Journal of Chromatography, 544 (1991) 267–280
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2192

Liquid chromatography–mass spectrometry in metabolic research

I. Metabolites of benzbromarone in human plasma and urine

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ABSTRACT

Seven benzbromarone metabolites were identified in human plasma and urine by electron-impact mass spectrometry after semipreparative high-performance liquid chromatographic fractionation and/or by liquid chromatography–mass spectrometry using a thermospray interface. The major metabolite in plasma and urine was a hydroxybenzofuranoyl species; the 1-hydroxyethyl entity was identified as a minor metabolite. Five urinary metabolites occurred in trace amounts, all of them carrying OH and/or C=O groups in different positions. The hydroxybenzofuranoyl metabolite has often been mistaken for benzarone in previous studies.

INTRODUCTION

Benzbromarone, 3,5-dibromo-4-hydroxyphenyl-2-ethyl-3-benzofuranyl ketone (BzB), is a uricosuric drug whose metabolism was first investigated by Broekhuysen *et al.* [1] using ^{3}H-labelled BzB in patients. They concluded that it is debrominated successively to form bromobenzarone (BBz) and benzarone (Bz) in phase I followed by appreciable glucuronidation in phase II. However, these authors failed to provide any information whatsoever about identification of the metabolites. Later, Vergin and Bishop [2], who developed a high-performance liquid chromatographic (HPLC) procedure to determine BzB and its putative metabolites in plasma and urine, partly corroborated these results by investigating BzB and Bz pharmacokinetics after oral application of 100 mg BzB in seven subjects [3]. These authors did not detect any BBz. Their only means of identifying analytes was HPLC retention times.

In a pharmacokinetic study using oral doses of 100 mg BzB in ten humans, we detected two plasma metabolites, one of which differed from Bz in its HPLC retention

0021-9673/91/$03.50

time by only 9 s. The retention time of the other metabolite did not coincide with any of the putative metabolites. BBz was below the limit of detection (20 ng/ml) in 240 plasma samples. Meanwhile, one of these metabolites has independently been identified as the 1-hydroxyethyl derivative of BzB by De Vries *et al.* [4] using gas chromatography–mass spectrometry (GC–MS) techniques after trimethylsilylation.

The work described in this paper was undertaken to identify this and six more BzB metabolites in human plasma and urine after the administration of therapeutic doses by applying HPLC and liquid chromatography–mass spectrometry (LC–MS) methods.

EXPERIMENTAL

Sample preparation

One male human subject received 100 mg BzB once daily for 4 days. Two hours after the first dose, a 60-ml blood sample was withdrawn. Plasma was obtained by centrifugation. The total urinary output was collected in 24-h fractions. The fractions were lyophilized and redissolved in 100 ml of 0.1 *M* acetate buffer (pH 5). Glucuronic acid conjugates in plasma and urine were hydrolyzed by digestion with β-glucuronidase from *Escherichia coli* (Boehringer Mannheim, Mannheim, Germany). A 50-μl sample of enzyme solution (200 U/ml at 37°C) was added to 1-ml samples of plasma or concentrated urine and kept at 37°C overnight.

Since only minor traces of free metabolites were found in urine and the highest concentration of conjugates proved to be in the fraction after the third dose, this fraction was treated entirely with β-glucuronidase for further investigations.

For clean-up, plasma and concentrated urine were acidified by addition of 0.25 ml of 0.1 *M* HCl per mililiter of sample and then extracted with 6 ml cyclohexane–*tert.*-butyl methyl ether (1:2, v/v) per mililiter of sample. The organic layers were centrifuged, evaporated to dryness in a gentle stream of nitrogen and redissolved in methanol for HPLC analysis.

High-performance liquid chromatography

Extracts of plasma and urine before and after digestion with β-glucuronidase were chromatographed on a Hewlett-Packard HP 1090M HPLC system using direct injection and precolumn enrichment techniques, respectively. Analytical separation was achieved by isocratic elution from a Nucleosil C_8 5-μm column (125 × 4.6 mm I.D.) at a flow-rate of 0.5 ml/min. The eluent consisted of acetonitrile and a 5 m*M* sodium dihydrogenphosphate buffer adjusted to pH 3.5 with phosphoric acid (60:40, v/v). Column temperature was maintained at 45°C. Detection was by UV absorption at 280 nm. UV spectra of unknown compounds were recorded on-line using an HP 1040 diode-array detector (Hewlett-Packard, Böblingen, Germany).

Extracts were fractionated by semi-preparative HPLC on a 250 × 8 mm I.D. column at a flow-rate of 3 ml/min, all other conditions being unchanged.

Fractions of urinary extracts were cut as follows: front cut, in 0.4-min steps; heart cut, one fraction per constituent of interest (controlled by UV detection); end cut, one fraction (including any BzB). Heart-cut and end-cut fractions were checked for purity by analytical HPLC as described above.

All fractions were further investigated by thermospray LC–MS and direct inlet probe electron-impact mass spectrometry (EI-MS) as follows.

