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SEPARATION OF PERMETHYLATED ISOMERIC GLUCURONIDES BY GAS CHROMATOGRAPHY AND ANALYSIS OF THE MASS SPECTRA

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SUMMARY

Glucuronic acid conjugates of several foreign compounds (1- and 2-naphthols, 2-, 3-, and 4-hydroxybiphenyls, *m*- and *p*-hydroxyphenylphenylhydantoins) were produced in the isolated perfused rat liver and identified in the bile as the permethyl derivatives. Permethylated glucuronide isomers were easily separated by gas chromatography on SE-30 and OV-17 columns. The mass spectra of permethylated glucuronide isomers were very similar. Gas chromatography proved to be most useful for the separation and identification of isomeric glucuronides.

INTRODUCTION

Many foreign compounds such as drugs, insecticides, carcinogens, and other chemicals undergo metabolism and detoxification in the liver by conjugation with glucuronic acid. This enzymatic process converts relatively less polar substances into more polar water-soluble compounds which can be excreted in the bile and urine. The formation of glucuronides is the most important conjugation reaction and occurs in most mammals, birds, reptiles, and amphibia[1]. Evidence for the presence of glucuronide metabolites in urine or bile has usually relied on identification of the aglycone after hydrolysis of the conjugate with acid or the enzyme β-glucuronidase. These hydrolytic techniques have inherent disadvantages which include: the destruction of the aglycone during treatment with strong acid, varying substrate affinities for β-glucuronidase, and the presence of inhibitors and alternative substrates in biologic samples. In the case of acid hydrolysis, aglycones from other conjugation reactions, *e.g.*, with glycine, may be released so that the evidence for glucuronide formation can only be regarded as tentative. Therefore, it is preferable to obtain direct evidence for the formation of glucuronic acid conjugates by analysis of intact molecules.

Gas chromatography (GC) and GC combined with mass spectrometry (GC–MS) are powerful techniques which have recently been used for the determination

of intact ether glucuronides. Pertrimethylsilyl (TMS)[2], trimethylsilyl ether-methyl ester (Me-TMS)[2–4], acetyl methyl ester[5], trifluoroacetyl-methyl ester (Me-TFA)[6] and permethyl[7,8] derivatives are all useful for the gas phase analysis of glucuronides. The Me-TMS, Me-TFA, and permethyl derivatives have the best chromatographic properties and are most suitable for the separation and identification of intact glucuronide molecules. The Me-TMS and permethyl derivatives have been used to identify intact glucuronides in biologic mixtures[4,7–9].

Previous reports from these laboratories have shown that the isolated perfused rat liver can be used to synthesize glucuronides which are excreted in the bile[7–12]. The identification of glucuronide metabolites was achieved by the evaporation of small aliquots of bile to dryness, permethylation of the residues with the methylsulfinylmethide carbanion and methyl iodide, and analysis of the permethyl deriva-

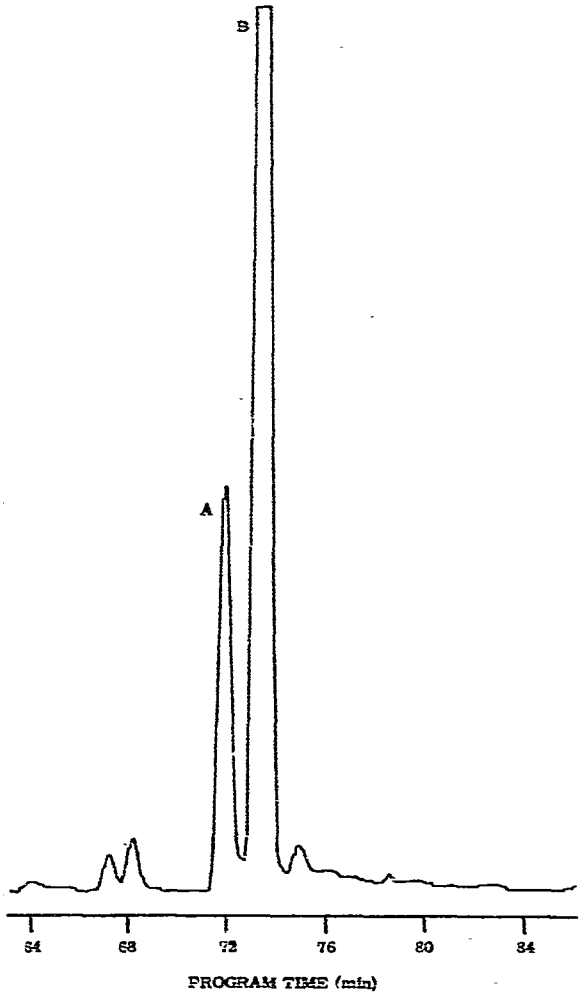

Fig. 1. GC separation of the permethylated glucuronides of 1-naphthol (A) and 2-naphthol (B) in rat bile. Conditions: Tracor Model 550; 6-ft. 3% SE-30 on Gas-Chrom Q (100–120 mesh); nitrogen flow-rate, 40 ml/min; inlet temperature, 250°; detector temperature, 300°; program rate, 2°/min from 80°.

