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Biotransformation of genistein in the rat: elucidation of metabolite structure by product ion mass fragmentology^n

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Abstract

Biotransformation of the phytoestrogen [^{14}C]genistein was investigated in male and female rats by application of narrow-bore radio-HPLC-MS^n (LCQ, Finnigan) to determine intermediates in metabolism. Urine contained five metabolites, Gm1–Gm5, 24 h after dosing by gavage with [^{14}C]genistein (4 mg kg^{-1}). Structural analysis following ESI revealed molecular ions $[M+H]^+$ of m/z 447, 449, 273, and 271 for metabolites Gm2, Gm3, Gm5 and genistein, respectively and an $[M-H]^-$ of m/z 349 for Gm4. Metabolite structure was deduced by evaluation of product ion spectra derived from unlabelled and [^{14}C]-labelled ions and sensitivity to treatment with β-glucuronidase. These studies indicated identity of metabolites with genistein glucuronide (Gm2), dihydrogenistein glucuronide (Gm3), genistein sulphate (Gm4) and dihydrogenistein (Gm5). Detection of the β-glucuronidase resistant major metabolite Gm1 by ESI was poor and so was analysed by negative ion APCI; this revealed a deprotonated molecular ion of m/z 165 which had chromatographic and mass spectral properties consistent with authentic 4-hydroxyphenyl-2-propionic acid, a novel metabolite of genistein. In vitro metabolism studies with anaerobic caecal cultures derived from male and female rats revealed metabolism of genistein to Gm1 via Gm5 and an additional metabolite (Gm6) which was identified from product ion spectra as 6′-hydroxy-*O*-desmethylangolensin. Biotransformation of genistein by both isolated hepatocytes and precision-cut liver slices was limited to glucuronidation of parent compound. Commonality of genistein metabolites found in rats with those reported in man suggest similar pathways of biotransformation, primarily involving gut micro-flora.

Keywords: Genistein; Biotransformation; Rat; Radio-HPLC-MS^n

1. Introduction

Genistein is a phytoestrogen found in high concentrations of about 1 g kg^{-1} in soya beans and associated food products, mainly as glycoside conjugates [1]. Epidemiological studies indicate that soya bean consumption is associated with beneficial health effects, including reduced rates of certain endocrine related diseases including cancers [2,3]. Experimental evidence has demonstrated diverse biological activities of phytoestrogens including oestrogenic [4,5] and anti-oestrogenic effects [6], modulation of enzymes involved in oestrogen metabolism [7] and intracellular signalling [8]

Abbreviations: Daidzein, 7,4′-dihydroxyisoflavone; Daidzin, 7′,4-dihydroxyisoflavone-7-glucoside; Dihydrodaidzein, 7,4′-dihydroxyisoflavanone; Dihydrogenistein, 5,7,4′-trihydroxyisoflavanone; Equol, 7,4′-dihydroxyisoflavane; Genistein, 5,7,4′-trihydroxyisoflavone; Genistin, 5,7,4′-trihydroxyisoflavone-7-glucoside; 6′-Hydroxy-*O*-desmethylangolensin, 1-(2′,4′,6′-trihydroxyphenyl)-2-(4″-hydroxyphenyl)-propan-1-one; *O*-Desmethylangolensin, 1-(2′,4′,-dihydroxyphenyl)-2-(4″-hydroxyphenyl)-propan-1-one; Tetrahydrodaidzein, 7,4′-dihydroxyisoflavanol; Tetrahydrogenistein, 5,7,4′tri-hydroxyisoflavanol; CID, collision induced dissociation.

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and suppression of the mammalian stress response [9]. Such diverse biological activity may be due to parent compound or metabolites since phytoestrogens are extensively biotransformed in many species. Studies with sheep have shown biotransformation of the related phytoestrogen daidzein to a range of metabolites including equol and *O*-desmethylangolensin [10]. Genistein is biotransformed in this species to 4-ethylphenol after administration of [^{14}C] or unlabelled biochanin A [10,11] although little is known of the metabolic intermediates. Biotransformation studies with rats [12–15] have shown conjugation of genistein with glucuronic and sulphuric acids and metabolism via unknown intermediates to 4-ethylphenol. Such glucuronide, and sulphate conjugates of genistein have also been identified in human plasma and urine [13] in addition to a range of urinary metabolites including dihydrogenistein and 6′-hydroxy-*O*-desmethylangolensin [16].

Although the liver is regarded as the major site of biotransformation for many xenobiotics, extensive metabolism may occur in other body compartments. Complex pathways of biotransformation may be readily evaluated by use of in vitro preparations representative of the major compartments, such as the liver and gut, to determine principal sites and intermediates of xenobiotic metabolism. In vitro studies with bovine rumen-liquor cultures have shown microbial metabolism of formononetin to equol via daidzein [17] and of biochanin A to genistein [10,17]. Similar biotransformation pathways may operate in humans since faecal cultures are also capable of converting daidzein to equol [18]. Other potentially important biotransformations in the gut include hydrolysis of glycoside forms of phytoestrogens by micro-flora since this process may play a critical role in facilitating absorption [16].

GC/MS has been used widely to detect and identify metabolites of phytoestrogens [19–21], although this approach is not well suited to on-line detection of water soluble radio-labelled metabolites or those which require prior derivatisation [22,34]. HPLC with on-line radiochemical detection and MS^n offers several advantages for such biotransformation studies, since conjugated metabolites may be analysed without prior hydrolysis or derivatisation and peaks from radio- and selected-ion mass chromatograms may be matched to enable selection of the appropriate parent ions for structural analysis by MS^n. Further, the ability to perform MS^n experiments on the molecular ions of metabolites enables acquisition of structural information for metabolite identification with minimal mass spectral contamination from co-eluting extractives. The use of appropriately labelled radiochemicals may offer further significant advantages since the mass differences between product ion pairs derived from the spectra of both radio- and unlabelled metabolites may enable the structural elucidation of fragments and thus metabolites [17].

The aim of the present study was to determine the metabolic fate of genistein in the rat by radio-HPLC-MS^n, using both in vitro and in vivo approaches, as a preliminary to similar studies in humans using genistein labelled with stable isotopes.

2. Methods

2.1. Animals

Wistar rats (5 females and 5 males; 200–250 g) were provided by the Central Toxicology Laboratory (Zeneca, Alderley, Macclesfield, UK). After an overnight fast the rats were dosed by gavage with genistein 4 mg kg^{-1} (1.833 MBq [^{14}C]genistein dissolved with unlabelled compound in DMSO (0.01 ml) and suspended in corn oil (1 ml)) and housed in silanised glass metabolism cages (Jencons Scientific, Leighton Buzzard, UK) constructed for separation and collection of urine and faeces which were stored frozen in silanised tubes at −20°C.

2.2. Chemicals

[4-^{14}C]Genistein (2.07 GBq mmol^{-1}; Amersham International, Little Chalfont, Bucks, U.K.) was synthesised from a deoxybenzoin intermediate [23] to a purity of 99%. Radiochemical purity was confirmed before use by radio-HPLC using conditions described below for analysis of genistein metabolites. Genistein, genistin, daidzein and β-glucuronidase (*Helix pomatia* digestive juices, contains β-glucuronidase and sulphatase activity) were obtained from Sigma (Poole, Dorset, UK) and daidzin from Apin Chemicals (Abingdon, Oxon, UK). *O*-Desmethylangolensin and equol were obtained from Plantech (Reading University, Berks, UK) and 4-hydroxyphenyl-2-propionic acid from Acros Organics (Fisher Scientific, Loughborough, Leicestershire, UK).

2.3. Preparation of hepatocyte and precision-cut liver slices

Isolated hepatocytes and precision-cut liver slices (200 μm thick; 8 mm diameter) were prepared from Wistar rats (200–250 g) using similar procedures to those described previously [24]. Isolated hepatocytes in suspension culture (1×10^6 cells ml^{-1}) and precision-cut liver slices were incubated with 10 μM genistein containing 2.2 kBq ml^{-1} [^{14}C] genistein. Culture medium was sampled at intervals up to 24 h, separated from cellular material by centrifugation at 2000× *g*, snap frozen on Cardice granules and stored at −20°C.

