Journal of Chromatography, 417 (1987) 420–427
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3651

Note

Analysis of benzbromarone in human plasma and urine by high-performance liquid chromatography and gas chromatography–mass spectrometry

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(First received November 15th, 1986; revised manuscript received February 11th, 1987)

Benzbromarone [(3,5-dibromo-4-hydroxyphenyl)-(2-ethyl-3-benzofuranyl)methanone, Fig. 1] is the main uricosuric drug used for treatment of gout [1–3], and it has been reported to be metabolized in humans to bromobenzarone and benzarone (Fig. 1) [4–6]. These two metabolites are themselves weakly uricosuric compounds, and they are thought to contribute to the therapeutic effects of the main drug [6]. Following the administration of benzbromarone to humans under various conditions, we investigated the in vivo levels of the parent drug and of the suspected metabolites by high-performance liquid chromatography (HPLC) and gas chromatography–mass spectrometry (GC–MS). Previously published assays for benzbromarone and benzarone following application of the parent drug have used gas chromatography (GC) [5], GC–MS [7], HPLC [8] and radioactivity [4]. Methods for the determination of the parent drug alone also have been published [9].

HPLC methods were reevaluated and found to be not specific enough for the simultaneous determination of benzbromarone and the reported metabolites benzarone and bromobenzarone. Therefore a new HPLC assay based on our previous work with benzarone [10] was developed for the measurement of plasma concentrations and urinary excretion of the compounds in humans following oral intake of benzbromarone; the clinical results obtained with this assay will be reported separately [11].

The suspected metabolites benzarone and bromobenzarone could not be detected either in plasma or in urine, either by liquid chromatography or by GC–MS after methylating the extracts (detection limit 5 ng/ml).

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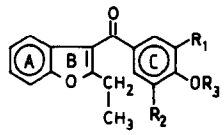

	R_1	R_2	R_3
1. Benzbromarone (BzBr)	Br	Br	H
2. Bromobenzarone (BrBz)	Br	H	H
3. Benzarone (Bz)	H	H	H
4. Benzbromarone methyl ether (BzBr-OMe)	Br	Br	CH_3
5. Bromobenzarone methyl ether (BrBz-OMe)	Br	H	CH_3
6. Benzarone methyl ether (Bz-OMe) -OMe	H	H	CH_3

Fig. 1. Structures of benzbromarone and related substances.

EXPERIMENTAL

Apparatus and reagents

The HPLC and GC–MS apparatus and reagents have been described previously [10].

Plasma and urine samples

Ten healthy normal volunteers (23–35 years) received a single oral dose of 100 mg of benzbromarone (Benzbromaron-ratiopharm®) tablets; a second available brand (Narcaricin®) was used as a reference preparation for studying bioequivalence. Blood and urine collection and storage conditions were analogous to those reported for benzarone [10]. Further details of the experimental design will be included in a separate communication [11].

Extraction

Plasma. In a 15-ml centrifuge tube, plasma (1 ml), warfarin (internal standard, 5 μg), 3 *M* hydrochloric acid (200 μl) and dichloromethane (6 ml) were added and processed as previously described for benzarone [10]. The extracts were dissolved in 100 μl of methanol, and 20 μl were injected for HPLC analysis.

Urine. Urine (1 ml) was incubated with β-glucuronidase/aryl sulfatase as described for benzarone [10]; extractions and work up were as described in the previous paragraph.

GC–MS analysis. For quantitation and validation of the HPLC method, extracts were methylated with diazomethane [10] and analysed by GC–MS in the electron-impact (EI) selected-ion monitoring (SIM) mode; ion settings were: *m/z* 280 (M^+) and 265 ($M^+ - 15$) for benzarone methyl ether, 358 (M^+) and 264 ($M^+ - 94$) for bromobenzarone methyl ether, 278 ($M^+ - 158$) for benzbromarone methyl ether and 313 ($M^+ - 43$) for the internal standard *p*-chlorowarfarin methyl ether.

HPLC peaks 1, 2, 3 and 4 and benzbromarone (Fig. 2) were fractionally collected after repetitive injection of plasma or urine extracts; the isolated fractions were evaporated and methylated as previously described [10]; each fraction was analysed by GC–EI-MS in the SIM and scanning modes.