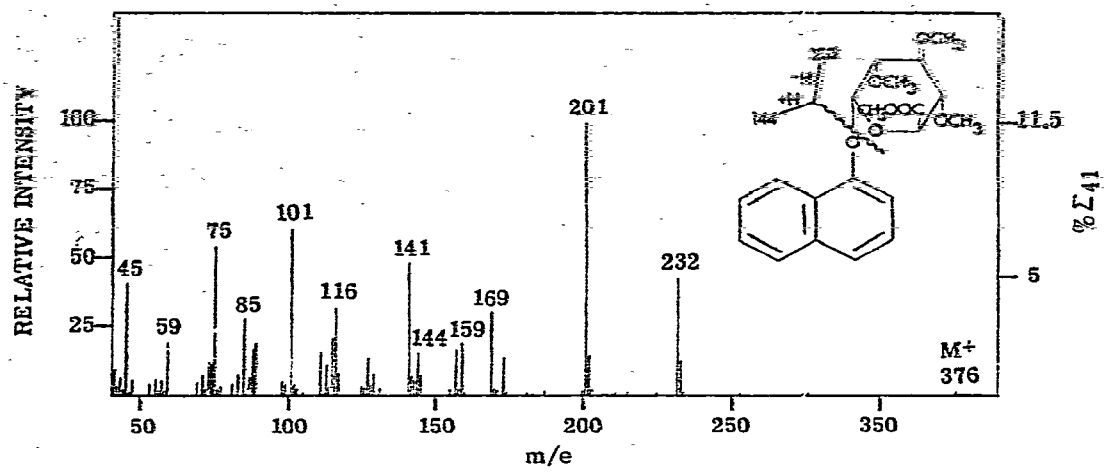

Fig. 2. 70-eV mass spectrum and structure of permethylated 1-naphthol glucuronide.

tives by GC and GC–MS on SE-30 and OV-1 columns. A diverse group of glucuronides was identified[7–9,11,12]. These techniques have now been applied to the analysis of three separate series of isomeric ether glucuronides. The permethyl derivatives of the isomers of each series were easily separated by GC. However, the mass spectra do not readily differentiate the isomers.

EXPERIMENTAL

The 1- and 2-naphthols were obtained from Aldrich (Milwaukee, Wisc., U.S.A.), the 2-, 3-, and 4-hydroxybiphenyls from Pfaltz and Bauer (Flushing, N.Y., U.S.A.), and *m*-hydroxyphenylphenylhydantoin (*m*-HPPH) from Parke-Davis (Detroit, Mich., U.S.A.). *p*-Hydroxyphenylphenylhydantoin (*p*-HPPH) was synthesized by Dr. Milton Bush, Vanderbilt University (Nashville, Tenn., U.S.A.).

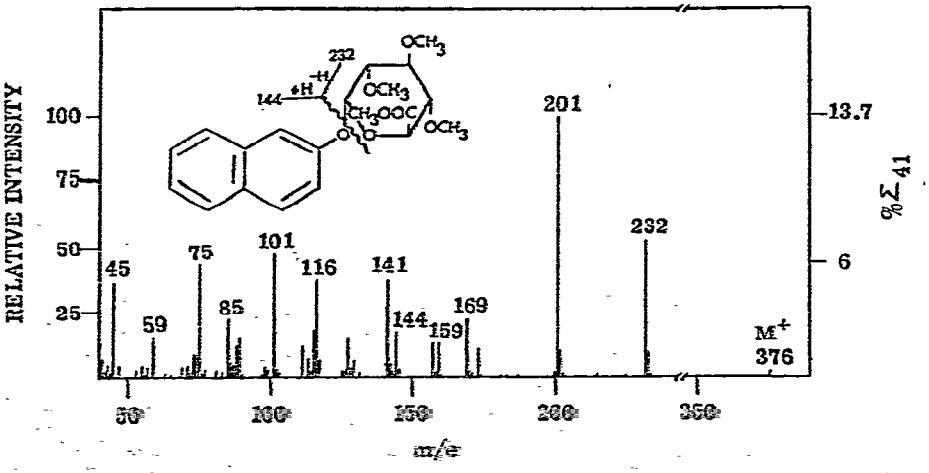

Fig. 3. 70-eV mass spectrum and structure of permethylated 2-naphthol glucuronide.