2.4. Caecal micro-flora preparation

Rat caecal micro-flora were prepared and incubated under anaerobic conditions to preserve the potential biotransformation capacity of oxygen sensitive microbes [25]. Wistar rats were euthanased by CO_2 asphyxiation and immediately transferred to an anaerobic cabinet (Forma Scientific, Ohio, USA) containing an atmosphere of H_2 (10% v/v), CO_2 (10% v/v) and N_2 (80% v/v). Caeca were excised, contents removed, diluted to 10% w/v in potassium phosphate buffer (0.1 M, pH 7.4) and cellular debris removed by centrifugation (500× *g* for 2 min). Aliquots of caecal contents supernatant (10 ml) were incubated at 37°C in tightly capped tubes with 10 μM genistein containing 2.2 kBq[^{14}C] genistein ml^{-1}. Samples (1 ml) were collected at intervals for up to 4 h, snap frozen on Cardice and stored at −20°C.

2.5. Metabolite extraction

Urine and culture media from hepatocyte, precision-cut liver slice and caecal content incubates (all 1 ml) were diluted with an equal volume of 0.2% (v/v) formic acid and applied to conditioned (methanol (10 ml) followed by 0.2% formic acid (10 ml)) C18 Bond Elut cartridges (1 g; Varian, Harbor City, CA, USA). Cartridges were washed with 0.2% (v/v) formic acid (3 × 10 ml), radiolabelled components eluted from the cartridges with methanol (5 ml) and taken to dryness under nitrogen at 45°C. Typical recoveries of radioactivity (mean ± 1 sd) by this procedure were determined to be 99 ± 1.0%. Cation and anion exchange chromatography were used to investigate the chemical nature of metabolite Gm1 by fractionation on the basis of functional groups [26].

2.6. β-Glucuronidase/sulphatase hydrolysis of metabolites

Extracts of urine, hepatocyte and precision-cut liver slice culture medium were dissolved in 50 mM sodium acetate buffer (pH 4.5, containing 100 mM NaCl) and incubated for 24 h at 37°C, with or without *H. pomatia* β-glucuronidase/aryl sulphatase (1.4 mg ml^{-1}), prior to analysis by radio-HPLC-MS^n.

2.7. Radio-HPLC of genistein metabolites

Metabolites of [^{14}C]genistein in extracts of urine, hepatocyte and precision-cut liver slice culture medium and caecal incubate were dissolved in mobile phase and separated by narrow-bore reverse phase HPLC. The effluent from the column was split (approximately 50:50) with a Y shaped tee (Upchurch Scientific, Anachem Ltd., Luton, Beds) and connected in parallel to an A525 radiodetector (Canberra Packard, Pangbourne, Berks) and LCQ mass spectrometer. The radiodetector was equipped for narrow-bore chromatography with a low volume scintillation cocktail/HPLC effluent mixing tee (10 μl) and a 35 μl liquid scintillation cell. [^{14}C]Genistein residues were chromatographed with a HP1050 HPLC system (Hewlett Packard, Bracknell, Hants) using a 2.1 × 150 mm Hypersil Elite (Hypersil, Runcorn, Cheshire; 5 μ particle size) C18 column and a linear gradient mobile phase ranging from acetonitrile: 0.1% (v/v) aqueous formic acid (15:85) to 45% acetonitrile over 20 min at a flow rate of 0.3 ml min^{-1}. Under these chromatographic conditions, with UV detection at 254 nm, reference solutions of isoflavones and potential metabolites were resolved (retention time, mins) in the order daidzin (6.1), 4-hydroxphenyl-2-propionic acid (7.4), genistin (9.3), *O*-desmethylangolensin (12.9), daidzein (14.2), 4-ethylphenol (18.0), equol (18.3) and genistein (18.5).

2.8. LC/MS^n

The structure of genistein metabolites was investigated using an ion-trap mass spectrometer (LCQ, Finnigan, Hemel Hempstead, UK), with positive-ion electrospray providing greatest sensitivity for the analysis of parent compound. Spectra were collected over the mass ranges *m/z* 110–600 and parent ions of genistein metabolites selected for MS^2 analysis by matching selected ion peaks of $[M+H]^+$ and the [^{14}C] isotope ions ($[M+2+H]^+$) with those from radio-HPLC chromatograms. MS^n experiments on genistein and metabolites were performed with CID energies of 26% and mass isolation width of 2 atomic mass units. Genistein metabolites Gm2, Gm3, Gm5 and Gm6 were analysed by positive ion electrospray whereas Gm4 was only detected in -ve ion mode. Electrospray was performed using the following settings; heated capillary temperature of 200°C, source voltage and current 2.3 kV and 100 μA, respectively, sheath gas and auxiliary gas flow rates 80 and 20 ml min^{-1} and a capillary voltage of 46 V (−46 V for analysis of Gm4). Analysis of Gm1 was performed using the APCI interface with capillary and source voltages of −26 V and 4 kV and heated capillary and vaporizer temperatures of 150 and 450°C, respectively. In both ionisation modes the maximum automatic gain control (AGC) ion storage time was 200 ms and 3 microscans were collected per spectrum. Analysis of genistein fragmentation by MS^n was determined by direct infusion of a 10 μM genistein standard at 10 μl min^{-1} into HPLC effluent (55% aqueous formic acid (0.1% v/v): 45% acetonitrile) at a flow rate of 0.3 ml min^{-1}.

Identification of all metabolites was established on the basis of susceptibility to hydrolysis by treatment

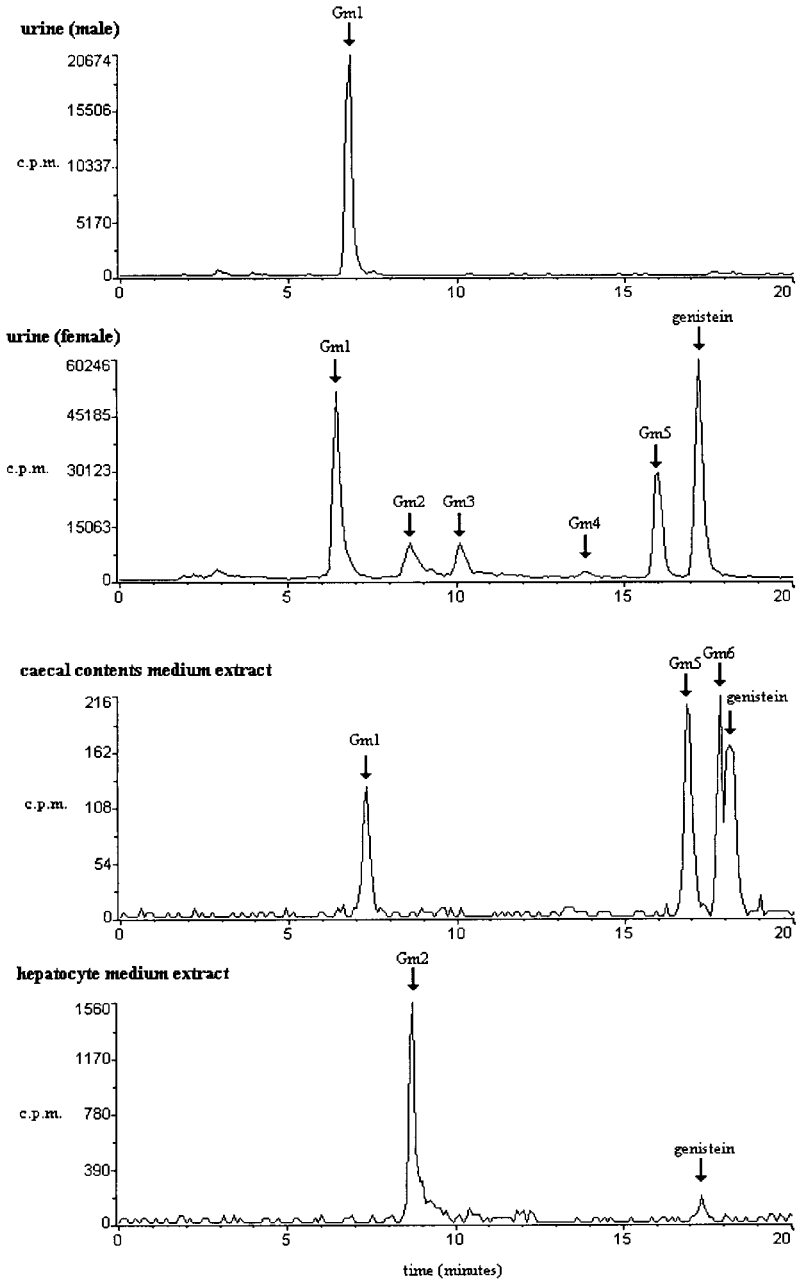


Fig. 1. Radio-HPLC metabolite profiles. Representative radio-HPLC traces illustrating the profiles of genistein metabolites found in extracts of urine (24 h samples) from male rats, female rats, a caecal contents incubate extract (1 min sample, male rat) and hepatocyte culture medium (15 min sample, male rat). Genistein metabolites were labelled Gm1–6 in order of elution from the HPLC column. Arrows above peaks in the radio-HPLC traces indicate location and retention time of metabolites Gm1–6 and genistein. Counts per minute (cpm) in the HPLC effluent are plotted against elution time in min.

with β-glucuronidase/aryl sulphatase, presence of a [^{14}C] isotope ion of approximately 25% abundance relative to the unlabelled molecular ion, daughter ion (MS^2) mass spectra and comparison of spectral and chromatographic characteristics with authentic standards, where available.