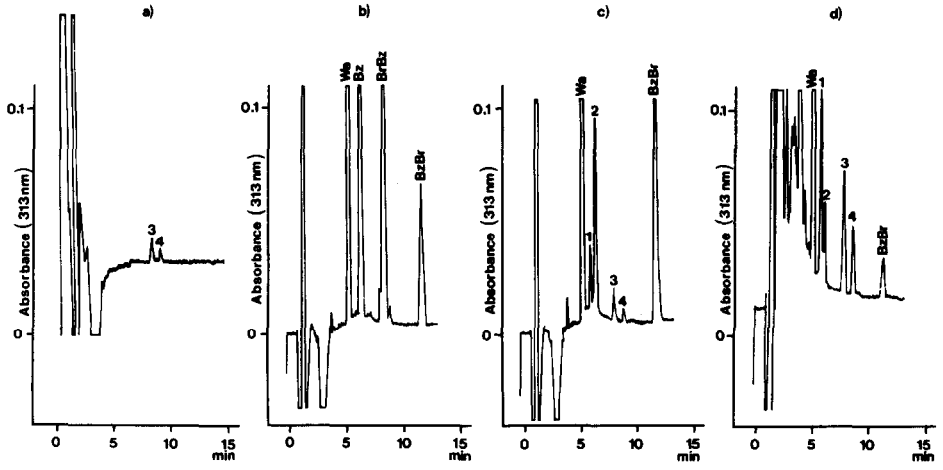

Fig. 2. HPLC profiles of plasma and urine. (a) Pretreatment plasma; (b) pretreatment plasma after addition of pure compounds (3 μg/ml); (c) plasma collected 2 h after oral administration of benzbromarone; (d) 0-24 h urine after hydrolysis and extraction. Peaks: Bz = benzarone; BrBz = bromobenzarone; BzBr = benbromarone; Wa = warfarin; 1, 2, 3 and 4 are peaks of unknown structure.

Chromatographic conditions

GC–MS settings and parameters and HPLC conditions were as previously described [10], except that the HPLC gradient elution controls and settings were modified (see Fig. 2). The initial and final conditions were 20 and 100% B, respectively; the gradient programme run was 40 min; convex gradient 3 ($y = 35x^{0.29}$ where y = gradient in % B, x = time in min); the run time was 13 min; the equilibration delay was 8 min.

Standard solutions and calibrations

Benzbromarone, bromobenzarone, benzarone and warfarin standard solutions (10 mg in 100 ml of methanol) were stable for several weeks in the cold and darkness. Warfarin (5 μg) and various amounts of benzbromarone, bromobenzarone and benzarone (0, 0.09, 0.3, 0.9, 3 and 6 μg) were added to 1 ml of control plasma or urine and extracted as described above. HPLC calibration curves were calculated from the regression line (Table I). The limit of HPLC quantitation was 0.02 μg/ml and the limit of detection for GC–MS was 5 ng/ml. Precision and accuracy are indicated in Table II. The calculated recoveries are shown in Table I. No interferences in the assay for plasma and urine were observed with the substances listed in ref. 10.

RESULTS

Plasma

HPLC analysis of various plasma extracts is shown in Fig. 2a–c; Fig. 2a is from a sample of pretreatment plasma, Fig. 2b from plasma after the addition of authentic substances and Fig. 2c from plasma from a volunteer 2 h after benzbro-

TABLE I

LINEAR REGRESSION COEFFICIENTS FOR PLASMA CALIBRATION CURVES

$y = ax + b$; y = area compound/area internal standard; x = concentration (μg/ml); range 0.09–6 μg/ml. Calculated extraction recoveries from plasma at a concentration of 3.0 μg/ml.

Compound	a	b	Correlation coefficient r	Calculated recovery (%)
Benzarone	0.836	−0.009	0.999	89.5 ± 3.0
Bromobenzarone	1.480	−0.003	0.998	91.7 ± 4.0
Benzbromarone	3.865	−0.001	0.999	93.2 ± 2.7
Warfarin				93.0 ± 3.5

marone administration; only benzbromarone and the internal standard could be identified on the basis of the retention times. Several plasma samples were hydrolysed prior to extraction, and these gave chromatograms identical with these from the non-hydrolysed sample shown in Fig. 2c, indicating that the plasma constituents were non-conjugated.

