m/z* 39, but there are even more background interferences owing to its very small amount. Since the nature of the metabolite was not proved, further analyses were performed: first, the peak disappeared after treatment of a urine sample with β-glucuronidase; second, analysis of urine from animals treated with an equimolar mixture of the drug and its N^1-trideuterated analogue confirmed that the peak belonged to a metabolite of the drug. In fact, its background-subtracted thermospray mass spectrum, shown in Fig. 6, shows two pairs of peaks differing by 3 u, at *m/z* 237 and 240 and *m/z* 413 and 416. Although the spectrum quality is poor owing to the high background, this demonstrates that this metabolite is a glucuronic acid conjugate of MX/2/120, whose molecular mass is 412. No information can be drawn about the position of the conjugation, the most logical ones being at the O-2 position of the drug in the enol form and at the N-3 position (Fig. 7(A) and (A')).

Fragments of *m/z* 253. Figure 8 depicts the CAD fragment ion spectra of the two chromatographic peaks at 20.72 and 25.36 min ((A) and (B), respectively) having *m/z* 253 as the putative molecular ion: they are similar, and both are in agreement with a structure having unchanged xanthine ring, as shown by the ions at *m/z* 167 (ion *a*) and 110 (ion *b*), and with a hydroxylated side-chain, a shown by the additional 16 u to the quasi-molecular ion mass of the drug, as well as by the ions at *m/z* 31 and 45 (CH_2OH^+ and $CH_2CH_2OH^+$, respectively). Accordingly, both spectra should indicate a structure as depicted in Fig. 7(B). Following incubation with β-glucuronidase of a urine sample, the first peak disappeared and the second peak was enhanced, indicating that the first peak corresponded to the glucuronic acid conjugate of the side-chain hydroxylated metabolite. Although no appreciable quasi-molecular ion at the expected *m/z* 429 could be observed in the thermospray mass spectrum of this compound, this could be easily explained by thermal degradation in the thermosrpay ion source, analogous to that observed for isbufylline.[1,2] The subsequent comparison with the HPLC retention time and the thermospray CAD fragment ion mass spectrum of synthetic 1-methyl-7-(2-methyl-2-hydroxymethyl)propylxanthine (the latter is shown in Fig. 8C) confirmed the assumptions made.

Fragments of *m/z* 267. Figure 9(A) shows the CAD fragment ion spectrum of the chromatographic peak at 24.34 min, having the supposed molecular ion of *m/z* 267: again, the ions at *m/z* 167 (ion *a*) and 110 (ion *b*) indicate an unchanged xanthine ring, while the relative molecular mass increase of 30 u is in agreement with a side-chain in which a methyl group has been oxidized to a carboxyl group. Hence the structure of this metabolite should be that shown in Fig. 7(D). The HPLC retention time and the CAD fragment ion spectrum of synthetic 2,2-dimethyl,3-(1-methylxanthin-7-yl)propionic acid shown in Fig. 9(B) confirmed the hypothesis made.

Fragments of *m/z* 223. The CAD fragment ion spectrum of the chromatographic peak at 28.86 min is shown in Fig. 10(A). The *m/z* 153 ion, which corresponds to ion *a* in the scheme in Fig. 3, is 14 u lower than the corresponding ion in the original drug and the metabolites examined so far, indicating an *N*-demethylated xanthine ring. Ion *b*, which is again at *m/z* 110 as in all the other compounds, is perfectly in accordance with the described fragmentation pattern, since in this case an isocyanic acid molecule (43 u) is lost from ion *a* instead of methyl isocyanate (57 u). The side-chain being unchanged, as shown by the initial loss of 70 u from the quasi-molecular ion and by the peaks in the low-mass region of the spectrum, this metabolite can be assigned the structure shown in Fig. 7(E). Again, the comparison with the HPLC retention time and the CAD fragment ion spectrum of synthetic 7-neopentylxanthine (Fig. 10(B)) confirmed the suggested structure.

Plasma and bile analysis

Analogous parent–neutral loss screening analyses, performed on plasma and bile samples, did not show