3. Results

3.1. Radio-HPLC of [^{14}C] genistein metabolites

Radio-HPLC profiles of metabolites present in solid phase extracts of male and female rat urine (24 h post

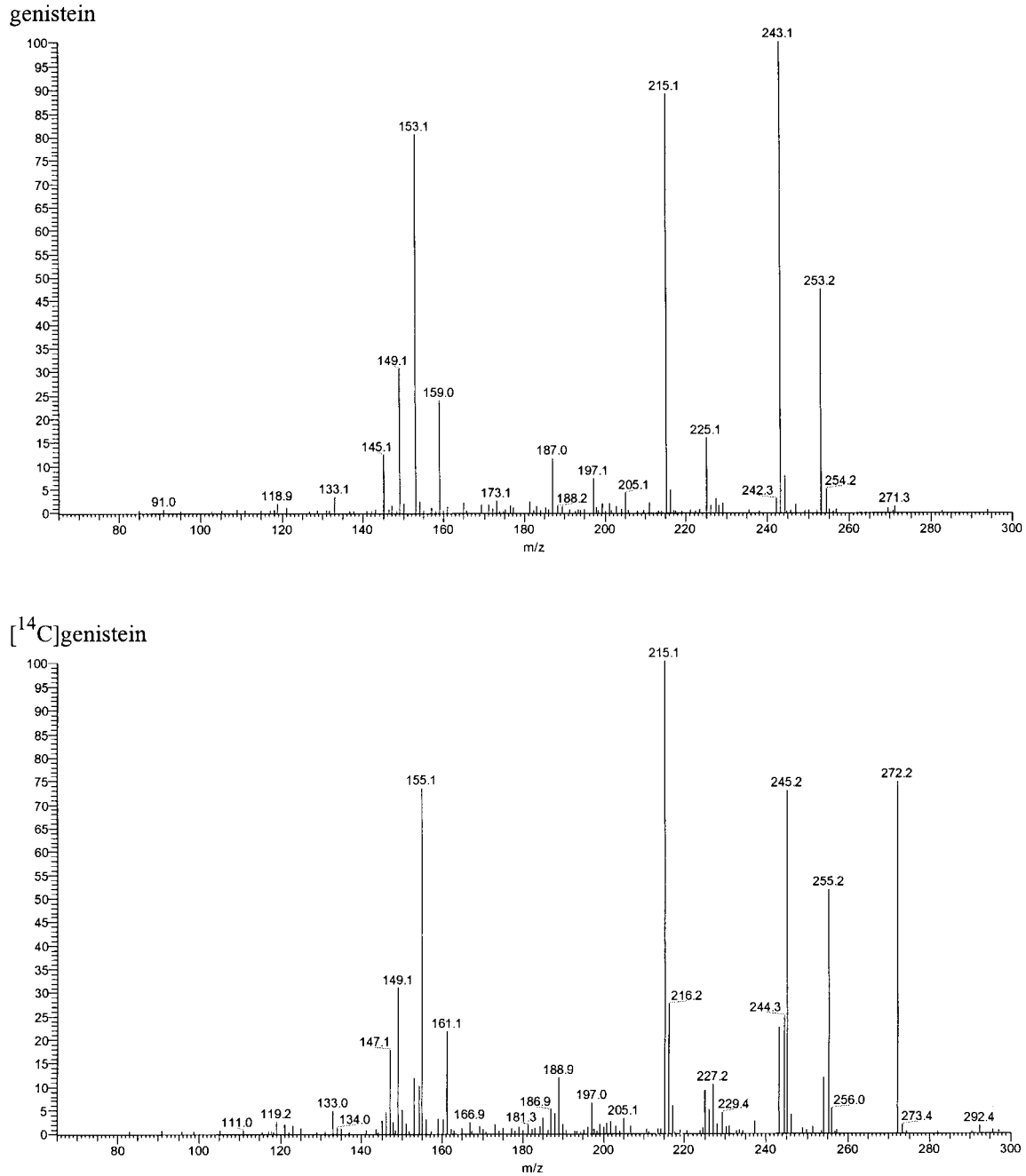
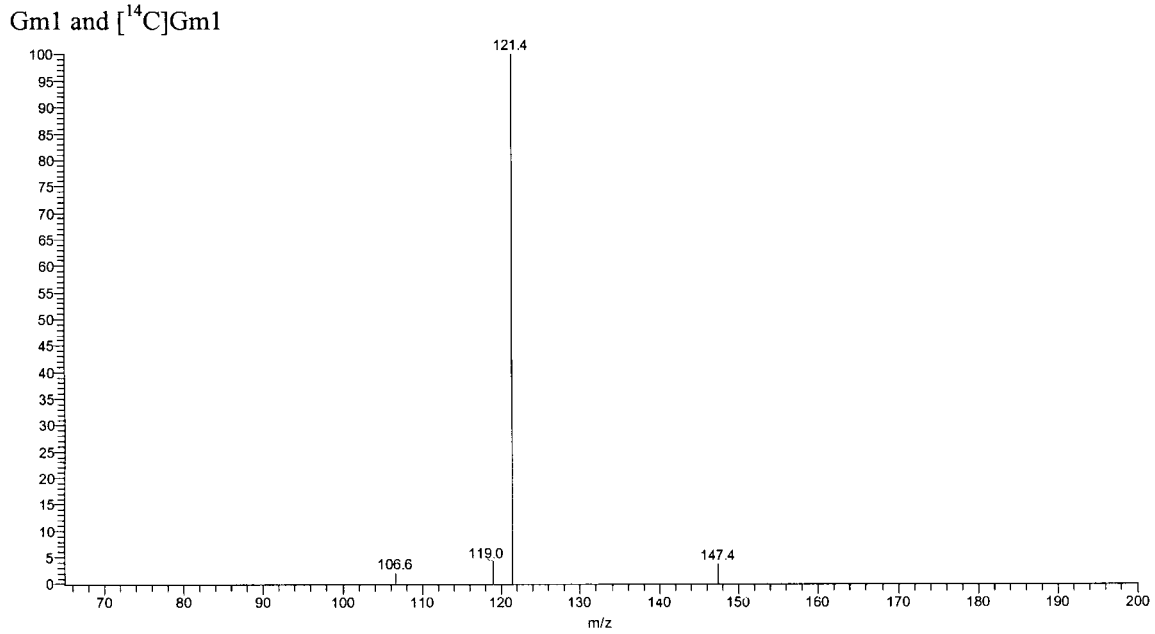


Fig. 2. Product ion spectra of genistein and metabolites. Representative product ion spectra generated from parent ions of: genistein ($[M+H]^+$ m/z 271); [^{14}C]genistein ($[M+H]^+$ m/z 273); Gm1 and [^{14}C]Gm1 ($[M-H]^-$ m/z 165 and 167); Gm5 ($[M+H]^+$ m/z 273); [^{14}C]Gm5 ($[M+H]^+$ m/z 275); Gm6 ($[M+H]^+$ m/z 275) and [^{14}C]Gm6 ($[M+H]^+$ m/z 277). Gm1 and [^{14}C]Gm1 both yielded essentially identical product ion spectra. Gm1 and [^{14}C]Gm1 were analysed by negative ion APCI all others with positive ion ESI. A CID of 26% was used to generate all product ion spectra.