Mass spectrometry

An HP 1090M high-performance liquid chromatograph was fitted to an HP5988A mass spectrometer (Hewlett-Packard, Palo Alto, CA, U.S.A.) by a Vestal-type thermospray interface. Using negative-ion mode, optimum signal-to-noise ratio was obtained with a 0.1 *M* ammonium acetate buffer adjusted to pH 3.5 with acetic acid, a source temperature of 230°C and a stem temperature of 110°C. In the positive-ion mode, optimum signal-to-noise ratio was obtained with a 1 *M* ammonium acetate buffer (pH 3.5) and a source temperature of 266°C. The analytical column was the same as above. Eluent (acetonitrile–ammonium acetate buffer) composition ranged from 43:57 to 58:42 depending on the polarity of the metabolites. Flow-rate was 0.7 ml/min. 'Filament-on' mode (1000 eV) was used throughout.

For EI and chemical ionization (CI) MS a direct inlet probe (DIP) technique was used. For EI-MS, source temperature was 200°C and DIP temperature programme was from 40 to 250°C at a rate of 30°C/min. Methane was used as a reagent gas for CI-MS at a source pressure of 1 Torr, an ionization potential of 200 eV and a multiplier voltage of 2000 V. Source temperature was 200°C for positive ion CI and 120°C for negative-ion CI.

Chemical synthesis

In analogy with benzarone metabolism, it was assumed that one of the metabolites was the 1-hydroxyethyl moiety [5], so this molecule was synthesized according to the procedure outlined by Grote and Sandrock [6]. The purity of the product was determined by GC and HPLC. Elemental analysis as well as ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were in accordance with the assumption of the 1-hydroxyethylbenzbromarone structure.

RESULTS

The most significant results were obtained using LC–MS fragmentation in the positive-ion mode and EI fragmentation. Negative-ion mode and CI proved to be less suitable because of lack of sensitivity and a lack of information on mass fragments.

In what follows the investigated metabolites are ranked according to conclusiveness of experimental findings.

Most reliable evidence is provided by chemical synthesis of the supposed molecule followed by comparison of HPLC retention times, UV spectra, mass fragments obtained by thermospray LC–MS and EI-MS of pure fractions. This was achieved for unchanged BzB and the 1-ethylhydroxylated metabolite (metabolite 1) in extracts of deconjugated urine.

HPLC retention times and diode-array UV spectra were identical for proposed and authentic BzB and metabolite 1. Thermospray and EI mass spectra of authentic BzB are depicted in Figs. 1 and 2. They are characterized by signals at m/z 425 ($[M + 1]^+$) and 424 (M^+) respectively, and by significant mass fragments (see Table I).

The corresponding results for authentic 1-hydroxyethylbenzbromarone are given in Figs. 3 and 4. Characteristic mass numbers are m/z 441 ($[M + 1]^+$) and 440 (M^+). LC and EI mass spectra of proposed and authentic substances were again identical. The position of the OH substituent of metabolite 1 in C-1 of the ethyl side-chain (as opposed to C-2) is proven by fragments of 173 and 145 a.m.u. (see Table II).

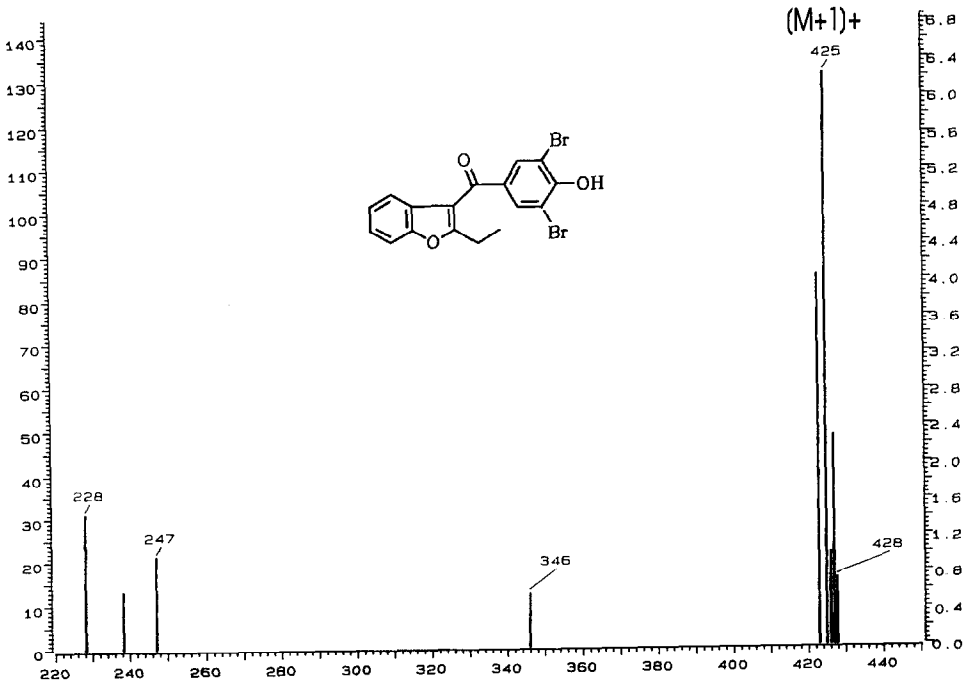


Fig. 1. LC–thermospray mass spectrum of authentic benzbromarone (BzB).