GC–EI-SIM-MS analysis of a plasma extract after methylation is shown in Fig. 3a and b. Characteristic and intense fragment ions in the mass spectra (see ref. 10 for benzarone and Fig. 4a and b for bromobenzarone and benzbromarone) were chosen for monitoring: Fig. 3a was obtained from control plasma with the addition of authentic substances and Fig. 3b from plasma from a volunteer 2 h after benzbromarone administrations. The latter analysis shows the benzbromarone peak but neither benzarone nor bromobenzarone was detectable. GC–MS analysis of extracts from plasma pooled 0–12 h following drug administration and during three different treatment days gave similar results. HPLC peaks 1, 2, 3 and 4 and benzbromarone (Fig. 2c) were fractionally collected and each was separately methylated and analysed by GC–MS in both the SIM and the scanning modes; again, only benzbromarone showed a peak with the same retention time and mass spectrum as that from the authentic substance (Fig. 3c; Fig. 4b and c).

TABLE II

REPRODUCIBILITY OF PLASMA AND URINE VALUES FOR BENZBROMARONE

Sample	Concentration added (μg/ml)	Concentration found (μg/ml)	Coefficient of variation ($n = 5$) (%)
Plasma	0.90	0.89	3.8
	3.0	3.02	3.0
	6.0	6.01	2.8
Urine	0.90	0.86	2.9
	3.0	2.86	1.9
	6.0	5.90	1.7