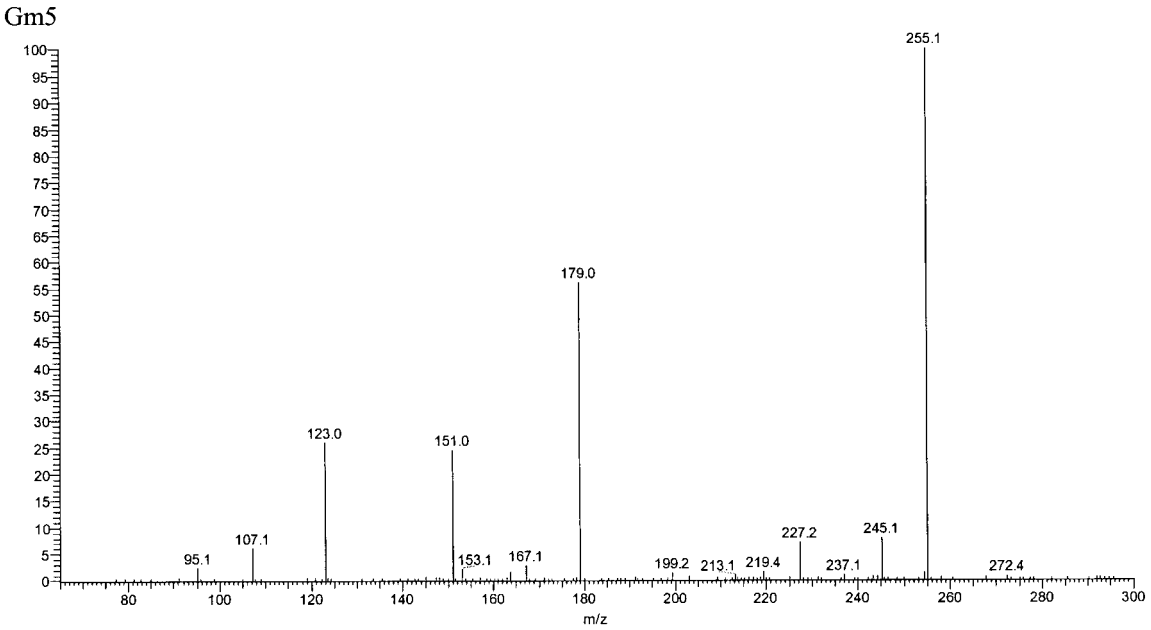
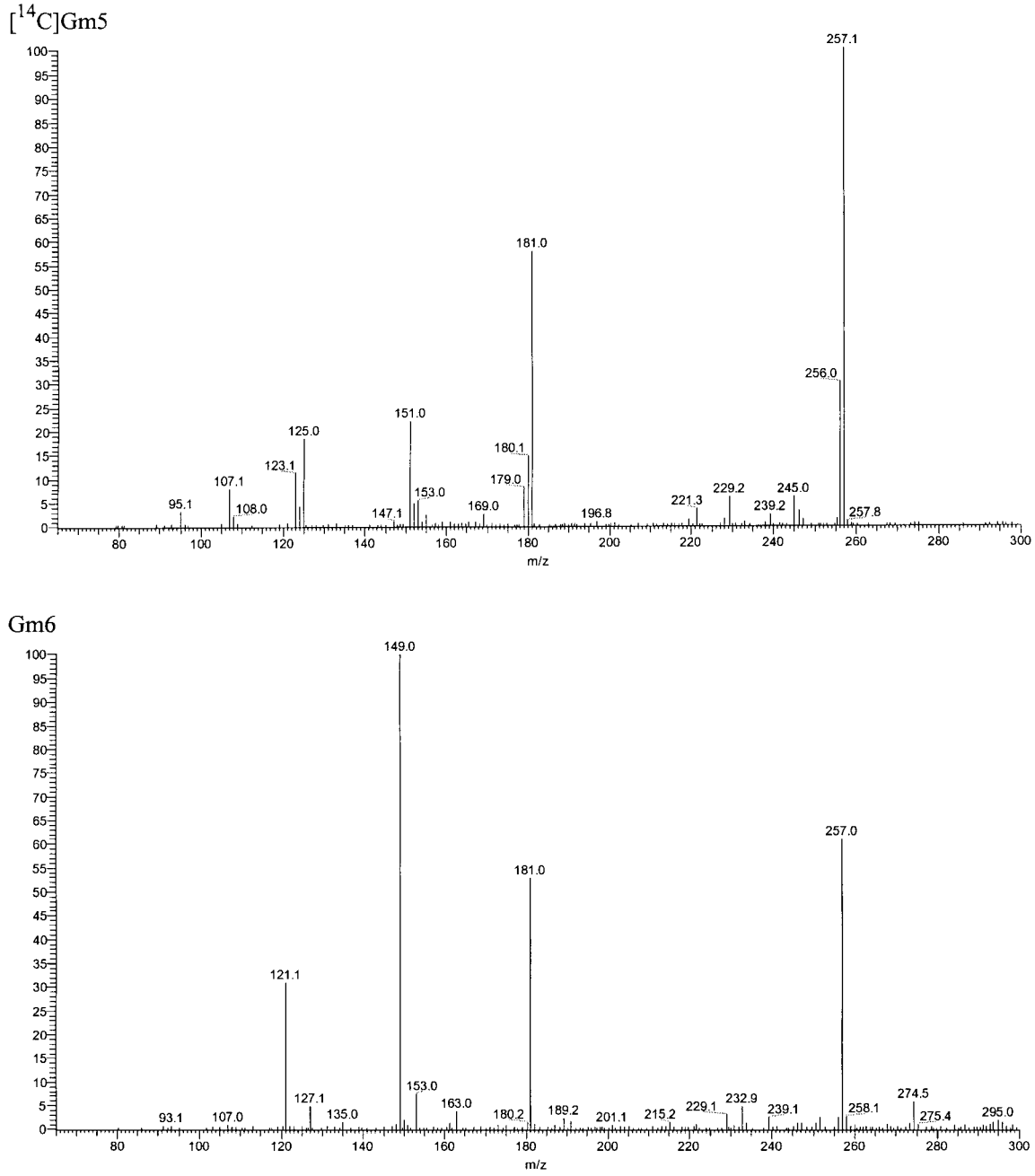


Fig. 2 (*continued*)



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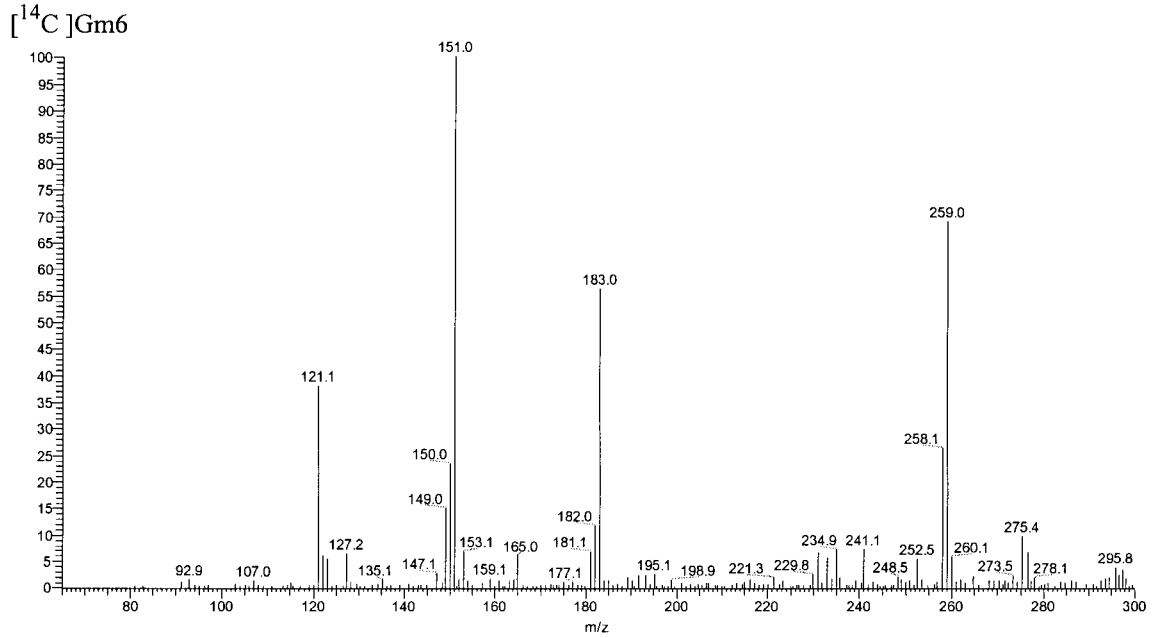