Fifty or 100 mg of each of the above compounds were dissolved in NaOH and added to a recirculating perfusate as previously described[10]. The bile was collected and permethylated as reported earlier[7].

The GC analyses were carried out with Tracor Models MT-220 and 550 gas chromatographs equipped with 6-ft. glass columns packed with either 1% or 3% SE-30 on Gas-Chrom Q (100–200 mesh), 1% OV-17 on Gas-Chrom Q (100–120 mesh), or 5% OV-1 on Gas-Chrom P (80–100 mesh). The nitrogen flow-rate was either 40 or 60 ml/min, the injector temperature 250°, the detector temperature 300°, and the column was programmed from 80° or 100° at 2 or 5°/min.

The mass spectra were obtained on an LKB-9000-S combination gas chromatograph–mass spectrometer with a 6-ft. 1% SE-30 column (100–120 mesh Gas-Chrom Q) as the inlet. The helium flow-rate was 30 ml/min, the accelerating and ionizing potentials were 3.5 kV and 70 eV, respectively, the trap current was 60 μA, and the source temperature was 270°. The column oven temperature was programmed from 150–175° at 4 or 5°/min.

RESULTS AND DISCUSSION

1- and 2-naphthols

Fig. 1 shows the analytical GC separation of the permethylated glucuronides

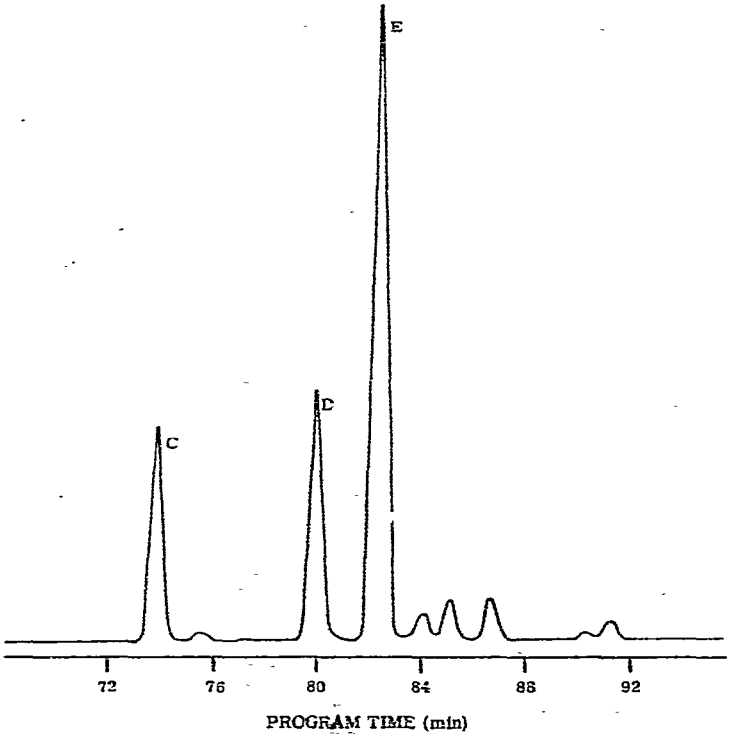

Fig. 4. GC separation of the permethylated glucuronides of 2-hydroxybiphenyl (C), 3-hydroxybiphenyl (D), and 4-hydroxybiphenyl (E) in rat bile. Conditions: Tracor Model 550; 6 ft. 3% SE-30 on Gas-Chrom Q (100–120 mesh); nitrogen flow-rate, 40 ml/min; inlet temperature, 250°; detector temperature, 300°; program rate, 2°/min from 80°.

of the naphthol isomers. The tracing was obtained by co-chromatography of the derivatized bile samples. Peak A represents the tetramethyl-1-naphthol glucuronide and peak B the tetramethyl-2-naphthol glucuronide. These compounds were clearly resolved with an SE-30 column, nearly to the baseline.