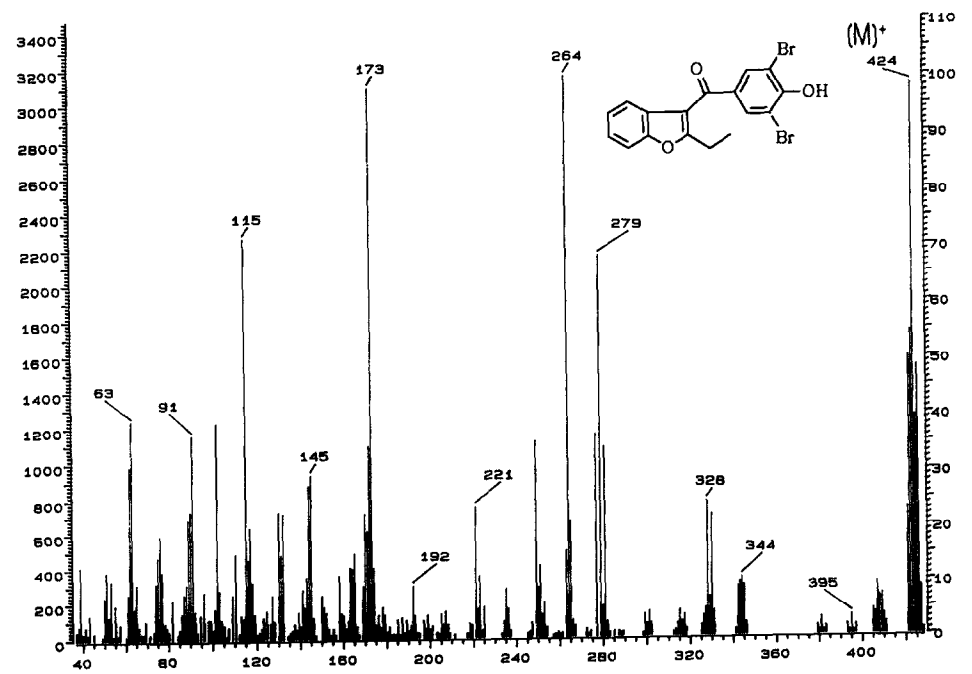


Fig. 2. DIP EI mass spectrum of authentic benzbromarone (BzB).

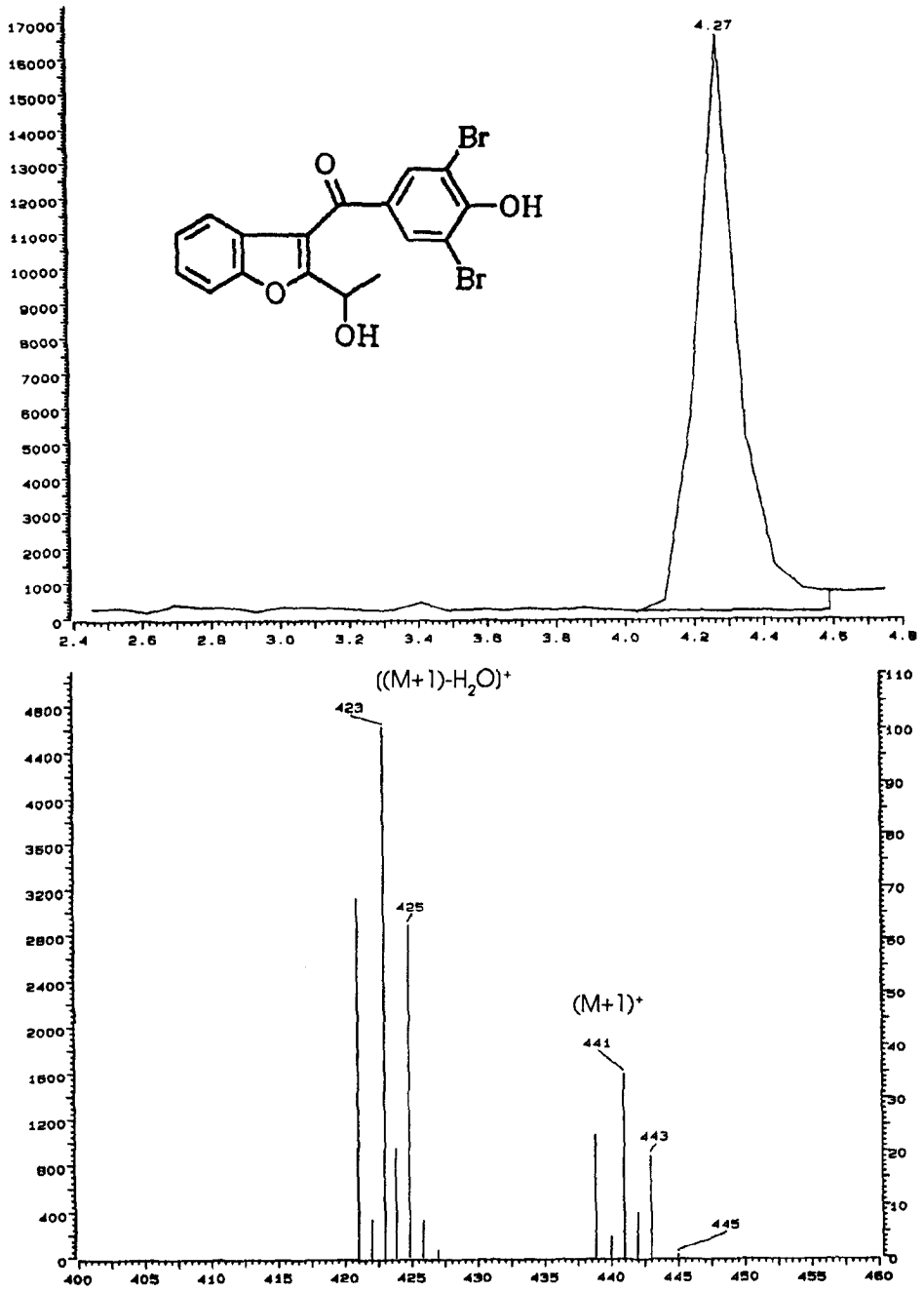
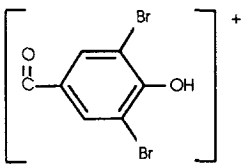
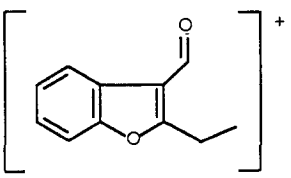