Fig. 2 (*continued*)

administration), caecal contents incubate (1 min incubation time) and isolated hepatocyte culture medium are shown in Fig. 1. Metabolites of genistein (Gm) were assigned numerals 1–6 corresponding to their order of elution from the HPLC column. Urine collected 4 h after dosing from both sexes contained metabolites Gm2–4 (chromatogram not shown). Urine from male rats collected 24 h after dosing comprised >95% of a single metabolite Gm1 whereas that from females contained the range of metabolites Gm1–Gm5. Caecal contents incubate from male and female rats produced three metabolites Gm1, Gm5 and Gm6 (Fig. 1). Time course studies of caecal contents incubate indicated sequential biotransformation of genistein to Gm5, Gm5 to Gm6 and Gm6 to Gm1; biotransformation of Gm6 to Gm1 was very rapid and complete after 15 min. Cultures of isolated hepatocytes and precision-cut liver slices both produced a single metabolite with similar HPLC retention times to Gm2 (Fig. 1). Metabolites Gm2 and Gm3 were converted to parent compound and Gm5 respectively by prior treatment of isolated hepatocyte and precision-cut liver slice culture media and urine extracts with *H. pomatia* β-glucuronidase solution. Metabolites Gm1, Gm5 and Gm6 derived from urine and caecal contents media extracts (Gm6 present in caecal contents only) were resistant to treatment with *H. pomatia* β-glucuronidase solution.

3.2. Daughter ion mass spectra of genistein and metabolites

Analysis of radio and selected ion chromatograms (MS^1) revealed co-elution of Gm1 and Gm4 with $[M-H]^-$ of m/z 165 and 349 and Gm2, Gm3, Gm5 and Gm6 with $[M+H]^+$ of m/z 447, 449, 273 and 275, respectively. Representative MS^2 spectra of molecular ions of genistein, [^{14}C]genistein, Gm1 and [^{14}C]Gm1, Gm5, [^{14}C]Gm5, Gm6 and [^{14}C]Gm6 derived from rat caecal content incubate extracts are shown in Fig. 2. Similar product ion spectra were found for equivalent metabolites present in urine and culture medium extracts. The product ion spectrum for [^{14}C]Gm1 was similar to that for Gm1 yielding a single product ion of m/z 121. Metabolites Gm2, Gm3 and Gm4 fragmented during MS^2 analysis to yield single product ions of 271, 273 and 269 (MS^2 spectra not shown). Further analysis of the aglycone product ions derived from Gm2 and Gm3 by MS^3 yielded spectra identical to those of genistein and Gm5. In negative ion mode the product ion spectra (MS^2) of Gm2 and Gm3 contained aglycone ions of m/z 269 and 271, respectively and both m/z 175. The origin of the daughter ion of m/z 272 in the MS^2 spectrum of [^{14}C]genistein (Fig. 2) is unknown but may arise through isobaric interferences in the ion-trap.

3.3. Structural elucidation of genistein product ions

Elucidation of the structures of genistein product ions (MS^2) was facilitated by comparison of spectra derived from genistein and [^{14}C]genistein since the presence of ^{14}C in a product ion increased the m/z ratio by two atomic mass units. Proposed structures of genistein product ions are shown in Fig. 3 and illustrate a

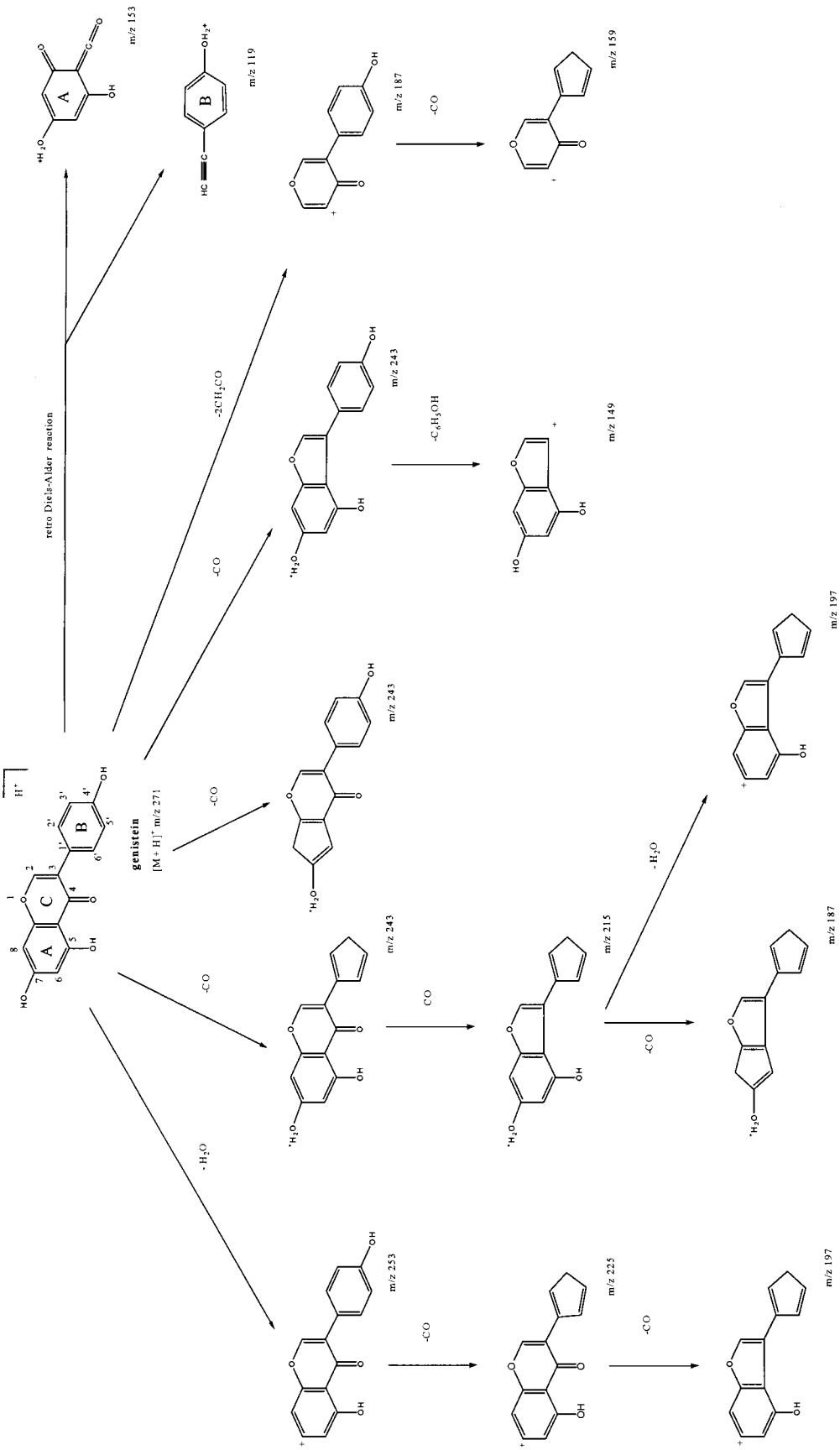

Fig. 3. Fragmentation pathway for genistein molecular ion $[M+H]^+$ m/z 271. The flavonoid rings are labelled (A–C) and carbon atoms numbered after Joannou et al. [16]. A limited number of structural isomers of product ions are shown since a loss of water could occur from several different sites. The site of protonation to form the molecular ion is not shown but the 5 and 4′ hydroxyls are plausible sites. The loss of the C4 was determined by comparison of product ion spectra derived from genistein and [^{14}C]genistein.

limited number of possible isomers, since losses of water and/or CO could occur from multiple sites of the molecule. Pathways of genistein fragmentation were subsequently confirmed by MS^n analysis of successive product ions (Table 1). Loss of a single (m/z 243) or double CO (m/z 215) were the most favoured CID fragmentation routes in the ion-trap. Fragmentation via a retro Diels Alder reaction gave product ions of m/z 153 and 119. The presence of product ions at both m/z 243 and 245 in the spectra of [^{14}C]genistein indicated loss of either CO or ^{14}CO. The unlabelled product ion at m/z 215 indicated that the loss of the second CO was from the ^{14}C carbonyl group at the C4 position.