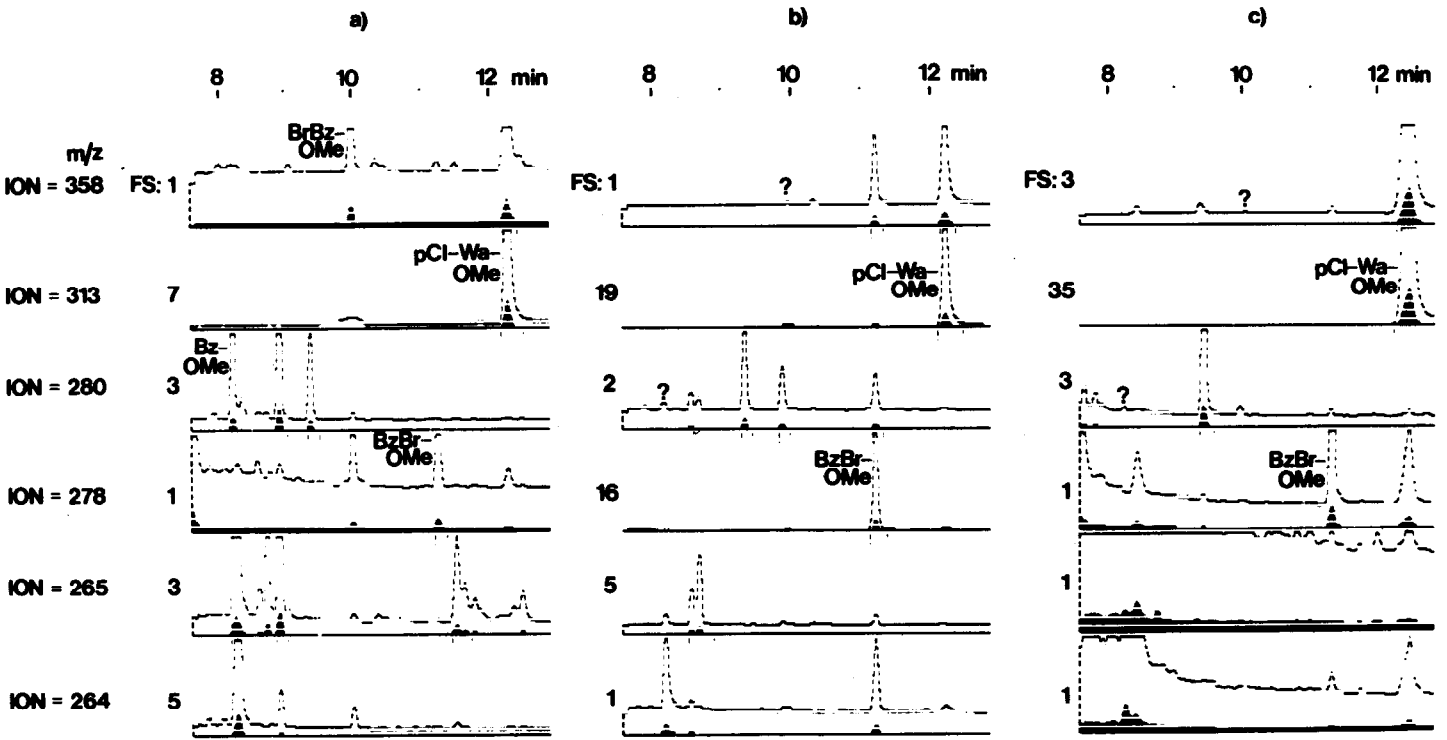

Fig. 3. GC-EI-SIM-MS profiles of plasma and urine extracts after methylation. (a) Pretreatment plasma after addition of 0.09 μg/ml or pure substances; (b) 2-h sample after benzbromarone application; (c) 0-24 h urine after benzbromarone administration. Abbreviations: Bz-OMe = benzarone methyl ether; BrBz-OMe = bromobenzarone methyl ether; BzBr-OMe = benzbromarone methyl ether; pCl-Wa-OMe = *p*-chlorowarfarin methyl ether; FS = full scale factor.

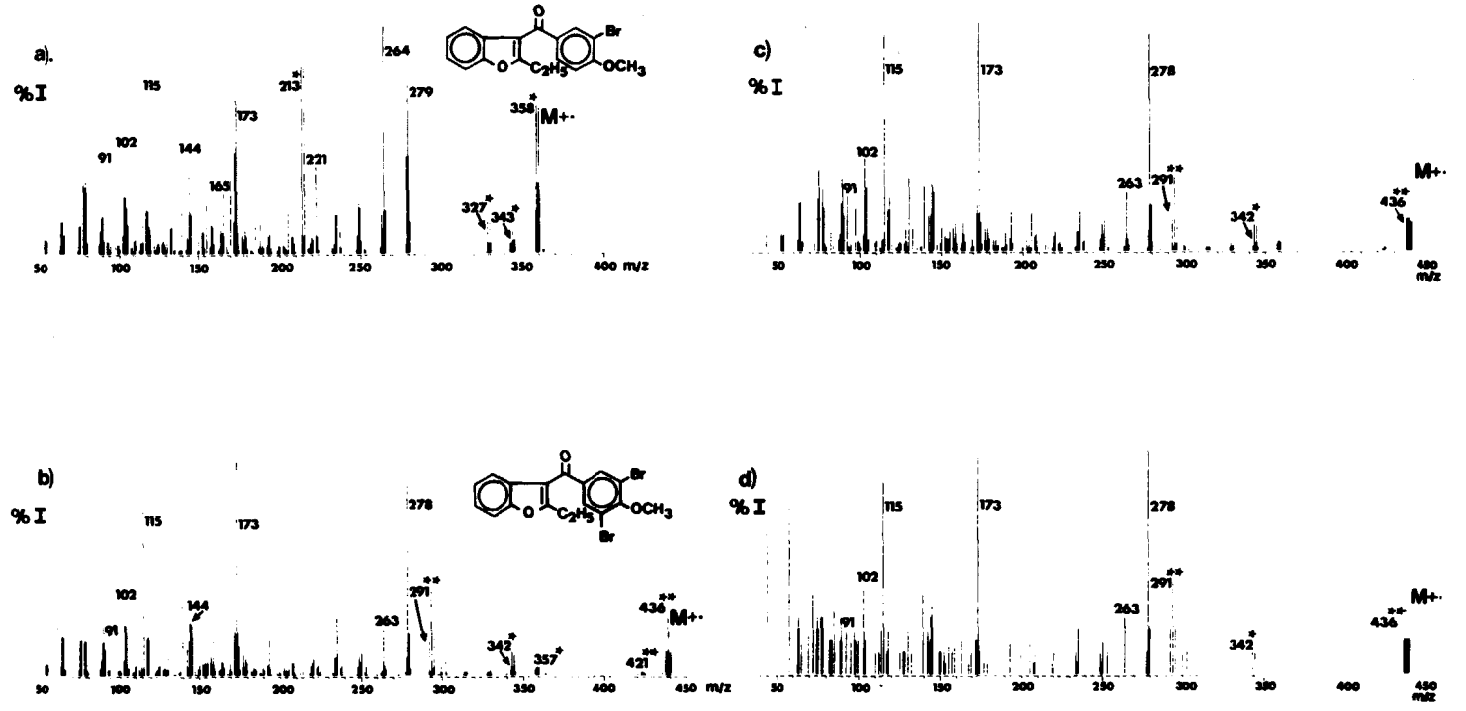

Fig. 4. GC-EI-MS scanning spectra of methylated compounds: (a) authentic bromobenzarone; (b) authentic benzbromarone; (c) and (d) benzbromarone from plasma and urine extracts after micropreparative HPLC. (⋆ fragments contain one bromine atom and ⋆⋆ two bromine atoms).