The mass spectra of components A and B are shown in Figs. 2 and 3, respectively. These spectra are nearly identical with molecular ions at *m/e* 376; peaks at *m/e* 75, 101, 116, 141, 169, 201, and 232 arising by fragmentation of the permethylated glucuronic acid moiety[7]; and ions at *m/e* 144 due to the aglycone and produced by the cleavages indicated in the structures included in the figures. Electron impact induced cleavage of the glycosidic bond with proton transfer from the permethylated glucuronic acid moiety to the aglycone as shown in Figs. 2 and 3 is typical for permethylated phenolic ether glucuronides[7]. Examination of the relative abundances of of the individual ions in these mass spectra could not be used to differentiate between these two isomers.

2-, 3- and 4-hydroxybiphenyls

Fig. 4 shows the GC separation of tetramethyl-2-hydroxybiphenyl glucuronide (C), tetramethyl-3-hydroxybiphenyl glucuronide (D), and tetramethyl-4-hydroxybiphenyl glucuronide (E) obtained by co-chromatography of the respective permethylated bile samples. These isomers were completely separated on an SE-30 column. Other biliary metabolites of the hydroxybiphenyls appear in the chromatogram. Four of the five peaks, eluting after E, are permethylated diol glucuronides. The structures of these metabolites have not been rigorously determined.

Figs. 5, 6, and 7 are the mass spectra of C, D, and E, respectively, and include the structure of each metabolite. Each has the following characteristics: a weak molecular ion at *m/e* 402; losses of CH_3O and CH_3OH; intense ions at *m/e* 75, 101, 116, 141, 169, 173, 201, and 232, due to fragmentation of the permethylated glucuronic acid moiety; and a peak at *m/e* 170, which is too intense to correspond entirely to the isotope peak of the *m/e* 169 ion and is partially due to the aglycone fragment as indicated in the structure. The major differences in these mass spectra are in the relative abundances of ions due to fragmentation of the permethylated glucuronic acid moiety, *e.g.*, there is an increase in the intensity of *m/e* 116 relative to *m/e* 101 as the series from *ortho* to *para* is examined. The ratio of *m/e* 169–170 decreases from

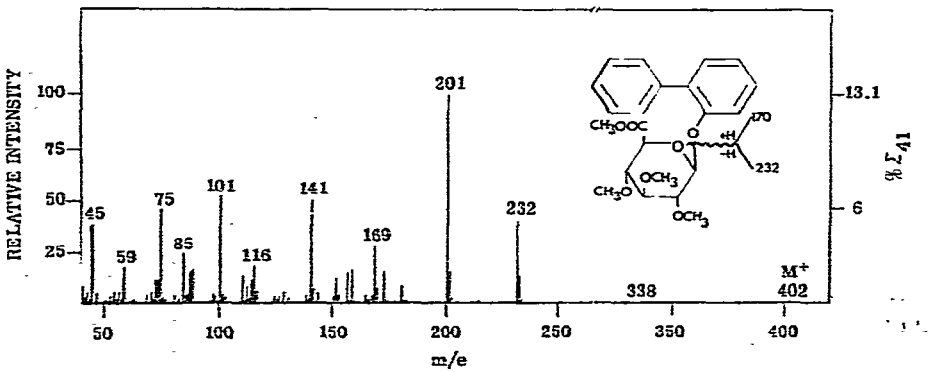

Fig. 5. 70-eV mass spectrum and structure of permethylated 2-hydroxybiphenyl glucuronide.

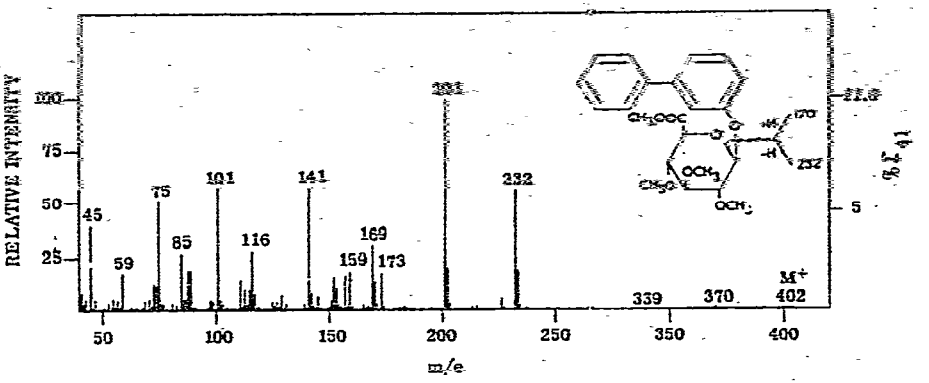

Fig. 6. 70-eV mass spectrum and structure of permethylated 3-hydroxybiphenyl glucuronide.