3.4. Structural elucidation of genistein metabolites

3.4.1. Gm1

Preliminary investigation of the structure of Gm1 by LC/MS was problematic due to poor sensitivity using positive ion electrospray. The resistance of Gm1 to treatment with β-glucuronidase and displacement by formic acid during anion exchange chromatography was consistent with an unconjugated acid. Subsequent analysis by negative ion APCI revealed co-elution of Gm1 and [^{14}C]Gm1 with ions of m/z 165 and 167 which both fragmented during MS^2 analysis to yield a single product ion m/z 121 (Fig. 2). Identical product ion spectra and HPLC retention time with authentic material confirmed the identity of Gm1 as 4-hydroxyphenyl-2-propionic acid.

3.4.2. Gm2 and Gm3

Treatment of Gm2 and Gm3 with β-glucuronidase yielded products which eluted at similar retention times as genistein and Gm5, respectively. Such sensitivity to β-glucuronidase, neutral losses of 176 mass units, MS^3 spectra of the aglycones and MS^2 product ions of m/z 175 daughter ions (negative ion mode) indicated that Gm2 and Gm3 were glucuronide conjugates of genistein and Gm5, respectively.

3.4.3. Gm4

This metabolite co-eluted with an ion of m/z 349 (negative ion mode) and fragmented during MS^2 analysis to yield a single daughter of m/z 269 (daughter ion spectra not shown) consistent with loss of sulphate (80 mass units) from the parent compound.

3.4.4. Gm5 and Gm6

These β-glucuronidase resistant metabolites co-eluted with ions of m/z 273 and 275, respectively, suggesting reductive biotransformation of parent. The most plausible sites for such reductions are the C ring at the C4 carbonyl, C2–3 olefin or fission of the C ring ether bridge. Product ion fragmentation spectra for

Table 1
Summary of major product ions formed following MS^2, MS^3 and MS^4 analysis of the genistein $[M+H]^+$ parent ion (m/z 271)[a]

MS^n n	Parent ions (m/z)	Product ions (m/z)
2	271	253 (48), 243 (100), 227 (3), 225 (16), 215 (90), 197 (9), 187 (12), 165 (3), 159 (24), 153 (80), 149 (32), 147 (2), 145 (13), 133 (4), 119 (3)
3	271, 253	225 (100), 197 (11), 183 (12)
4	271, 253, 225	197 (100)
3	271, 243	225 (11), 215 (100), 149 (25), 165 (17), 197 (10), 159 (12), 133 (6), 187 (6)
4	271, 243, 215	197 (100), 187 (75), 147 (62)
3	271, 187	159 (100)

[a] The relative abundance of the product ions is shown in parentheses. For all MS^n anlayses a CID of 26% was optimal. This information was used to elucidate a pathway for genistein fragmentation shown in Fig. 3.

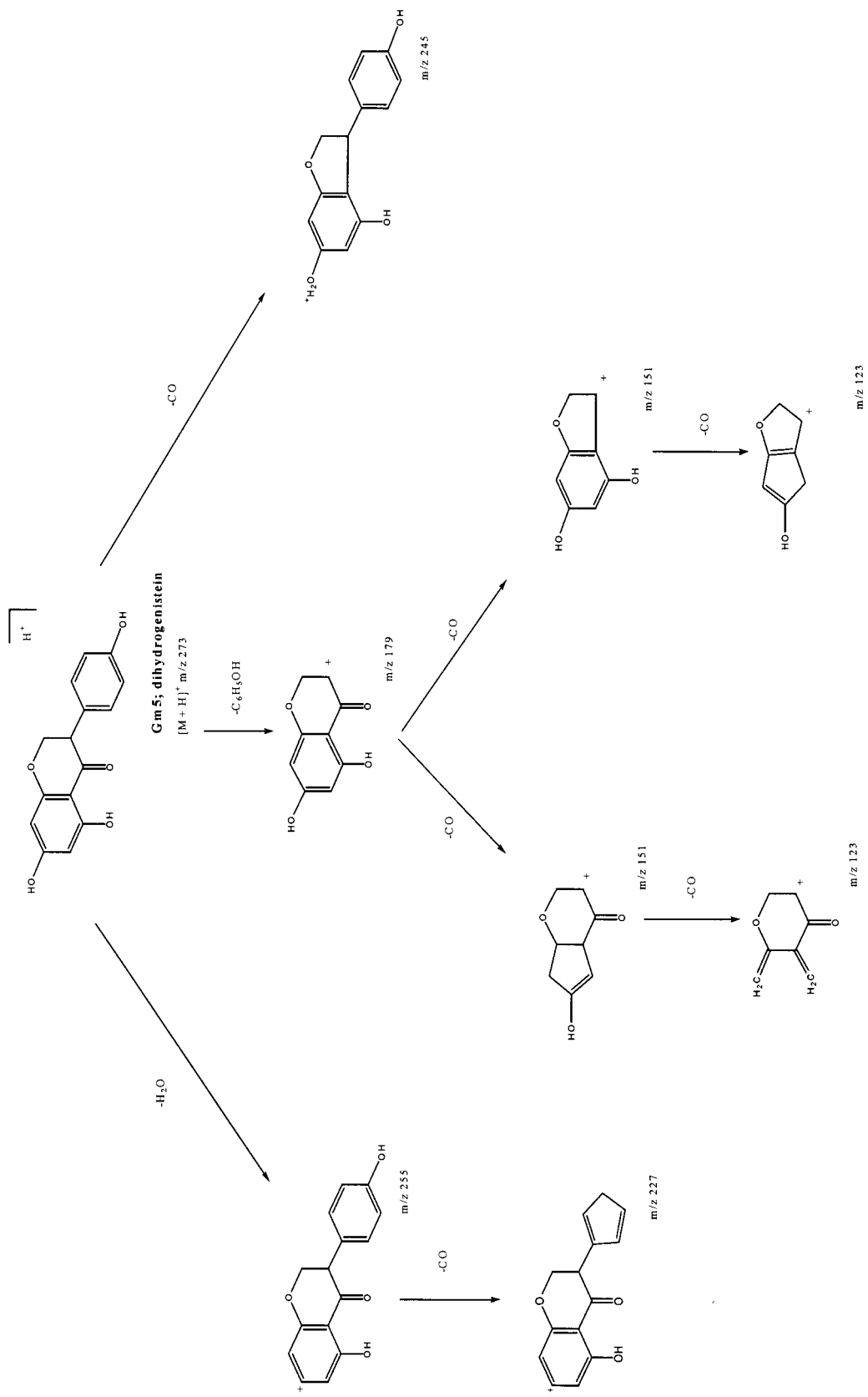

Fig. 4. Proposed fragmentation pathway for Gm5 molecular ion $[M+H]^+$ m/z 273. A limited number of structural isomers of product ions are shown since a loss of water could occur from several different sites. The loss of the C4 was determined by comparison of product ion spectra derived from Gm5 and [^{14}C]Gm5.