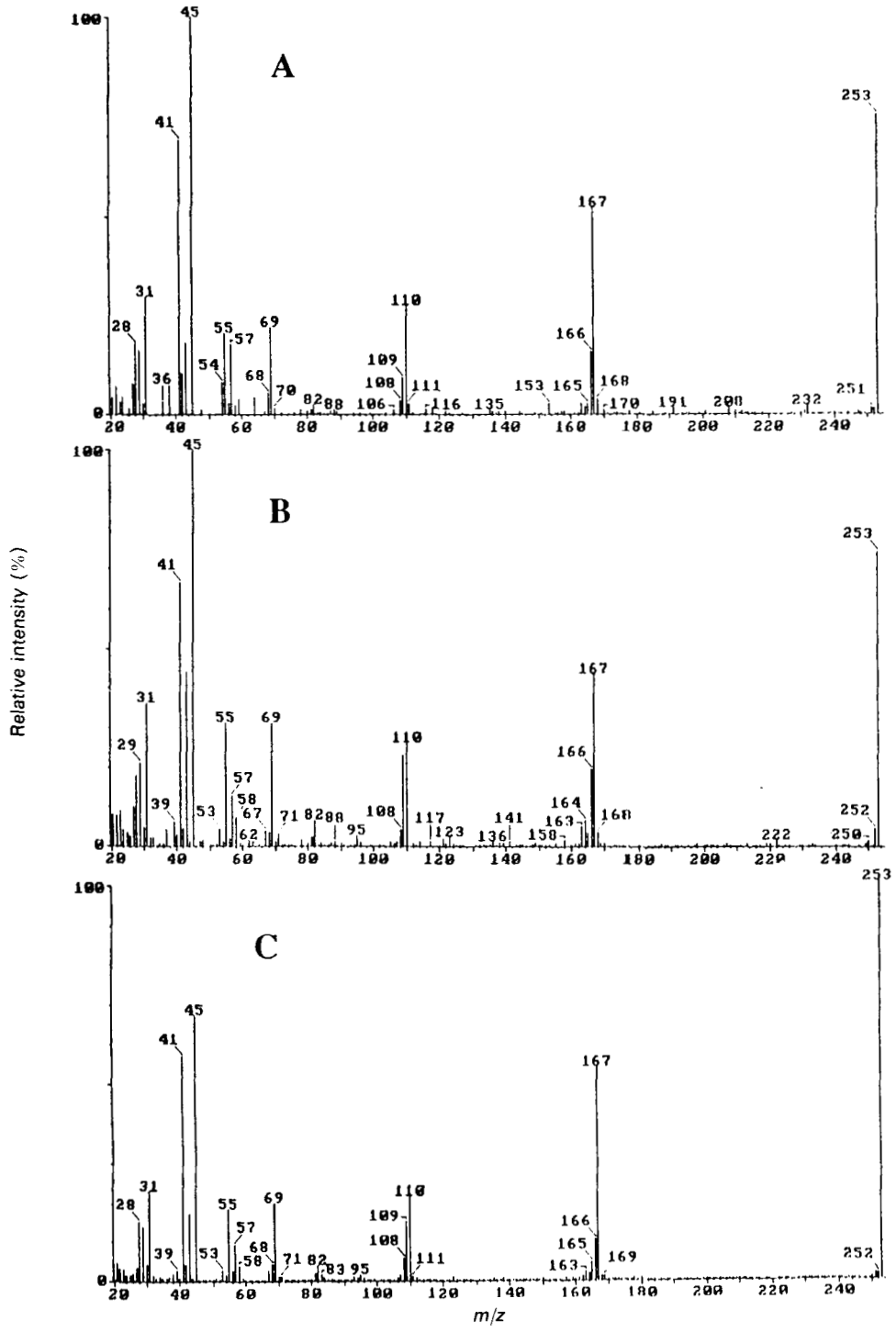

Figure 8. CAD MS/MS fragment ion spectra of the two chromatographic peaks having *m/z* 253 and the possible quasi-molecular ion ((A) peak at 20.72 min and (B) peak at 25.36 min) and of synthetic 1-methyl-7-(2,2-dimethyl-3-hydroxypropyl)xanthine (C).

detectable levels of metabolites; only the unmodified drug was found in appreciable amounts in both biological fluids, as demonstrated by retention time and CAD fragment ion spectra (data not shown).

DISCUSSION

The parent–neutral loss screening technique permits the rapid identification of drug metabolites with very little sample preparation; in a few hours it is possible to obtain information that can otherwise be achieved only after long extraction and fractionation procedures. However, some disadvantages have to be taken into account: first, it is possible to miss some metabolites formed via unusual metabolic processes, or giving unexpected CAD MS/MS fragmentation pathways;[12] second, isomeric species cannot be distinguished because of their usually very similar fragmentations and the superimposition of their signals, although they can be detected if some separation between them occurs, such as during heating in a direct insertion probe;[7] third, artifacts can result from reactions on a FAB