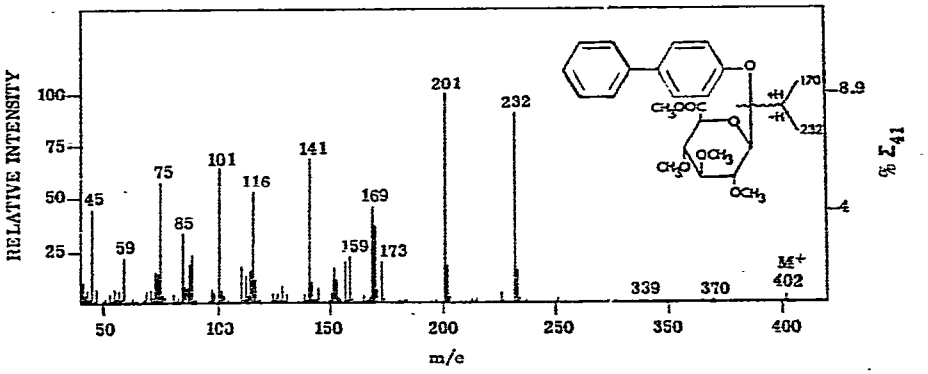

Fig. 7. 70-eV mass spectrum and structure of permethylated 4-hydroxybiphenyl glucuronide.

ortho to para and may be a more reliable mass spectral difference among these isomers because ions due to the aglycones are being used to establish their identities. These are the only parts of the total glucuronide molecules which are different. As in the case of the naphthol glucuronides, GC analysis of the permethyl derivatives of the hydroxybiphenyl glucuronides separates the isomers satisfactorily and the mass spectra are needed only to confirm their identities.

m-HPPH and p-HPPH

A third example of the separation by GC of a more complex pair of permethylated glucuronide isomers is presented in Fig. 8. Peak F represents the hexamethyl glucuronide of *m*-HPPH, a metabolite of the anticonvulsant drug 5,5-diphenylhydantoin (DPH) in the dog and an artifact of the acid hydrolysis of the urine of other species produced by dehydration of the dihydrodiol metabolite of DPH: 5-(3,4-dihydroxy-1,5-cyclohexadien-1-yl)-5-phenylhydantoin. Peak G is the hexamethyl glucuronide of *p*-HPPH, the major metabolite of DPH in species other than the dog. Again, GC analysis by co-chromatography of the permethylated bile samples on an SE-30 column completely separated these glucuronide isomers. Peak H is the heptamethyl glucuronide of a catechol metabolite of both *m*-HPPH and *p*-HPPH in the isolated perfused rat liver and is the same catechol glucuronide as from DPH[9].

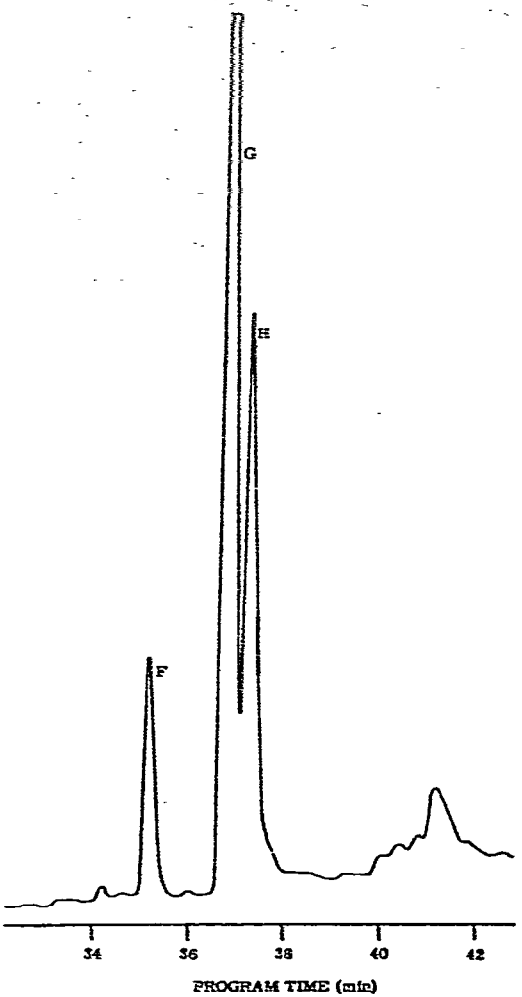

Fig. 8. GC separation of the permethylated glucuronides of *m*-hydroxyphenylphenylhydantoin (F) and *p*-hydroxyphenylphenylhydantoin (G) in rat bile. H is a permethylated catechol glucuronide described previously (ref. 9). Conditions: Tracor MT-220; 6 ft. 5% OV-1 on Gas-Chrom P (80–100 mesh); nitrogen flow-rate, 60 ml/min; inlet temperature, 250°; detector temperature, 300°; program rate, 5°/min from 100° after 5 min initial hold.