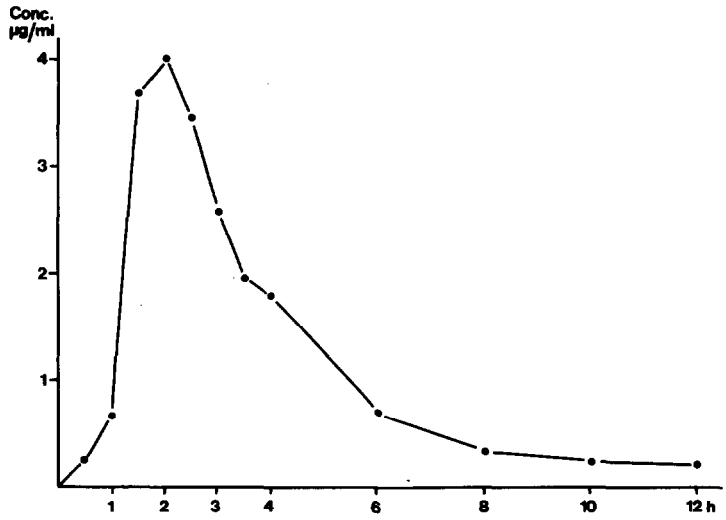

Fig. 5. Plasma benzbromarone concentration–time curve in a volunteer after administration of 100 mg of the drug.

GC–MS analysis of the isolated peaks 1–4 was different from those of benzarone and bromobenzarone methyl ethers.

Benzbromarone concentrations were measured from plasma samples obtained from ten normal volunteers after a single oral administration of 100 mg of benzbromarone. Fig. 5 shows a concentration–time curve for plasma in one individual. Plasma benzbromarone concentrations obtained by HPLC correlated well with these revealed by GC–EI-SIM-MS analysis after methylation.

Urine

HPLC analysis of a urine extract is shown in Fig. 2d. Only 0.38% of the ingested dose was excreted in urine as benzbromarone conjugate; free drug was present as a trace. Analysis of urines by GC–MS is shown in Fig. 3c for an extract and in Fig. 4d after HPLC peak collection; only benzbromarone could be identified.

DISCUSSION

So far benzarone has been suspected to be an active metabolite in part responsible for the therapeutic effect of benzbromarone [5,6]; however, our results indicate that benzarone is not detectable in vivo.

An HPLC peak in plasma and urine (peak 2; Fig. 2c and d) with chromatographic properties almost identical with those of benzarone was shown by GC–MS to be different from the authentic substance; previous reports [4–6,8] in which benzarone was assumed to be an in vivo metabolite of benzbromarone in humans, took for granted that a chromatographic peak with very similar retention times was benzarone, but without validation. A report describing the simultaneous GC–MS determination of benzarone, bromobenzarone and benzbromarone in plasma after trifluoroacetylation showed [7] chromatograms for in vitro spiked

control plasma samples; however, determination of these substances in human plasma after in vivo administration of benzbromarone was neither shown nor mentioned [7].

HPLC peaks 1 and 2 were found in both plasma and urine (Fig. 2c and d). They probably represent metabolites as they were not detected in control plasma or urine. Their peak height–time curves in plasma after benzbromarone administration are characteristic for metabolites with elimination half-lives higher than that of the parent drug. The chemical nature of these compounds is still unknown and will be subject of future investigations. Therefore the biotransformation of benzbromarone and the possible uricosuric activity of the metabolites remain to be reinvestigated.

In conclusion, a specific and sensitive method for the analysis of benzbromarone by HPLC has been described which showed the absence of benzarone and bromobenzarone as metabolites of benzbromarone in humans. The assay may be useful for further investigations of benzbromarone kinetics.

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