Fig. 3. Thermospray total ion current (top) and mass spectrum (bottom) of authentic 1-hydroxyethylbenzbromarone (metabolite 1).

TABLE I

EI MASS FRAGMENTS OF BENZBROMARONE (BzB)

m/z	Fragment	Relative intensity (%)
424	M^+	90
422		50
426		48
409	$[M-CH_3]^+$	8
411		
407		
344	$[M-Br]^+$	11
		
279		68
281		34
277		38
264	$[M-2Br]^+$	100
		
173		98

Using HPLC retention times, UV spectra and LC mass spectra recorded on-line, these two substances were also identified in plasma extract. By interpretation of LC and EI mass fragmentograms, the major metabolite (metabolite 2) in purified fractions of urinary extracts could be identified as benzofuran ring-hydroxylated BzB. Its EI-MS is depicted in Fig. 5. The molecular peak appears at 440 a.m.u. as in metabolite 1; however, the fragment at 422 a.m.u. in Fig. 4, which is formed by elimination of water, is missing here. This result is also confirmed by LC–MS. The position of the OH substituent in the benzofuran system (as opposed to the phenolic ring) is proven by fragments at 161 and 189 a.m.u. (see Table III).

Again, using retention times, UV spectra and on-line LC–MS, metabolite 2 was also identified in plasma. It is this metabolite that has been mistaken for Bz (mol. wt. = 266) in previous studies because of their almost identical HPLC retention times.

On reversed-phase HPLC the more polar metabolites frequently elute with (polar) interfering substances, which often prevent or at least hinder their detection by UV absorption. For this reason front eluates were cut "blindly" into 0.4-min fractions, which were further assayed by LC–MS. In this way three more metabolites were detected. DIP EI-MS was impossible with these samples because of low amounts of analytes and the need for pure fractions.

The LC–mass spectrum of metabolite 3 is depicted in Fig. 6. The $[M + 1]^+$ peak at 457 a.m.u. provides evidence of two hydroxyl substituents. Loss of H_2O

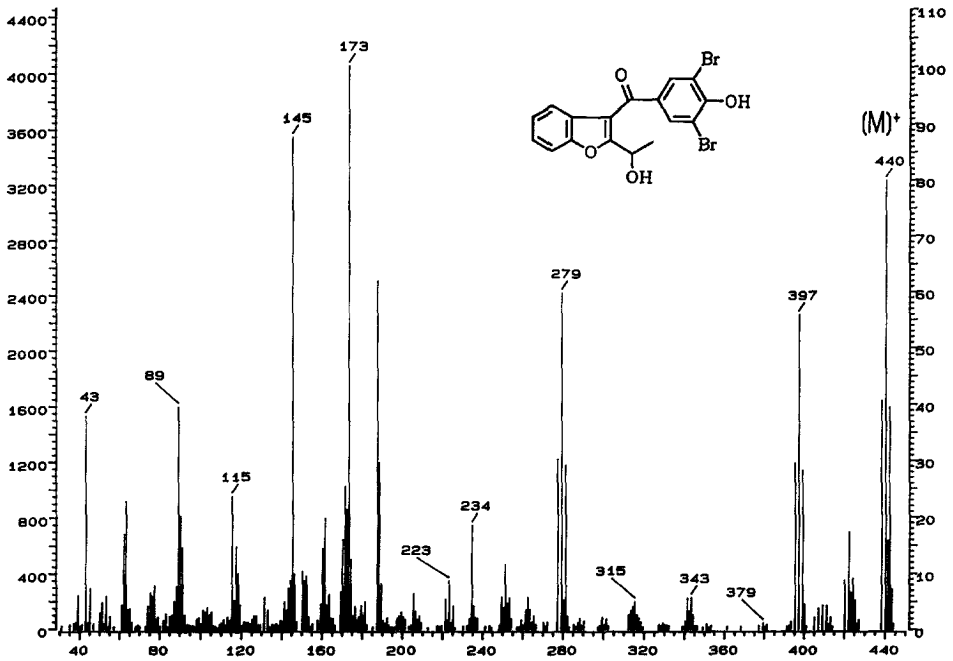


Fig. 4. DIP EI mass spectrum of authentic 1-hydroxyethylbenzbromarone (metabolite 1).