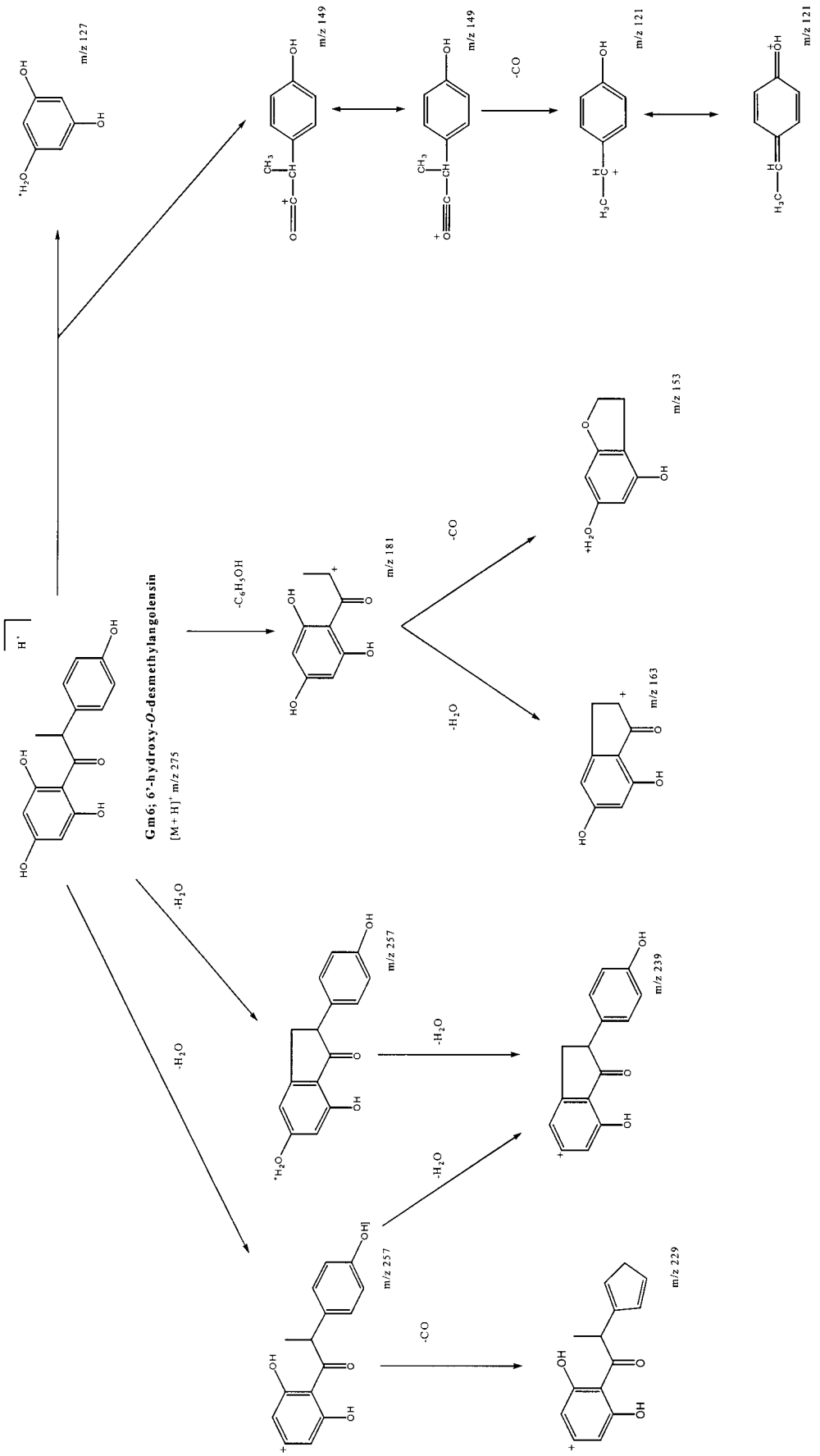

Fig. 5. Proposed fragmentation pathway for Gm6 molecular ion $[M+H]^+$ m/z 275. A limited number of structural isomers of product ions are shown since a loss of water could occur from several different sites. The loss of the C4 was determined by comparison of product ion spectra derived from Gm6 and [^{14}C]Gm6.

Gm5, [^{14}C]Gm5, Gm6 and [^{14}C]Gm6, as shown in Fig. 2, enabled deduction of the molecular site of reduction. The proposed structures of Gm5 and Gm6 and associated product ions are shown in Figs. 4 and 5, respectively. A limited number of structural isomers are shown, since losses of water and/or CO could occur from multiple sites of the molecule. The loss of ^{14}CO from Gm5 and Gm6 indicated that the C4 carbonyl remained unchanged by metabolism. Prominent product ions of m/z 179 and 181 and further loss of ^{14}CO from these, were consistent with metabolism in the C ring in both metabolites. Significant differences in the spectra between these two metabolites were evident since the opening of the C ring to form Gm 6 enabled fragmentation to produce a base product ion of m/z 149 and 127 which were derived from the C + B and A rings, respectively. Corresponding product ions of m/z 147 and 127 were not evident in the spectra of Gm5 since, with the C ring intact, this fragmentation route would be less favoured.

4. Discussion

Genistein was extensively biotransformed in the rat. Structural analysis by LC/MS^n enabled identification of all major radiolabelled metabolites in urine and media derived from caecal contents, isolated hepatocytes and precision-cut liver slice cultures. The combination of HPLC with on line parallel radio-detection and MS^n proved a highly effective means for structural analysis of metabolites, requiring neither prior hydrolysis of conjugates nor derivatisation of samples. This analytical approach is particularly advantageous since, in the event of extensive biotransformation, metabolites may evade detection during GC/MS analysis as a consequence of resistance to chemical derivatisation or poor chromatography under the conditions selected for parent and structurally similar compounds. Further, identification of metabolite ions during MS^1 analysis was simplified by matching peaks from radio- and selected ion chromatograms. Benefits of using the [^{14}C]-radiolabel were realised during mass spectral analysis since additional confirmation was provided both by chromatographic association of the molecular ion $[M+H]^+$ with the ^{14}C isotope ion ($[M+2+H]^+$) and by provision of information regarding product ion fragmentation. Analysis of product ion spectra by sequential ion-trap mass spectrometry to MS^4 enabled the pathways of genistein fragmentation to be followed and confirmed and facilitated subsequent application to the identification of genistein metabolites.

The location of the ^{14}C-label at the C4 position was particularly informative for deduction of the structures of Gm5 and Gm6. The loss of ^{14}CO during metabolite fragmentation in the ion trap supported the proposition that the radiolabelled carbonyl group had not undergone biotransformation and thus that reductive metabolism was occurring at other sites. Clearly, location of the ^{14}C in the A, and/or B rings would extend the ability to detect, by radio-HPLC, A ring derived metabolites and those, such as the putative product 4-ethylphenol, which would not be possible following loss of the C4 [^{14}C] by decarboxylation of 4-hydroxyphenyl-2-propionic acid. Time course studies of [^{14}C]genistein biotransformation by caecal microflora did not indicate further metabolism of 4-hydroxyphenyl-2-propionic acid. However, [^{14}C]-decarboxylation of 4-hydroxyphenyl-2-propionic acid to 4-ethylphenol cannot be completely excluded since this metabolite has been found in rat excreta [13] and glucuronide and sulphate conjugates detected by negative ion LC/MS in rat urine [22]. Such biotransformation may occur in other areas of the gut or body compartments such as liver.

The major metabolite of genistein in male and female rats was 4-hydroxyphenyl-2-propionic acid (Gm1) which was found in urine and in cultures of caecal contents. This indicated that this novel metabolite is produced as a result of reductive metabolism of genistein by gut micro-flora, firstly to dihydrogenistein (Gm5), followed by fission of the C ring to form 6′-hydroxy-*O*-desmethylangolensin (Gm6) and further fission to liberate the A ring, and formation of 4-hydroxyphenyl-2-propionic acid (Gm1). Differential rates of metabolism by gut micro-flora or biliary elimination may provide an explanation for the preponderance of Gm1 in urine collected 24 h after dosing from male compared with female rats.

Confirmation that Gm1 was 4-hydroxyphenyl-2-propionic acid was substantiated through five separate lines of evidence. Resistance to *H. pomatia* β-glucuronidase treatment precluded a glucuronide or sulphate conjugate and displacement by formic acid during anion exchange chromatography was consistent with a weak acid [26]. Co-elution of radiolabelled Gm1 with $[M-H]^-$ and ^{14}C-isotope ions ($[M+2\text{-}H]^-$) of m/z 165 and 167, respectively, and fragmentation of both parents to yield a product ion of m/z 121 were consistent with 4-hydroxyphenyl-2-propionic acid and location of the radiolabel at the carboxyl. Product ion spectra and chromatographic properties were identical to authentic material. Together, these data provide unequivocal identification of Gm1 as 4-hydroxyphenyl-2-propionic acid. Moreover, this metabolite is consistent with known pathways of flavonoid metabolism by gut micro-organisms; apigenin (an isomer of genistein) quercitin, (+)-catechin and hesperidin are all similarly degraded by heterocyclic ring fission to yield aromatic acids including 4-hydroxyphenylpropionic, 4-hydroxycinnamic, and 4-hydroxybenzoic acids [14,27,31,32]. Similar pathways of flavonoid biotransformation,