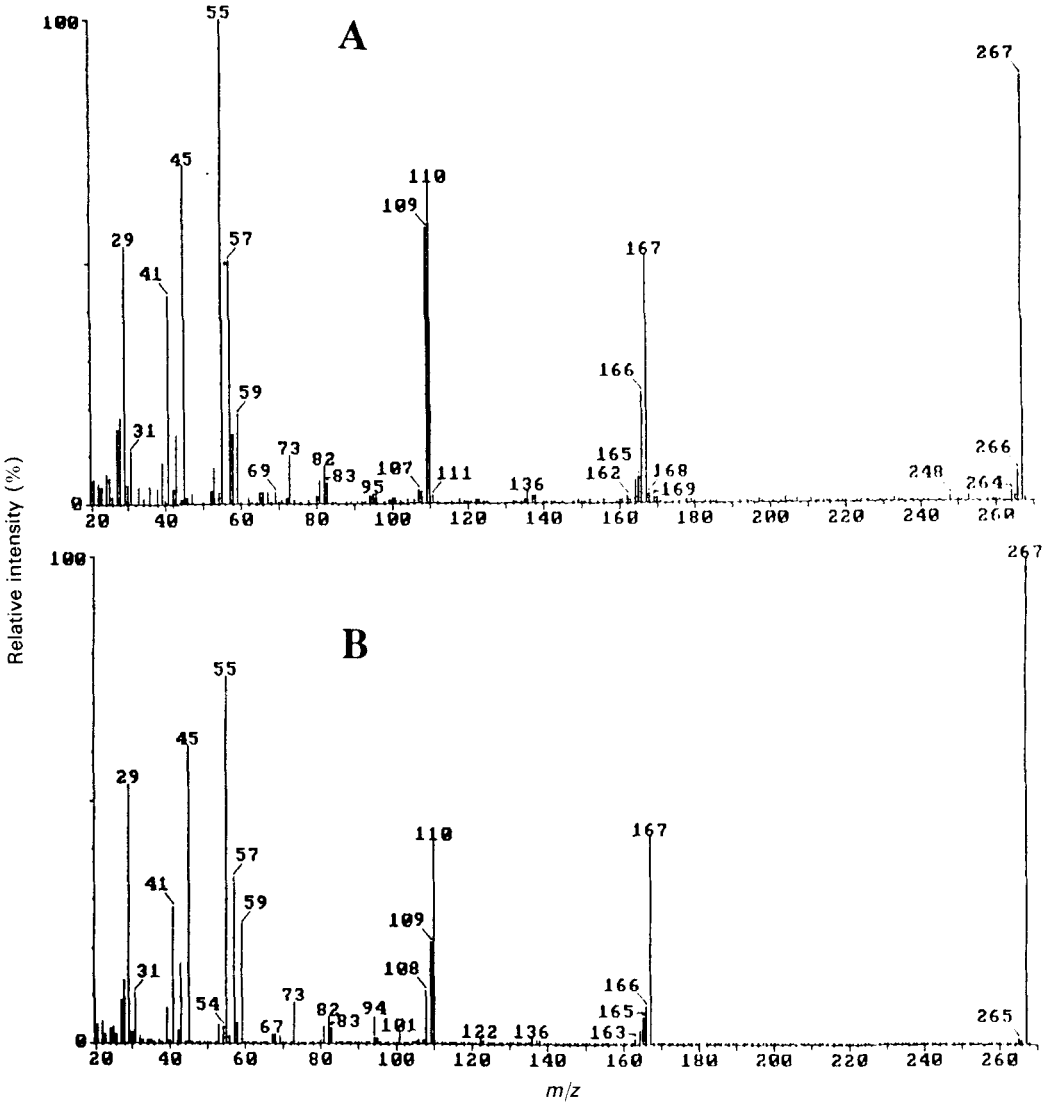

Figure 9. CAD MS/MS fragment ion spectra of the chromatographic peak at 24.34 min having *m/z* 267 as the possible quasi-molecular ion (A) and of synthetic 2,2-dimethyl,3-(1-methylxanthin-7-yl)propionic acid (B).

probe tip during the ionization process,[12,13] or from the presence of endogenous substances having some fragment ions or neutral losses in common with the drug and its metabolites.

The on-line coupling of a chromatographic technique with the parent–neutral loss screening technique can be very useful in order to overcome some of the above-mentioned drawbacks. The addition of a separation step minimizes interferences from the biological matrix and artifacts originating in the ion source. The retention time information permits the detection and possibly characterization of isomeric species, and adds confidence to the postulated structures if synthetic standards are available.

Although this method is capable of characterizing previously undetected metabolites, as shown by Korfmacher *et al.*[17] for the HPLC/MS/MS determination of pyrilamine metabolites in rat urine, the risk of missing some 'unusual' metabolites potentially still remains. To reduce this possibility, it is useful to perform multiple precursor ion and neutral loss experiments, also including those related to theoretically possible metabolites, in order to obtain cross-confirmations and complementary information. For instance, referring to Table 2, the N^1-demethylated metabolite, which was detected through the precursors of *m/z* 153 experiment, would have been missed if the screening had been based only on the original drug fragmentation, because the neutral losses of *m/z* 70 experiment, which could have been able to detect it, lacked sufficient sensitivity. Of course, complementary techniques such as radioactive or stable isotope labelling,[18,19] can be successfully applied in order to confirm metabolite identification in doubtful cases.