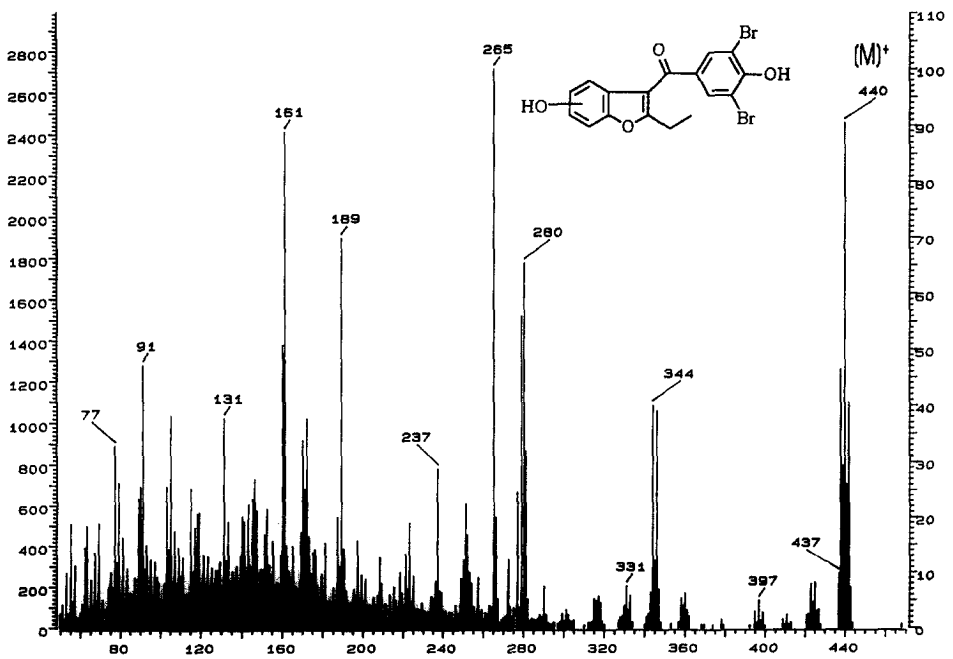
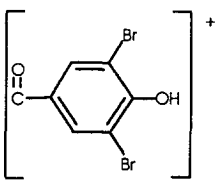
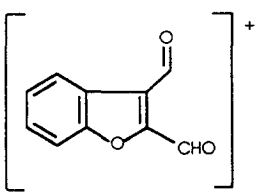
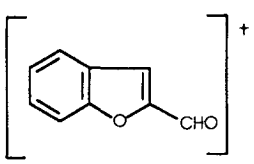


Fig. 5. DIP EI mass spectrum of hydroxyarylbenzbromarone (metabolite 2).

TABLE II

EI MASS FRAGMENTS OF 1-HYDROXYETHYLBENZBROMARONE (METABOLITE 1)

m/z	Fragment	Relative intensity (%)
440	M^+	80
442		36
438		37
422	$[M-H_2O]^+$	18
424		9
420		10
397	$[M-CH_3-CO]^+$	56
399		29
395		30
279		60
281		30
277		31
173		100
145		88

(fragment at 439) demonstrates that at least one OH group is located in the ethyl side-chain. In analogy with metabolite 1, it is assumed that it will be in position C-1. Because of the lack of fragmentation, the position of the other OH group cannot be determined exactly, however it will most probably be in the benzofuran system, since ring hydroxylation is the major metabolic pathway for BzB.

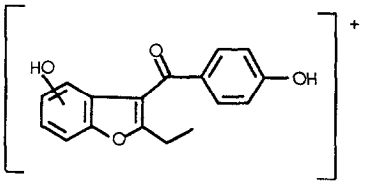
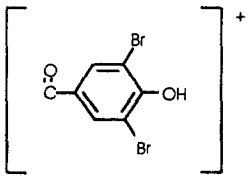
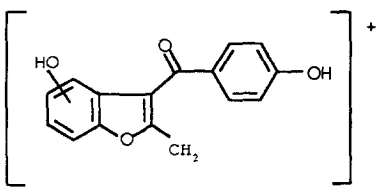
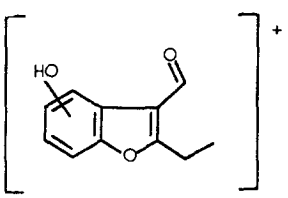
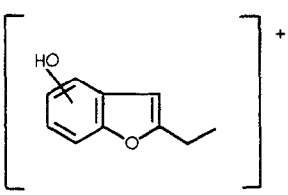
The LC–MS of metabolite 4 is shown in Fig. 7.

The $[M + 1]^+$ peak at 439 a.m.u. and the absence of water loss are consistent with a keto group in position 1 of the ethyl side-chain. A mass number of 439 a.m.u. could also be interpreted as water loss from metabolite 3. However, the $[M + 1]^+$ peak at 457 a.m.u. is missing in the LC–MS of metabolite 4. Moreover, metabolites 3 and 4 differ in HPLC retention times by 0.4 min.

Metabolite 5 ($[M + 1]^+$ at 455 a.m.u.) nearly coelutes with metabolite 3 ($[M + 1]^+$ at 457 a.m.u.). Its discovery was additionally complicated by the isotope distribu-

TABLE III

EI MASS FRAGMENTS OF METABOLITE 2 (ARYLHYDROXYBENZBROMARONE)

m/z	Fragment	Relative intensity (%)
440	M^+	91
442		41
438		47
280		66
279		57
277		
281		
265		100
189		71
161		90

tion of the two bromine atoms, giving rise to two more signals at $[M + 1]^+ \pm 2$ a.m.u. In spite of this impairment, metabolite 5 could be detected by stepwise display of mass spectra which were recorded repeatedly over the entire peak (Fig. 8).