The mass spectra of these isomers (Figs. 9 and 10) do not contain molecular ions at *m/e* 528 but do show weak M — CH_3OH ions at *m/e* 496. The ions due to the permethylated glucuronic acid moiety are present as in the other examples, as are ions at *m/e* 296 caused by cleavage of the glycosidic bond with hydrogen transfer. These two spectra do not clearly distinguish between these two permethylated glucuronides.

The above analyses indicate that permethylated isomeric glucuronides can be separated by GC. A major requirement of the procedure is the stability of the aglycone to the permethylation reaction and to GC operating temperatures of about 300°.

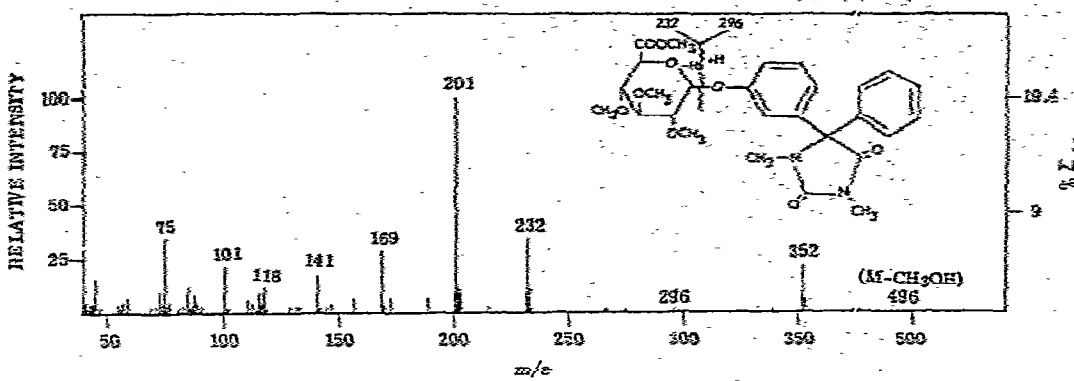

Fig. 9. 70-eV mass spectrum and structure of permethylated *m*-hydroxyphenylphenylhydantoin glucuronide.

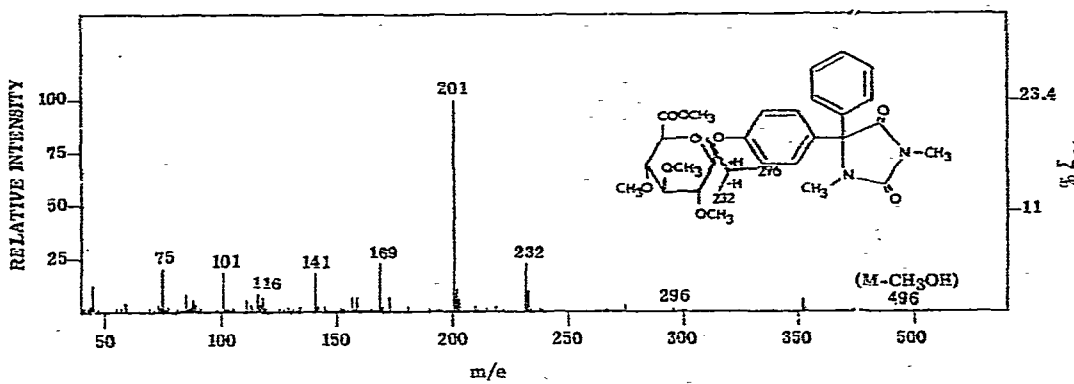

Fig. 10. 70-eV mass spectrum and structure of permethylated *p*-hydroxyphenylphenylhydantoin glucuronide.

GC–MS is required in order to confirm that a component is actually a glucuronide. The glucuronides are identified by the characteristic ions caused by fragmentation of the permethylated glucuronic acid moiety[7]. We are currently using these techniques to examine the conjugated metabolites of many different classes of drugs, including anticonvulsants and narcotic analgesics[13,14].

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