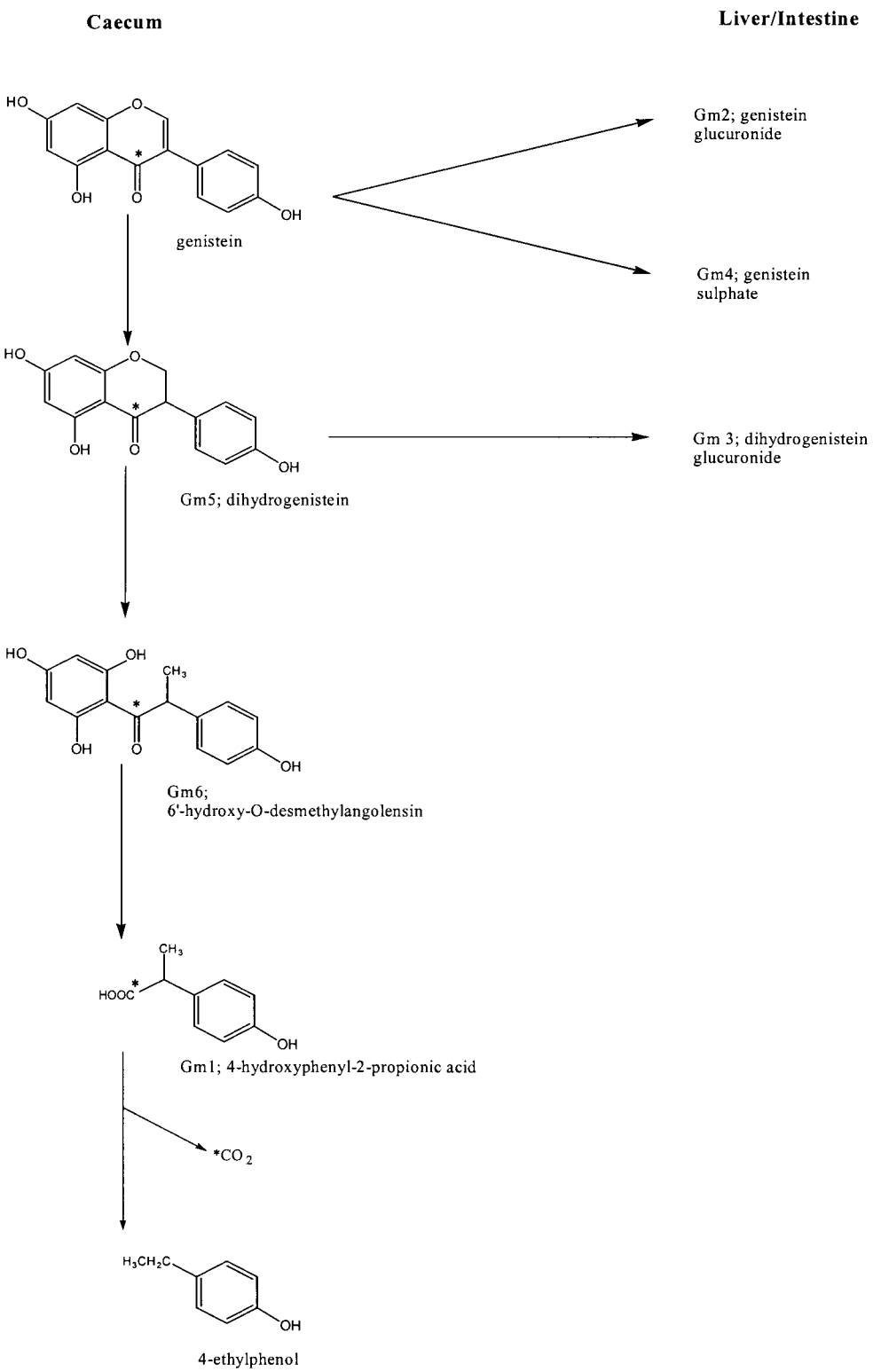

Fig. 6. Proposed pathway of genistein metabolism in the rat. In vitro preparations indicated that reductive biotransformation of genistein to Gm6 and heterocyclic ring fission to form Gm1 occurred in the caecum and phase II conjugation in the liver. Conjugation of genistein with glucuronic acid is also reported [13] to occur in the intestinal wall. *Indicates the position of the [^{14}C]-radiolabel. The precise molecular site of glucuronidation of Gm2 and Gm3 and sulphation of Gm4 could not be determined by mass spectrometry. Biotransformation of 4-hydroxyphenyl-2-propionic acid to 4-ethylphenol was not detected in the present study possibly due to loss of the radiolabel, but this metabolite has been found previously in the rat [13,22].

involving heterocyclic C ring cleavage, occur in humans during hesperedin metabolism [33]. Negative ionisation provided relatively little fragmentation for structural identification; Gm1 yielded essentially a single product ion of m/z 121 compared with the wide range observed in the positive ion spectra of genistein, Gm5 and Gm6.

Identification of other genistein metabolites, for which authentic material is not commercially available, proceeded by interpretation of product ion mass spectra. Although only 4 mass units separate genistein, dihydrogenistein (Gm5) and 6′-hydroxy-*O*-desmethylangolensin (Gm6) significant changes in product ion spectra were evident as a consequence of metabolism which were used for identification. Reduction of the C2–3 double bond to produce Gm5 prevented the retro Diels Alder reaction, which was prominent in the spectra of genistein, and promoted the loss of a B ring fragment to yield an ion of m/z 179 followed by ejection of the unchanged radiolabelled carbonyl to form the m/z 151 product ion. Opening of the C ring altered the fragmentation further, such that A and B ring fragments of m/z 127 and 149, respectively, were evident in the spectrum of Gm6. Dihydrogenistein (Gm5) and 6′-hydroxy-*O*-desmethylangolensin (Gm6) and the daidzein equivalents, dihydrodaidzein and *O*-desmethylangolensin, have all been found in human urine following administration of soya food products [17]. However, these studies also revealed more extensive biotransformation of daidzein including metabolism at the C4 carbonyl to produce equol and tertrahydrodaidzein. Further degradation of daidzein to 4-ethylphenol has not been reported. The most plausible explanation for the apparent resistance of genistein to reduction at the C4 position, and consequent absence of analogous metabolites such as tetrahydrogenistein, is protection of the carbonyl from metabolism by hydrogen bonding with the C5 hydroxyl group [10].

Phase II metabolites were readily identified as genistein (Gm2) and dihydrogenistein (Gm3) glucuronides and genistein sulphate (Gm4) since fragmentation via neutral losses of 176 or 80 atomic mass units were evident yielding aglycone ions which could be further analysed by MS^3 and compared with the product ion spectra (MS^2) of unconjugated metabolites and parent compound. Such neutral losses have been reported previously during collision induced dissociation in ion-trap instruments [28]. Although the position of conjugation could not be determined by mass spectrometry, 7-*O*-glucuronides and 4-*O*-sulphates of genistein have been found in the rat [13–15] and human breast cancer cells have been shown to produce a 7-*O*-sulphate [29]. Curiously, the order of elution of glucuronides of genistein and Gm5 from the HPLC column was reversed compared with the parent substances.

In vitro preparations have been widely used to predict the biotransformation of xenobiotics [18,24,28]. The findings of the present study with caecal contents, isolated hepatocyte and precision-cut liver slice cultures serve to emphasise both the utility and limitations of these preparations. Biotransformation of genistein by liver preparations was limited to glucuronidation of the parent compound which, if taken in isolation, suggested only phase II biotransformations in rats. In vivo, the primary site of such glucuronidation is likely to be the intestinal wall since following infusion of the duodenum with [^{14}C]genistein, portal vein blood contains predominantly the 7-*O*-glucuronide [13]. In contrast to liver, caecal contents cultures were most informative, since extensive biotransformation of genistein through to 4-hydroxyphenyl-2-propionic acid was evident, indicating that the gut was a major compartment of genistein metabolism. The explanation for the absence of 6′-hydroxy-*O*-desmethylangolensin (Gm6) from rat urine was revealed by time course studies with caecal contents cultures, since this metabolite was rapidly metabolised to Gm1 within 15 min despite dilution of the supernatants to 10% v/v. Human faecal stool cultures are also capable of similar biotransformations producing dihydrogenistein [30] and equol [18] from genistein and daidzein, respectively.

In conclusion, genistein was extensively metabolised in the rat by reductive metabolism, heterocyclic ring fission and phase II conjugation with glucuronic and sulphuric acids to produce a range of products including the novel metabolite 4-hydroxyphenyl-2-propionic acid. Biotransformation by in vitro liver preparations was limited to phase II metabolism, whereas reduction and heterocyclic ring fission occurred in caecal microflora cultures. A pathway for genistein metabolism through to 4-ethylphenol in the rat has been proposed and is presented in Fig. 6. Further studies with the radio-label located in the A and B rings may reveal the metabolic fate of the former providing a complete picture of genistein biotransformation and excretion. The pivotal role of gut micro-flora in phytoestrogen metabolism in rats and humans is likely to have a significant effect on biological activity in both species and on potential beneficial health effects in man.

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