Metabolite 5 is a hydroxy derivative of metabolite 4, with the OH group again most probably in the benzofuran system.

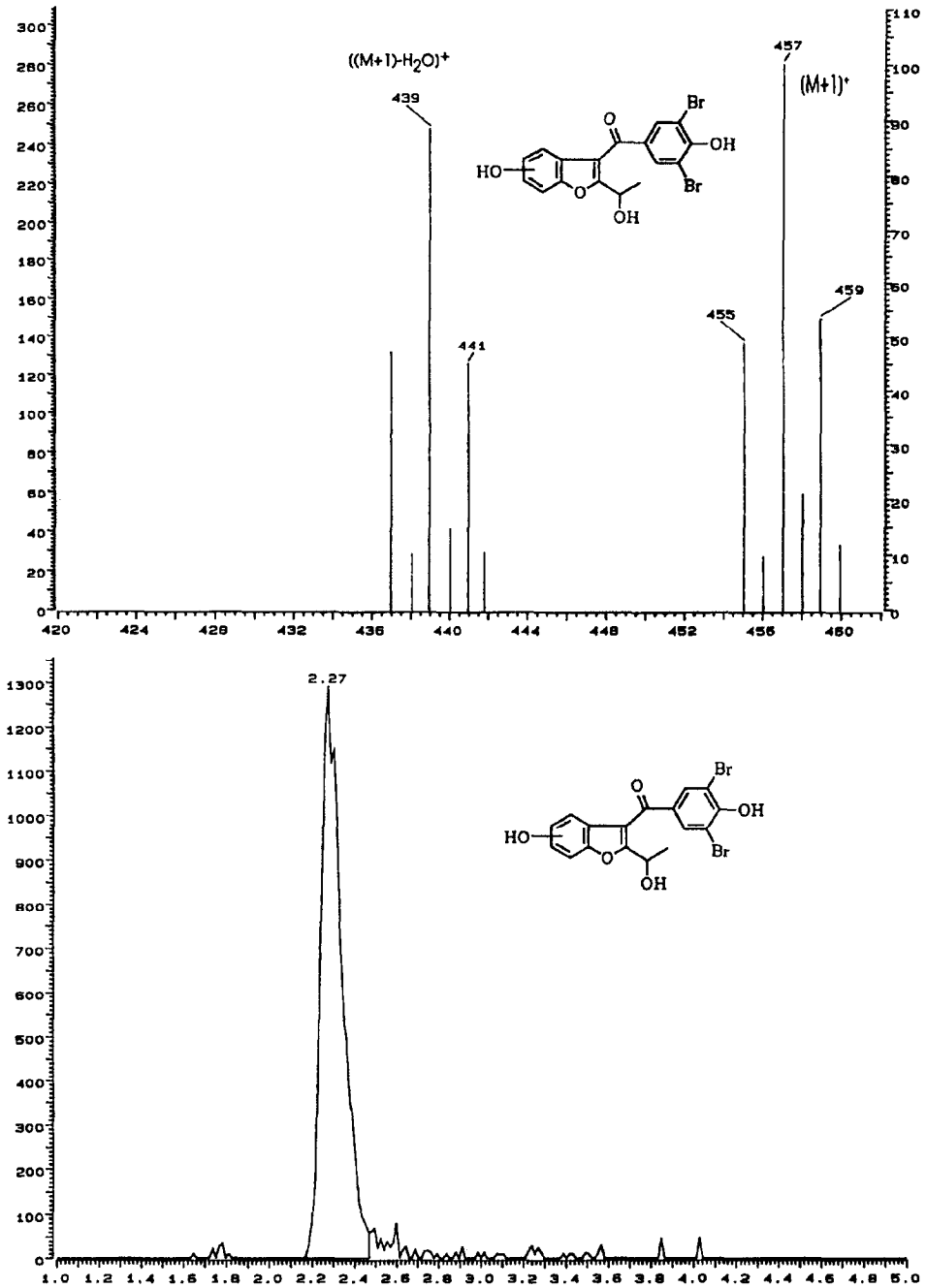


Fig. 6. LC–thermospray total ion current (bottom) and mass spectrum (top) of hydroxyarylhydroxyethylbenzbromarone (metabolite 3).