CONCLUSIONS

The biotransformation of the new anti-bronchospastic drug MX/2/120 in the guinea-pig is depicted in Fig. 7. The drug undergoes principally neopentyl side-chain oxidation to alcohol, which is in turn oxidized to carboxylic acid or conjugated with glucuronic acid. Two other minor metabolization pathways are demethylation of the xanthine ring and conjugation of the drug itself with glucuronic acid.

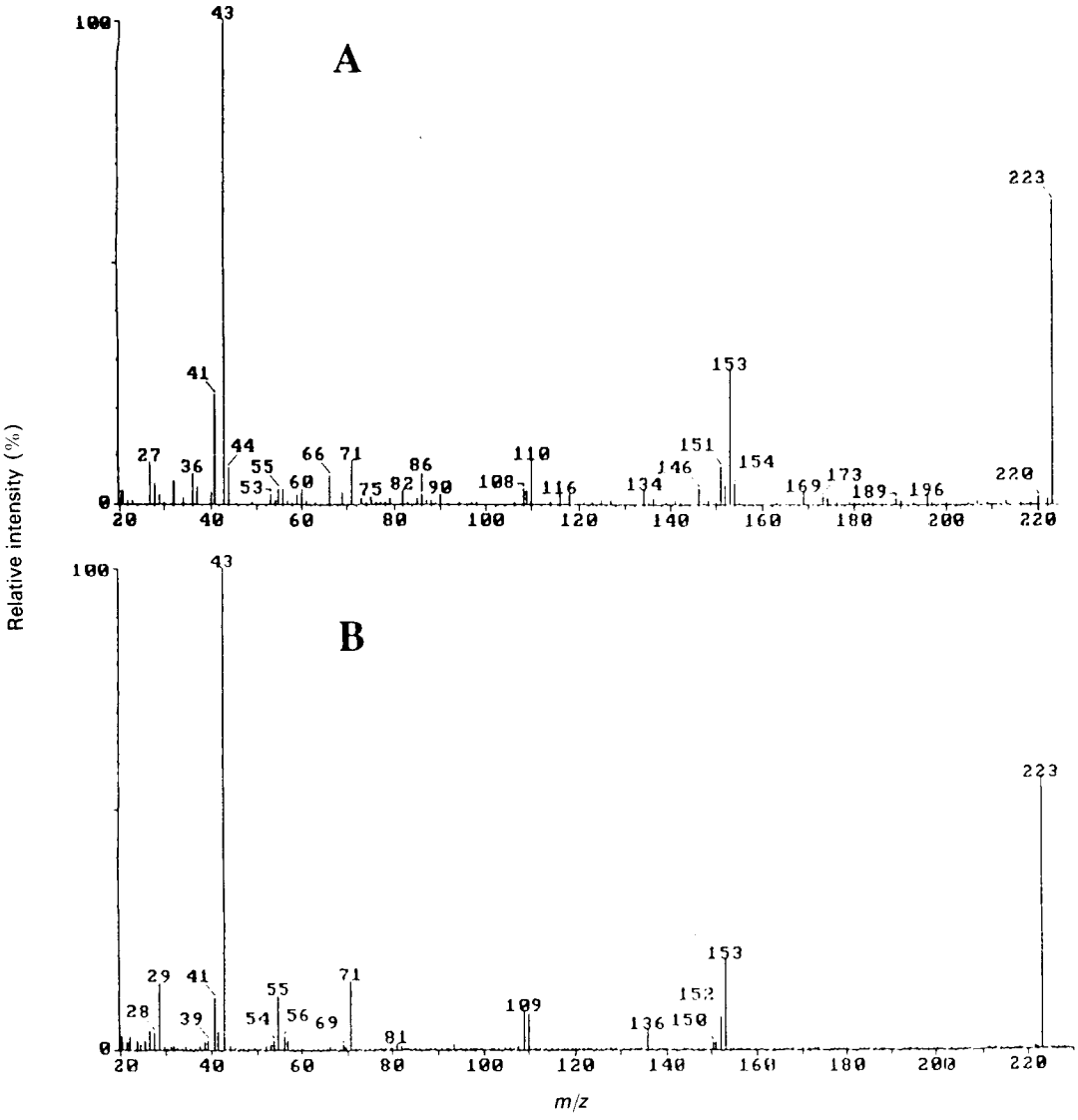

Figure 10. CAD MS/MS fragment ion spectra of the chromatographic peak at 24.34 min having *m/z* 223 as the possible quasi-molecular ion (A) and of synthetic 7-neopentylxanthine (B).

All of the above metabolites were identified in urine; plasma and bile samples were found to contain the original drug and no detectable traces of biotransformation products.

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