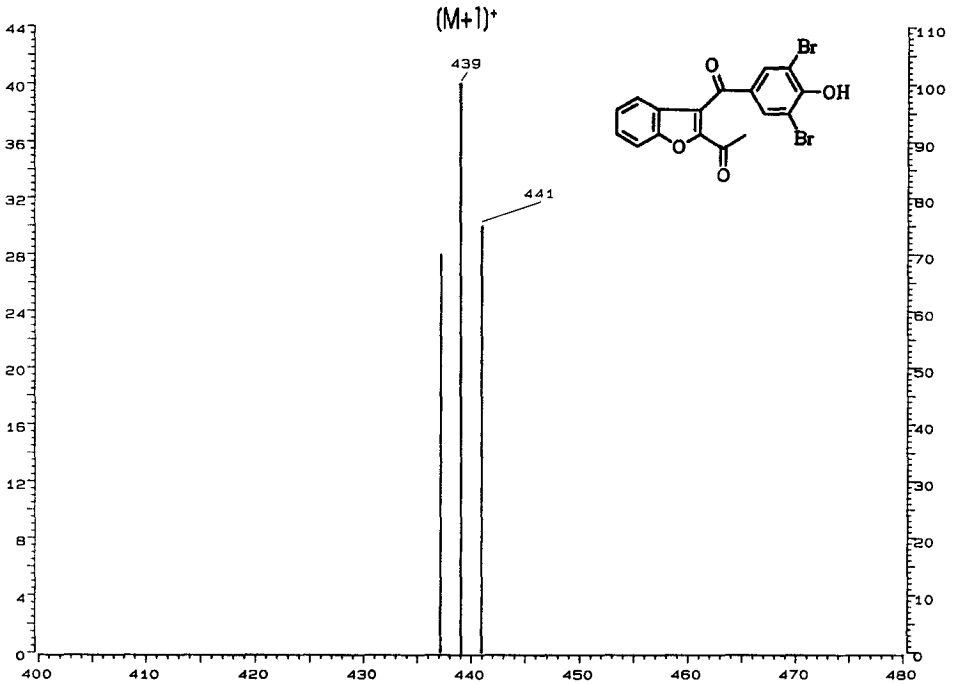


Fig. 7 LC–thermospray mass spectrum of oxoethylbenzbromarone (metabolite 4).

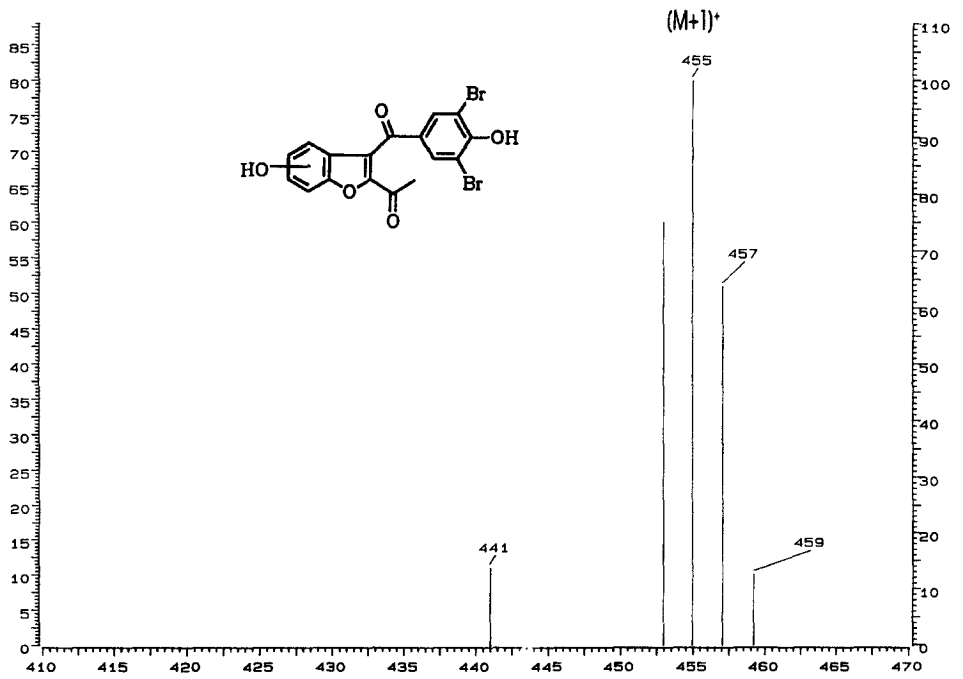


Fig. 8. LC–thermospray mass spectrum of hydroxyaryloxoethylbenzbromarone (metabolite 5).

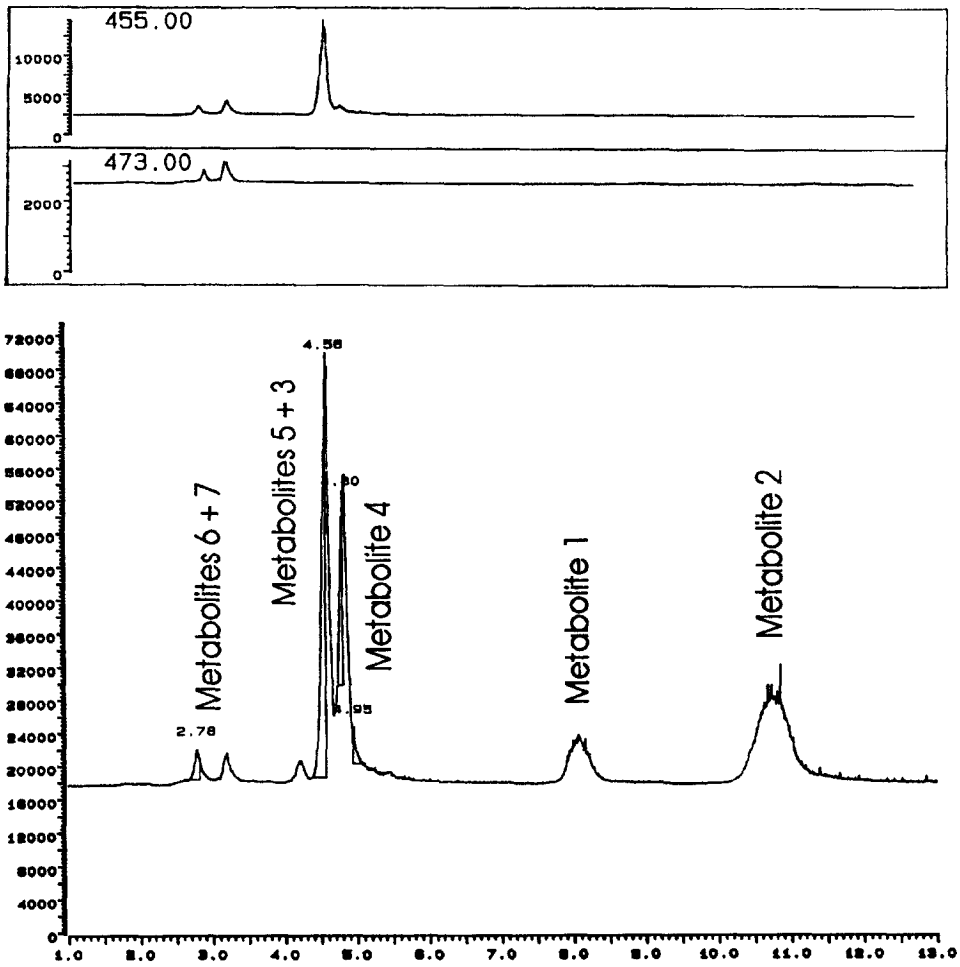


Fig. 9. SIM LC–MS chromatograms, m/z 473 and 455 (top) and total ion current (bottom) of deconjugated urinary extract.

Some metabolites occurred in such small concentrations that even on-line LC mass spectra were impossible to obtain. To search for any trihydroxylated metabolites, selected-ion monitoring (SIM) LC–MS runs on mass numbers 473 and 455 were recorded from the total urinary extract. The results are shown in Fig. 9. Evidently, two more species at $[M + 1]^+ = 473$ exist, both of them consistent with one OH group at C-1 of the ethyl side-chain with two more OH substituents at different positions whose locations could not be determined (metabolites 6 and 7).

Finally, the entire metabolic pathway of benzbromarone is depicted in Fig. 10.

Metabolite 2 proved to be the major metabolite, while metabolite 1 occurred in minor amounts in plasma and urine. Metabolites 3–7 were not detected in plasma. In urine they appeared in trace amounts only. All metabolites underwent Phase II conjugation with glucuronic acid. Only small amounts of free metabolites 1 and 2 were

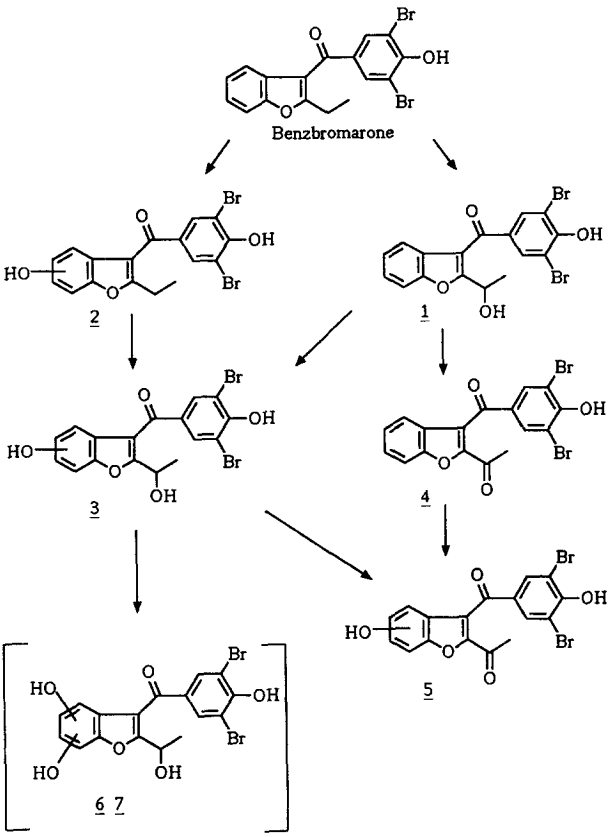


Fig. 10. Metabolic pathways of benzbromarone in man.

found in urine. In contrast, in plasma, metabolites 1 and 2 occurred in the free form and no conjugates could be detected.

DISCUSSION

Since most drug molecules can be analyzed in biological material by HPLC, this is also a suitable separation technique for metabolic research. However, structural identification of unknown substances remained difficult as long as no fully developed interfaces to mass spectrometers were available.

In our work we used EI ionization with a DIP as an off-line technique and thermospray for on-line interfacing.

Comparing these techniques, one can conclude that EI is suitable for identification of major metabolites which can be isolated by semi-preparative means. It requires relatively large amounts of analytes but supplies a large amount of mass spectral information. Identification of minor constituents is the domain of direct LC–MS coupling, which can work with smaller amounts and does not require additional clean-up but provides very little structural information.

Very recently, Maurer and Wollenberg [7] investigated BzB metabolites by GC–MS after derivatization in the urine of suicide victims who had ingested high doses of the drug. Our finding of unchanged BzB and metabolites 1, 2 and 4 in urine, mainly in conjugated form, corresponds with their results. However, unlike them we could not separate two different ring-hydroxylated metabolites nor could we detect any methoxylated entities. In addition we found metabolites 3 and 5–7. These differences may have been caused by the toxicity of the high doses taken by Maurer and Wollenberg's patients.

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